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Role neutrofilních granulocytů v rozvoji zánětu

HABILITAČNÍ PRÁCE
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Poděkování

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Obsah

Abstract

Polymorphonuclear neutrophils (PMN) are considered the first line of host defense against pathogen attack. However, their activation may also cause damage to its own tissues. Therefore, PMN are considered potentially dangerous for the host and an important therapeutic target in the treatment of inflammatory diseases. Even though our knowledge about the importance of PMN in the inflammatory processes greatly increased in the recent years range of functions particularly related to their ability to influence the course of the inflammatory process remains unknown. A better understanding of this complex interplay can provide new therapeutic targets of manipulating PMN functions for the treatment of pathological inflammations. The presented habilitation thesis is a comprehensive collection of papers focused particularly on the role of PMN in the inflammatory processes in the arteries and cardiovascular diseases. In our studies, we contributed to clarification of the relationship between partial and full activation of PMN by polysaccharides with various structures interacting with receptors that recognize structures associated with pathogens. This activation may contribute to the immunostimulatory effects of these polysaccharides *in vivo*. In contrast, the negative activation of PMN associated with the pathologic acute or chronic inflammatory process was studied in our other studies focused on the activation and pathological role of PMN in the inflammatory response to the extensive surgical procedures associated with organ transplantation, or with the use of extracorporeal systems, either in patients during open heart surgeries or hemodialysis. In our studies we have shown that opposing positive and negative features of PMN during an inflammatory response are regulated by many factors, including the type of stimulus and the local or systemic cytokines and chemoattractants that regulate the PMN extravasation extend and their interaction with other cells. In other studies, we focused on the myeloperoxidase (MPO) as the one of the most PMN abundant enzymes released from PMN during activation. The formation of reactive metabolites catalyzed by MPO contributes not only to immune defenses, but it may have a significant impact on the promotion of inflammatory processes, including pathologies of the cardiovascular system. Our studies have shown that not only the ability of MPO to catalyze the formation of reactive oxygen and nitrogen intermediates, but also the effects of MPO independent of its catalytic activity affect the activation state of the endothelial cells and platelets and PMN extravasation into peripheral tissues. Our *in vivo* studies together with studies by other authors have shown that MPO may participate in a series of events involved in the initiation, propagation and subsequent complications in the pathology of cardiovascular diseases. Overall we described several scenarios that support a pro-inflammatory role of MPO. Therefore, MPO and PMN in general represent attractive targets for development of prognostic biomarkers and therapeutic interventions not only in the treatment of cardiovascular diseases as well as other diseases associated with inflammation related pathological processes.

Abstrakt

Polymorfonukleární neutrofily (PMN) jsou považovány za první obrannou linii hostitele proti útoku patogenů. Avšak aktivace PMN může také vést k poškození vlastních tkání. Proto jsou považovány za potenciálně nebezpečné pro vlastní organismus a jako zajímavý terapeutický cíl v léčbě zánětlivých onemocnění. I když se znalostí o významu PMN při zánětlivých procesech v posledních letech značně zvýšily, řada funkcí, zejména spojených se schopností PMN modulovat průběh zánětlivého procesu, zůstává neznáma. Lepší pochopení této komplexní souhry může poskytnout nové cesty pro ovlivnění funkce PMN v léčbě patologických zánětů. Předkládaná habilitační práce je komplexním souborem prací, které se zabývají touto problematikou se zaměřením na význam PMN v zánětlivých procesech v cévách a kardiovaskulárních onemocněních. V našich studiích jsme přispěli k objasnění vztahu mezi částečnou a kompletní aktivací PMN polysacharidovými strukturami interagujícími s receptory rozpoznávající struktury asociované s patogeny. Tato aktivace může přispívat k imunostimulačním efektům těchto polysacharidů *in vivo*. Naproti tomu negativní aktivace PMN spojená s rozvojem poškozujícího akutního a chronického zánětlivého procesu byla studována v našich dalších pracích, kde byla popsána intenzivní aktivace PMN v reakci na rozsáhlé operační výkony spojené s transplantacemi orgánů, či s využitím mimotělních systémů, ať už u pacientů během otevřených operací srdce nebo hemodialýzy. V našich studiích jsme ukázali, že pozitivní a negativní funkce PMN během zánětlivé odpovědi jsou regulovány řadou faktorů, včetně typu stimulu a místní či systémové tvorby cytokinů a chemoatraktantů, které regulují intenzitu extravazace PMN a jejich interakce s jinými krevními a imunitními buňkami. V dalších studiích jsme se zaměřili na problematiku myeloperoxidázy (MPO) jako jednoho z nejabundantnějších enzymů PMN uvolňovaném během jejich aktivace. Tvorba reaktivních metabolitů katalyzovaná MPO přispívá nejen k imunitní obraně, ale může mít značný dopad na podporu zánětlivých procesů, včetně patologických procesů kardiovaskulárního systému. Naše studie ukázaly, že nejen schopnost MPO katalyzovat tvorbu reaktivních kyslíkových a dusíkových meziproduktů, ale také její účinky nezávislé na katalytické aktivitě, ovlivňují aktivační stav endoteliálních buněk i krevních destiček a zvyšují extravazaci PMN do periferních tkání. Naše *in vivo* studie spolu se studiemi jiných autorů ukázaly, že MPO se může účastnit řady událostí zapojených do iniciace, propagace i následným komplikacím v patologii kardiovaskulárních onemocnění. Celkově bylo popsáno několik scénářů podporujících prozánětlivou roli MPO. Díky tomu MPO a také obecně PMN v současnosti představují atraktivní cíle pro rozvoj prognostických biomarkerů a terapeutických zásahů nejen v léčbě kardiovaskulárních nemocí, ale i dalších onemocnění spojených s patologickými procesy indukovanými zánětem.

Úvod

V kontextu současného poznání obranných mechanismů u živočichů, lze zánět definovat jako prvotní reakci organismu na infekci nebo poškození tkání. Zánětlivý proces vede k odstraňování patogenů, toxinů a poškozených buněk organismu s cílem obnovit homeostázu prostřednictvím indukce procesu hojení. Lokalizovaná akutní zánětlivá reakce je tedy běžná a nutná pro udržení normálních funkcí organismu. Má zásadní význam pro obranyschopnost proti infekcím a pro minimalizaci poškození a opravu narušené tkáně. Naproti tomu chronický zánět představuje přetrvávání zánětlivých procesů spojených s infiltrací imunitních buněk včetně PMN, makrofágů a lymfocytů v původně infikovaných nebo poškozených tkáních. Chronický zánět je tedy abnormální patologický proces, který je odchylkou od fyziologické odpovědi hostitele (Alessandri et al. 2013). Během posledních desetiletí bylo publikováno velké množství dat, která ukazují, že chronické zánětlivé procesy významně přispívají k rozvoji řady chorob, jako například revmatoidní artritida, lupus a kardiovaskulární onemocnění. Proto hledání nových, účinných farmakologických přístupů ovlivňujících průběh zánětlivých procesů má významný potenciál pro léčbu většiny civilizačních chorob (Alessandri et al. 2013).

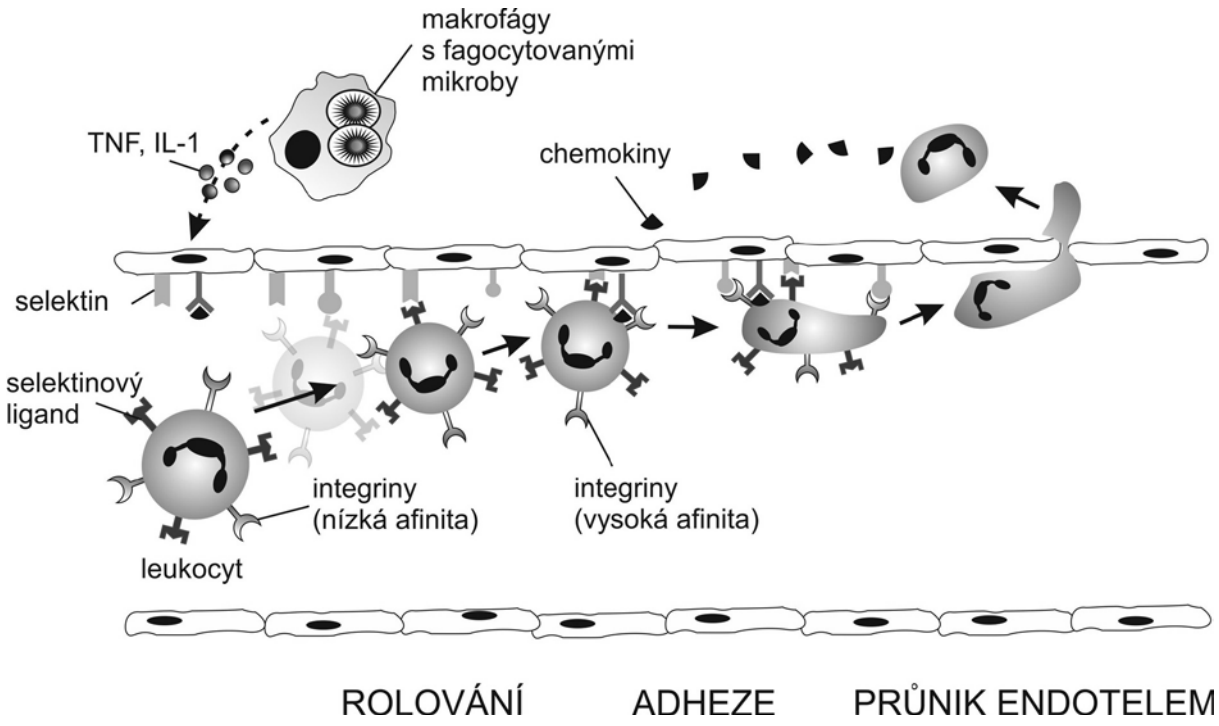
Předkládaná habilitační práce představuje výběr 19 publikací, které shrnují výsledky našeho studia zaměřeného na mechanismy regulující funkce PMN, primárních buněk zánětlivých procesů, a význam myeloperoxidázy (MPO), vysoce abundantního enzymu PMN, v rozvoji a regulaci zánětlivých pochodů v organismu. V jednotlivých kapitolách jsou stručně uvedeny základní poznatky v této problematice a výsledky obsažené v článcích jsou prezentovány v jejich kontextu.

Leukocyty jako klíčové buňky v zánětlivé odpovědi

Zánět probíhá jako kaskáda na sebe navazujících dějů vedoucích k aktivaci a akumulaci obraných buněk v místě zánětu s cílem odstranění patogenů nebo poškozených buněk. Proces je podrobně popsán v různých souborných článcích včetně například recentních (Alessandri et al. 2013; Arnhold and Flemmig 2010; Bardoel et al. 2014; Fournier and Parkos 2012; Nauseef 2014; Nauseef and Borregaard 2014; Pittman and Kubes 2013; Winterbourn and Kettle 2013). Primárně dochází k aktivaci buněk v odpovědi na přítomnost patogenů, přesněji řečeno struktur asociovaných s patogeny (pathogen-associated molecular patterns, PAMPs) (Pittman and Kubes 2013). Buňky mohou být také aktivovány díky přímému poškození buněk organismu, kdy jako aktivační signál působí biomolekuly, které se uvolňují z poškozených buněk (např. DNA, RNA, močové krystaly, atd.) (damage-associated molecular patterns, DAMPs). PAMPs a DAMPs pak aktivují intracelulární nebo povrchové receptory rozpoznávající tyto specifické struktury (pattern recognition receptors, PRRs). Aktivované buňky nespecifické imunity nacházející se v tkáních – makrofágy, žírné a dendritické buňky spolu s fibroblasty - produkují prozánětlivé cytokiny a další mediátory, jako např. matrix metaloproteinázy (MMP), které zahajují rozvoj zánětu v daném místě. Spolu s aktivovanými složkami komplementu a faktory krevního

srážení indukují aktivaci endoteliálních buněk přilehlých cév, zejména kapilár, prostupující danou tkáň. Tato aktivace endoteliálních buněk umožňuje průnik imunitních buněk ze systémové cirkulace do místa zánětu.

Extravazace leukocytů z krevní cirkulace do okolní tkáně je jedním z hlavních mechanismů zánětu. Zahrnuje dobře definovanou kaskádu dějů řízených komplexní souhrou interakcí adhezních molekul, receptorů na povrchu buněk a chemokinů. Ve většině tkání lze během extravazace pozorovat následující po sobě jdoucí děje: zachycování, rolování, přilnutí/adhezi, pohyb po povrchu endotelu a finálně transmigraci přes endotel (obrázek 1).



Obrázek č. 1: Extravazace PMN v cévách (Podhorec a Kubala 2015)

Primárně je tento proces iniciován změnami na povrchu endotelu, kdy se po aktivaci endoteliálních buněk během několika minut indukuje na jejich povrchu exprese adhezních molekul. Počáteční zachycení a rolování cirkulujících leukocytů na aktivovaném endotelu jsou zprostředkovány zejména P-, E- a L-selektiny, které umožňují interakci se selektinovými ligandy, zejména P-selektinového glykoproteinového ligandu 1. To vede k zachycení volně pohyblivých leukocytů na povrch endotelu a jejich následné rolování podél endotelu ve směru toku krve. V průběhu rolování jsou leukocyty dále aktivovány chemokiny, pozitivně nabitými molekulami imobilizovanými na endotelu vazbou na negativně nabité glykosaminoglykany. Další aktivace leukocytů, ke které dochází díky aktivaci receptorů chemokinů spřažených s G proteiny, vede ke konformační změně integrinů, které následně vykazují vyšší afinitu pro jejich ligandy. Fagocyty konstitutivně exprimují vysoké hladiny integrinů včetně lymfocytárního antigenu 1 ([LFA-1] také známý jako α1β2; β2), integrinu CD11a v komplexu s CD18 a

makrofágový antigen-1 (MAC-1, také známý jako αMβ2; CD11b/CD18). Aktivace integrinů umožní jejich vazbu na endoteliální adhezní molekuly z imunoglobulinové rodiny (např. intercelulární molekula buněčné adheze [ICAM]-1 a ICAM-2). Tato vazba zprostředkovává pevné zastavení leukocytů na stěny endotelu a následnou migraci přes endotel. Vazba LFA-1 na ICAM-1 je klíčová pro pevnou adhezi.

Role PMN v zánětlivé reakci

Migrace leukocytů z cév do okolní tkáně je tedy primárním znakem zánětu. Mezi prvními buňkami migrujícími do místa zánětu jsou PMN, které patří mezi klíčové mechanismy vrozeného imunitního systému. Během zánětu se počet PMN v tkáních řádově zvyšuje. Během ukončení zánětu pak PMN odumírají a jsou odstraněny makrofágy a dendritickými buňkami. Údaje z experimentálních a klinických studií ukazují, že akumulace PMN v místě zánětu je rozhodující pro vymizení infekce. Význam PMN v antimikrobiální obraně je také odvozován z vysokého počtu nových PMN, které jsou denně generovány, aby byl zajištěn dostatečný počet nutný k odstranění infekce. Výrazný pokles počtu PMN v krvi nebo mutace spojené s poruchou funkce proteinů důležitých pro úlohy PMN vede u lidí k závažným imunodeficitům.

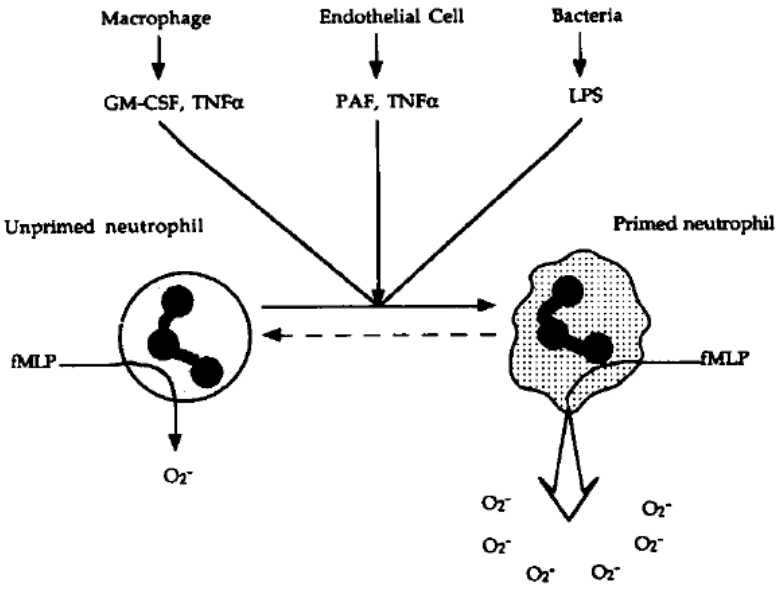
PMN vznikají v kostní dřeni z myeloidních prekurzorů. Během zrání prochází PMN několika vývojovými stupni, a to myeloblasty, promyelocyty, myelocyty, metamyelocyty, až diferencovanými nezralými PMN – tyčkami a nakonec zralými PMN s vysoce segmentovaným jádrem. Denní produkce PMN uvolňovaných do periferní cirkulace může dosáhnout až 2×10^{11} buněk. Z hlediska významu výsledků získaných na myších modelech je potřeba si uvědomit, že lidé a myši se zásadně liší v zastoupení PMN v periferní cirkulaci, kdy u lidí PMN představují 50-70% cirkulujících leukocytů, zatímco u myší tvoří pouze 10-25%.

Lidské PMN, o velikosti 7-10 µm, mají segmentované jádro a jejich cytoplazma je bohatá na granula a sekreční vezikuly, které obsahují různé typy enzymů a mikrobicidních peptidů. Tři typy PMN granulí jsou tvořeny postupně v průběhu jejich zrání, od stádia promyelocytu. Jedná se o azurofilní (primární) granula, která obsahují zejména MPO, specifická (sekundární) granula, ve kterých se nachází zejména laktoferin, a nakonec gelatinázová (terciární) granula, které jsou charakteristická obsahem MMP-9 (gelatináza).

Mechanismy aktivace PMN

Kompletní aktivace PMN může probíhat dvoustupňově. Nejprve může dojít k částečné aktivaci, označované jako „priming“, indukované prozánětlivými cytokiny, jako je například faktor nekrotizující nádory (TNF-α) a interleukin (IL)-1β, nebo při styku s aktivovaným endotelem či expozicí k PAMPs, chemoatraktantům nebo růstovým faktorům (obrázek 2). Již během této částečné aktivace začíná proces degranulace. PMN uvolňují granula a sekreční vezikuly, které mohou rychle přenášet obsah na povrch

buňky, kde jsou membránové struktury váčku začleněny do povrchové membrány. Díky tomu se na povrchu zvyšuje zastoupení receptorů a adhezivních molekul, které jsou nezbytné pro interakci s endotelem popsanou výše. Uvolnění proteáz z granulí dále podporuje narušení bazální membrány endotelu a také následně extracelulární matrix, což usnadňuje transmigraci PMN.



Obrázek č. 2: Aktivace PMN (Condliffe et al. 1998)

Plné aktivace dosahují PMN po setkání s patogenem nebo poškozenou buňkou, kdy spouští obranné zabíjecí mechanismy. PMN mohou eliminovat patogeny více způsoby a to jak uvnitř fagozómu, tak extracelulárně. Granula fagocytů obsahují nejen enzymy vytvářející vysoce reaktivní kyslíkové metabolity (RKM), ale také obsahují řadu degradačních enzymů a účinných antimikrobiálních proteinů. V klasickém případě jsou mikroorganismy fagocytovány, což může být zprostředkováno PRRs. Tato vazba je však nízko afinitní a je významně umocněna opsonizujícími látkami (zejména IgM, IgG a štěpy aktivovaného komplementu). Ty se navážou na povrch pohlcované částice a urychlují mechanismy spojené s rozpoznáním a pohlcením patogenu prostřednictvím receptorů, které lze rozdělit na receptory pro imunoglobuliny (zejména receptor pro Fc IgG [FcγR] a Fc IgA [FcαR] a na receptory pro komplement (především pro štěp C3b komplementu [CR1, CD11b/CD35]).

Poté, co je patogen uzavřen ve fagozómu je likvidován mechanismy závislými na kyslíku tvořící různé formy RKM a mechanismy na kyslíku nezávislými zahrnujícími antibakteriální proteiny a škálu proteolytických enzymů (např. defensiny, laktoferin a lysozym). Tvorba RKM je nazývána „oxidativní vzplanutí“, kdy klíčovou roli hraje enzym nikotinamidadenindinukleotidfosfát oxidáza (NADPH oxidáza), která je lokalizována v membráně fagozómu. Skládá se ze dvou membránově vázaných (gp91phox a p22phox) proteinů a tří proteinů lokalizovaných primárně v cytoplazmě (p40phox, p47phox a p67phox), které se spojí s membránově vázanými jednotkami již v průběhu částečné aktivace (Winterbourn and

Kettle 2013). NADPH oxidáza pak katalyzuje přeměnu kyslíku na superoxidový radikál, ze kterého dalšími reakcemi vznikají singletový kyslík, peroxid vodíku a hydroxylový radikál. Vzniklý peroxid vodíku je substrátem pro tvorbu kyseliny chlorné (HOCl) katalyzované enzymem MPO (Winterbourn and Kettle 2013).

Mikrobicidní mediátory mohou působit i na extracelulární patogeny. Vysoce aktivované PMN mohou také eliminovat extracelulární mikroorganismy tím, že uvolňují tzv. extracelulární pasti (neutrophil extracellular traps, NETs) (Bardoel et al. 2014). NETs se skládají z vláken DNA, na kterou jsou navázány histony, mikrobicidní peptidy a proteiny (např. laktoferin a katepsiny) a enzymy (např. MPO a neutrofilní elastáza). Předpokládá se, že NETs znehybňují patogeny, čímž brání jejich šíření, usnadňují následnou fagocytózu zachycených mikroorganismů a také se přímo podílí na zabíjení patogenů díky přítomnosti antimikrobiálních peptidů a enzymů (Bardoel et al. 2014). Cekově lze tedy shrnout, že PMN jsou vybaveny širokou škálou překrývajících se velmi účinných mikrobicidních mechanismů.

Aktivátory PMN dle mechanismu působení

Současné poznatky ukazují, že aktivace PMN je komplexní proces, který závisí nejen na typu stimulu, ale na řadě dalších faktorů ovlivňujících v daném okamžiku PMN. Z pohledu funkční mikrobicidní odpovědi je důležité rozpoznat, zda dané stimuly indukují pouze částečnou aktivaci PMN nebo indukují plnou aktivaci spojenou s oxidativním vzplanutím PMN.

V jedné z našich studií jsme se zaměřili na sledování potenciálu typických zástupců PAMPs a to různých typů (1 -> 3)-β-glukanů indukovat částečnou či úplnou aktivaci PMN (příloha č. 1) (Kubala et al. 2003). Obecně jsou β-glukany známé jako silné induktory humorální a buněčné imunitní odpovědi u lidí a zvířat. Pro farmaceutické využití se používají zejména (1->3)-β-D-glukany izolované z různých zdrojů. Díky tomu se tyto glukany liší v jejich chemických strukturách a fyzikálních parametrech. To může následně ovlivnit jejich imunostimulační potenciál vůči PMN. Byla studována stimulační aktivita dvou vybraných (1->3)-β-D-glukanů a to schizophylanu (SPG) a karboxymetylglukanu (CMG), které mají odlišné chemické a fyzikální parametry. S využitím *in vitro* modelu inkubace těchto látek s plnou lidskou krví bylo prokázáno, že oba testované glukany, SPG a CMG, indukovaly zvýšenou produkci RKM a vybraných prozánětlivých cytokinů - IL-6, IL-8, a TNF-α. Dále byla aktivace PMN prokázána zvýšenou expresí CD11b a sníženou expresí CD62L na PMN a monocytech. Aktivace PMN však byla jen částečná, významně nižší oproti stimulaci PMN klasickými aktivátory oxidativního vzplanutí jako je například opsonizovaný zymosan. SPG i CMG aktivovaly také lymfocyty, což bylo prokázáno na základě zvýšené povrchové exprese CD69. Je zajímavé, že SPG prokázal významně vyšší potenciál stimulovat krevní fagocyty a produkci vybraných prozánětlivých cytokinů než CMG. Vyšší účinnost SPG stimulovat krevní lidské fagocyty může být dána různými faktory, jako je například vyšší frekvence větvení polysacharidového řetězce SPG, neutrální náboj polymeru SPG, nebo odlišné konformace v roztoku ve srovnání s CMG. Význam rozdílné struktury potvrdli také jiní autoři porovnávající imunostimulační efekty

polysacharidů různých struktur (Noss et al. 2013). V další práci jsme se přímo zaměřili na mechanismy jak polysacharid glukomannan, který je dalším typickým zástupce molekul rozpoznávaných PRRs, může indukovat částečnou aktivaci PMN a tak je předpřipravit na intenzivnější odpověď vůči dalšímu stimulu (příloha č. 2) (Hajkova et al. 2009). Byl použitý rozpustný glukomannan, u kterého se přepokládají významné imunostimulační účinky indukující intenzivnější imunitní odpověď a zvyšující odolnost organismu (viz. souborné články (Descroix et al. 2006; Magnani and Hernan Castro-Gomez 2008; Soltanian et al. 2009)). Rozpustný glukomannan nestimuloval přímo oxidativní vzplanutí fagocytů, ale výrazně zesiloval oxidativní vzplanutí indukované následnou stimulací opsonizovaným zymosanem. Schopnost PMN produkovat RKM je závislá na aktivaci NADPH oxidázy, kdy musí být aktivovány jednotlivé podjednotky. Bylo ukázáno, že částečná aktivace PMN je spojena s fosforylací podjednotky p47phox, což umožňuje rychlejší a intenzivnější indukci oxidativního vzplanutí jinými aktivátory, jako je N-formylmethionylleucylfenylalanin nebo phorbol-12-myristát-13-acetát (El Benna et al. 2002). V naší studii jsme jako první ukázali, že rozpustný glukomannan indukuje fosforylaci podjednotky NADPH oxidázy p47phox na Ser345. To může být mechanismus zodpovědný za potenciální intenzivnější odpověď na následný stimul. Dále, stimulace glukomannanem indukovala částečnou degranulaci vyznačující se zvýšenou povrchovou expresí receptorů primárně uložených v granulích PMN (CD10, CD11b, CD14, CD35, a CD66b). Degranulace byla dále spojena s uvolněním enzymu neutrofilní elastázy do okolního média. Zvýšení povrchové exprese receptorů rozpoznávající opsonizované částice patogenů také může přispívat k intenzivnější obranné odpovědi PMN. Celkově práce ukazuje význam aktivování PRRs pro částečnou aktivaci PMN, která se projevuje dílčí degranulací a zvýšenou povrchovou expresí vybraných receptorů ve srovnání s plnou aktivaci PMN vedoucí k oxidativnímu vzplanutí. Avšak na rozdíl od našich výsledků, jiní autoři, jako například Drabikova et al., pozorovali snížení aktivace PMN detekované na základě snížené produkce RKM fagocytů jak *in vitro*, tak na modelu artritických potkanů *in vivo* (Drabikova et al. 2009). Jinými autory však byla stimulační aktivita rozpustného glukomannanu *in vivo* na imunitní odpověď včetně aktivity fagocytů prokázána například u drůbeže (Brito Darpossolo et al. 2010).

V průběhu imunitní odpovědi organismu jsou klíčovými mediátory regulujícími částečnou aktivaci PMN prozánětlivé cytokiny pro mají klidové PMN receptory. Tyto cytokiny hrají hlavní roli při regulaci zánětlivých reakcí včetně regulace aktivity fagocytů. V následující studii jsme se tedy zaměřili na potenciál různých prozánětlivých cytokinů (IL-6, IL-8 a TNF-α) indukovat částečnou nebo úplnou aktivaci PMN spojenou s degranulací a produkcí RKM (příloha č. 3) (Gallova et al. 2004). Byl také sledován potenciál IL-10, který patří mezi cytokiny s významnými inhibičními účinky na buňky specifické imunity, inhibovat aktivaci PMN indukovanou těmito vybranými prozánětlivými cytokiny. Výsledky ukázaly, že TNF-α, IL-6 i IL-8 v závislosti na koncentraci zvyšovaly produkci RKM a degranulací, které byly detekované na základě zvýšení povrchové exprese CD15 a CD11b jak na PMN, tak na monocytech. Dále bylo ukázáno, že IL-10 neměl významný inhibiční účinek na tvorbu RKM a degranulaci, protože aktivační účinky

testovaných prozánětlivých cytokinů IL-6, IL-8 nebo TNF-α nebyly významně modifikovány společným ovlivněním s IL-10. Získané výsledky naznačují, že TNF-α, IL-6 i IL-8 mohou významně přispívat k aktivaci PMN během zánětlivé odpovědi a prostřednictvím této aktivace zánětlivý proces amplifikovat. Na druhou stranu bylo zjištěno, že IL-10 nemá potenciál přímo regulovat aktivaci krevních fagocytů a to ani PMN ani monocytů. V následující studii naproti tomu autoři Dang et al. pozorovali potenciál IL-10 inhibovat částečnou aktivaci PMN indukovanou GM-CSF, která byla spojena s výše popsanou aktivací podjednotky p47phox (Dang et al. 2006). Dle výsledků těchto autorů byl tento efekt IL-10 zprostředkován inhibicí aktivace kinázy ERK1/2 indukovanou u PMN GM-CSF.

Aktivace PMN během systémového sterilního zánětu

Jak je uvedeno výše, na základě významu role PMN v rozvoji zánětlivé reakce se předpokládá, že PMN se také podílejí na rozvoji systémového zánětu. Systémový zánět může být vyvolán nejen masivním průnikem patogenu do organismu, ale také masivním poškozením vlastních tkání hostitele. V druhém případě často hovoříme o tzv. sterilním zánětu, jež typicky doprovází nejen poškození tkání v důsledku mechanického úrazu, ale také poškození tkání v důsledku dočasného přerušení krevního zásobení tkáně spojeného s ischemicko-reperfuzním poškozením dané tkáně. Další významnou příčinou indukce systémového sterilního zánětu jsou rozsáhlé operace a použití mimotělního oběhu krve ať již během otevřených operací srdce pro udržení krevní cirkulace a okysličení krve nebo v rámci mimotělní dialýzy u pacientů s nefunkčními ledvinami. Všechny výše uvedené příklady systémového sterilního zánětu mají významný dopad v každodenní klinické praxi. Přes značné úsilí stále nejsou známé optimální farmakologické přístupy, které by limitovaly poškození organismu spojené s akutním systémovým zánětem. Proto jsme se v našich pracích zaměřili na aktivaci PMN během těchto patologických procesů.

Byl studován vzorek pacientů podstupujících orgánové transplantace jater, otevřené operace srdce a transplantace srdce a pacientů s chronických selháním ledvin podstupující pravidelnou hemodialýzu.

Zánětlivá odpověď během transplantace jater

U pacientů podstupujících transplantaci jater byla ve vybraných intervalech, od počátku před započetím chirurgického zákroku až do 7. dne po transplantaci, sledována aktivace PMN na základě změny potenciálu spontánní a aktivované produkce RKM (příloha č. 4) (Kubala et al. 2001). Spolu s těmito parametry byly sledovány změny plazmatických koncentrací IL-6, IL-8, IL-10 a TNF-α. Bylo zjištěno, že hladiny IL-6, IL-8 a IL-10 se zvýšily již během časné reperfuze a poté se vrátily do normálu většinou v rámci prvního pooperačního dne. Plazmatická hladina TNF-α během celého sledovaného období vzrůstala. Zajímavé bylo, že jak spontánní, tak i aktivovaná produkce RKM v plné krvi byla snížena od začátku reperfuze a zůstala nízká během celého sledovaného období. Avšak byla pozorována pozitivní

korelace mezi cytokiny IL-6 a IL-8 a mezi IL-6 a IL-8 s produkcí RKM. Lze spekulovat, že toto snížení je způsobeno extravazací částečně aktivovaných PMN do periferních orgánů, zejména jater a plic, a současným vyplavením nezralých forem PMN z kostní dřeně. Tyto nezralé PMN mají sníženou schopnost produkce RKM i přes to, že v periferní cirkulaci jsou vysoké koncentrace prozánětlivých cytokinů indukujících částečnou aktivaci PMN a potencující produkci RKM. Zajímavého zjištění bylo dosaženo, když byla získaná data hodnocena s ohledem na výsledek transplantace. Plazmatické koncentrace IL-8, IL-10 a TNF-α byly výrazně zvýšeny během prvního týdne po operaci především ve skupině pacientů, u kterých se vyvinuly vážné komplikace během prvního měsíce po transplantaci. Unikátně získaná data ukázala na přímé spojení mezi aktivací nespecifické imunity a uvolňováním jak prozánětlivých, tak protizánětlivých cytokinů. Výsledky také ukázaly možnost využití sledování hladin těchto cytokinů, jako predikčních faktorů klinického vývoje po transplantaci. K podobným výsledkům dospěli také další autoři, kteří ukázali nárůst jak prozánětlivých cytokinů IL-6 a TNF-α, tak protizánětlivého IL-10 během transplantace jater (Jerin et al. 2003). Dále Bezinover et al., kteří stanovovali změny hladiny celkem 17 cytokinů v průběhu transplantace jater, podobně jako v naší studii prokázali významné zvýšení většiny prozánětlivých cytokinů (včetně TNF-α) zejména během reperfuze transplantovaných jater, které mohou významně přispět k aktivaci PMN během jaterních transplantací (Bezinover et al. 2011). Obdobné zvýšení IL-6 a IL-8 bylo zjištěno s využitím modelu ischemicko / reperfuzního poškození perfundovaných prasečích jater *ex vivo* (Gravante et al. 2009). Tento model, potvrdil, že klíčovým zdrojem těchto cytokinů jsou cirkulující leukocyty a perfundovaná játra.

Zánětlivá odpověď během otevřené operace a transplantace srdce

V dalších našich dvou studiích jsme se zaměřili na pacienty podstupující transplantaci srdce a otevřenou operaci srdce spojenou s vytvořením bypasu. Společným znakem těchto postupů je rozsáhlý chirurgický zákrok, který je spojený s otevřením hrudního koše a napojením pacienta na mimotělní oběh zajišťující udržení průtoku krve a její okysličování. Z pohledu indukce zánětlivé odpovědi je během těchto operačních zákroků klíčové, že leukocyty cirkulující v krvi se dostávají do přímého kontaktu s umělými membránami mimotělního oběhu a stěnami spojovacích hadic. Lze tedy předpokládat, že jak rozsáhlý chirurgický zákrok, tak použití mimotělního oběhu bude indukovat částečnou aktivaci PMN cirkulujících v periferii, což následně může přispět k amplifikaci negativní zánětlivé odpovědi během těchto rozsáhlých operačních zákroků.

Cílem první studie tedy bylo charakterizovat aktivaci PMN spojenou s indukcí systémového zánětu během otevřených operací srdce s použitím mimotělního oběhu (příloha č. 5) (Pavelkova et al. 2006). Vzorky krve byly odebrány v průběhu operace až do 24 hodin po operaci. Výsledky ukázaly velmi intenzivní aktivaci komplementu již na začátku použití mimotělního oběhu. Následovalo zvýšení počtu PMN v periferní cirkulaci spojené s jejich aktivací, k čemuž došlo po začátku reperfuze. To se projevilo

zvýšenou produkcí RKM jak spontánní, tak aktivovanou opsonizovaným zymosanem. Aktivace PMN byla potvrzena zvýšením povrchových receptorů zejména CD11b a CD31 podílejících se na adhezi PMN na endotel a následnou extravazaci PMN. cDNA microarray analýza genové exprese u leukocytů v periferní krvi potvrdila prozánětlivou aktivaci leukocytů 4 hodiny po reperfuzi. Celkově výsledky ukázaly, že otevřené operace srdce s využitím mimotělního oběhu jsou spojeny s rychlou a intenzivní aktivací krevních fagocytů a komplementu, která však není plně závislá na počátku mimotělního oběhu. Lze spekulovat, že rozsáhlý chirurgický zásah spojený s masivní aktivací komplementu v periferní cirkulaci má zásadní význam pro aktivaci PMN ještě před použitím mimotělního oběhu.

Cílem další studie bylo porovnat systémovou zánětlivou reakci a aktivaci PMN v periferní cirkulaci u otevřených operací srdce a transplantací srdce, kdy v obou typech operací byl použit mimotělní oběh (příloha č. 6) (Kubala et al. 2002, Cytokine). Výsledky ukázaly, že hladiny IL-6, IL-8 a IL-10 se zvýšily u obou skupin pacientů již v průběhu časné reperfuze a vrátily se na předoperační úroveň v rámci prvního pooperačního dne ve skupině transplantovaných pacientů. Avšak u pacientů podstupujících otevřenou operaci srdce zůstaly zvýšeny až do sedmého dne po operaci. Tento paradoxní výsledek lze vysvětlit podáváním intenzivní imunosupresivní terapie s potenciálem snižovat produkci těchto prozánětlivých cytokinů u transplantovaných pacientů. Protizánětlivý cytokin IL-10 byl zvýšen spolu s prozánětlivými a jeho hladiny v plazmě byly během reperfuze vyšší ve skupině pacientů s transplantaci srdce. Zajímavé bylo zjištění, že aktivace PMN byla zvýšena od chvíle reperfuze až do konce sledovaného období pouze u netransplantovaných pacientů. Během transplantace srdce se aktivita PMN pohybovala na předoperační úrovni, nebo dokonce snížila. Tento trend byl obdobný jako během transplantace jater a jak je uvedeno výše, lze ho vysvětlit extravazací plně maturovaných aktivovaných PMN do periferních orgánů a také použitím imunosupresivní léčby u těchto pacientů. Avšak ukazatel celkového oxidativního stresu organismu, úroveň peroxidace lipidů stanovená v séru, nebyl významně odlišný během obou typů operací. Celkově lze tedy shrnout, že oba typy srdeční operace jak s transplantací srdce, tak bez transplantace jsou spojeny se zvýšeným oxidačním stresem a zvýšenou produkcí prozánětlivých a protizánětlivých cytokinů.

K obdobným výsledkům dospěli také autoři Canbaz et al., kteří ukázali významné zvýšení prozánětlivých cytokinů jako IL-6 tak i protizánětlivého IL-10 u pacientů podstupujících otevřené operace srdce (Canbaz et al. 2008). Nicméně nebyla identifikována korelace mezi parametry zánětu a výskyty negativních procesů po operaci jako například fibrilace síní. Obdobně jako v našich studiích se negativním významem aktivace PMN během transplantace srdce intenzivně zabývali autoři Healy et al. Ve svých studiích ukázali, že aktivace PMN během transplantace indukuje infiltraci PMN do periferních orgánů včetně samotného myokardu a může indukovat jeho poškození spojené s větší pravděpodobností odvržení transplantovaného orgánu (Healy et al. 2006). Podobně jako v naší studii prokázali zvýšení aktivace PMN částečnou degranulací spojenou se zvýšením exprese CD11b. Tato aktivace pak pozitivně korelovala s výskytem odvržení transplantovaného orgánu u těchto pacientů. V

další studii ukázali, že předoperační aktivační stav PMN determinovaný na základě povrchové exprese CD11b a také relativní nárůst během operace pozitivně koreluje s pooperačními komplikacemi po otevřených operací srdce (Healy et al. 2007).

Zánětlivá odpověď u hemodialyzovaných pacientů

Další skupinou pacientů, na které jsme se zaměřili, a u kterých se předpokládá patologický význam aktivace PMN spojené s nadměrnou produkcí RKM a extravazací PMN do periferních orgánů, jsou pacienti podstupující pravidelnou hemodialýzu. Oxidační stres je jedním z důležitých komplikací vyskytujících se během hemodialýzy, který je spojován s aktivací PMN díky jejich přímému kontaktu s dialyzačními membránami, s dialyzační tekutinou a také aktivací komplementu v dialyzační soupravě (Rysz et al. 2006, CMI; Rysz et al. 2006, AITE-W). Cílem naší studie tedy bylo zjistit, jak hemodialýza indukuje aktivaci PMN spojenou s tvorbou RKM a současně, jak se mění protektivní antioxidační kapacita plazmy u pacientů v průběhu hemodialýzy (příloha č. 7) (Soska et al. 2007). Aktivita PMN stanovená na základě spontánní a aktivované produkce RKM byla vyšší u pacientů před dialýzou oproti aktivitě PMN u zdravých dobrovolníků. Během dialýzy došlo ke snížení aktivace PMN v periferní cirkulaci, což lze pravděpodobně přičíst extravazaci aktivovaných PMN do periferních orgánů a jejich adherenci na membrány dialyzační jednotky. V periferní cirkulaci tedy zůstaly méně aktivované a také zcela nedozrálé PMN se sníženou schopností produkovat RKM. Výrazný nárůst antioxidačního potenciálu plazmy, zejména díky vysoké hladině kyseliny močové, byl zjištěn oproti zdravým kontrolám u pacientů před hemodialýzou. Po hemodialýze se hodnoty významně snížily. Lze tedy předpokládat, že současná aktivace PMN během dialýzy a snížení antioxidačních vlastností plazmy přispívá k oxidačnímu stresu a nedostatečné antioxidační obraně u pacientů s chronickým selháním ledvin. Aktivace PMN během hemodialýzy byla potvrzena také v jiných studiích. Autoři Rysz et al. ukázali zvýšení produkce RKM krevními PMN u pacientů s chronickým selháním ledvin podstupujících pravidelné hemodialýzy oproti kontrolní skupině již před hemodialýzou (Rysz et al. 2006, CMI; Rysz et al. 2006, AITE-W). Jak spontánní, tak aktivovaná produkce RKM se pak dále zvýšila v průběhu hemodialýzy. V následné studii tito autoři ukázali, že klíčovou roli hraje částečná aktivace krevních fagocytů indukovaná TNF-α, která je u těchto pacientů zvýšena (Rysz et al. 2006, AITE-W). Dále došlo u pacientů během hemodialýzy ke zvýšení plazmatické koncentrace TNF-α spolu dalšími prozánětlivými cytokiny IL-1, IL-6, IL-8 a to jak s ohledem na před dialyzační úroveň, tak v porovnání se zdravými kontrolami (Rysz et al. 2006, AITE-W). Naše výsledky i výsledky jiných autorů ukázaly, že zásadní význam pro aktivaci PMN během hemodialýzy má interakce PMN s hemodialyzačními membránami, případně jejich adherence na tyto membrány v hemodialyzační jednotce. Cílem další studie tedy bylo porovnat vliv hemodialyzačních membrán z materiálů hemophan a polysulfon na aktivaci PMN. To bylo testováno jak *in vitro* s využitím diferencovaných buněk myeloidní linie HL-60, tak in vivo porovnáním aktivace PMN v průběhu

hemodialýzy u pacientů s hemodialýzou na těchto vybraných membránách (příloha č. 8) (Kubala et al. 2002, GPB). Na rozdíl od předchozí studie, pacienti podstupující chronickou hemodialýzu měli oproti kontrolním jedincům sníženou schopnost produkce RKM již před započetím hemodialýzy. Avšak celkový aktivační status PMN byl zvýšený, jak ukázala povrchová exprese receptorů, např. CD11b. Rozdílné vlastnosti pro aktivaci fagocytů mezi membránami potvrdily výsledky, které ukázaly, že k dalšímu snížení produkce RKM a zvýšení exprese CD11b bylo pozorováno zejména u pacientů podstupujících hemodialýzu s využitím membrány hemophan. Snížení produkce RKM a zvýšení povrchové exprese CD11b bylo pozorováno také po inkubaci PMN s membránami hemophan *in vitro*. U PMN byla také detekována změna mitochondriálního membránového potenciálu, který reflektuje energetický metabolismus PMN spojený s jejich životaschopností. Také v tomto případě došlo k poklesu pouze po inkubaci PMN s membránami hemophan, což dále potvrdilo nežádoucí účinky membrán z tohoto materiálu ve srovnání s polysulfonovými membránami. Celkově lze tedy shrnout, že byly prokázány negativní účinky hemodialýzy na metabolickou aktivitu PMN, kdy chronická aktivace PMN díky expozici k hemodialyzačním membránám zejména z materiálů hemophan, vede k jejich dlouhodobé částečné aktivaci PMN, což může zvyšovat PMN extravazaci do periferních orgánů. Zároveň tyto chronicky aktivované PMN mají sníženou schopnost účinné obranné odpovědi vůči modelovým aktivátorům. Tyto faktory tedy mohou přispívat ke klinicky popsanému fenoménu zvýšeného chronického zánětlivého stavu u hemodialyzovaných pacientů kombinovaným se sníženou schopností obrany proti bakteriálním patogenům. Vedlejším výsledkem této studie byla optimalizace komplexní metodiky kombinující stanovení RKM, povrchové exprese receptorů a mitochondriálního membránového potenciálu u PMN inkubovaných s testovaným materiálem jako nástroj pro analýzu biokompatibility materiálů. Opakovaná aktivace PMN během hemodialýz vede u dlouhodobě hemodialyzovaných pacientů k obdobím se zvýšenými ukazateli systémového zánětu přetrvávajícímu nejen v krátkém období po ukončení hemodialýzy. Příspěvek PMN k tomuto fenoménu je však nejasný. Proto jsme se v naší další studii, u pacientů se selháním ledvin podstupujících dlouhodobě hemodialýzu, zaměřili na vztah mezi obecnými ukazateli systémového zánětu - hladinami C-reaktivního proteinu (CRP) a IL-6 v plazmě a aktivací endotelu stanovovaného na základě detekce plazmatických hladin solubilní intracelulární adhezivní molekuly (sICAM) a vaskulárního endoteliálního růstového faktoru (VEGF) spolu s determinací aktivace PMN danou úrovní degranulace v systémové cirkulaci stanovené na základě sérové hladiny MPO (příloha č. 9) (Kaysen et al. 2006). Epizodické zvýšení CRP u těchto pacientů bylo spojeno s vyššími hladinami sICAM a MPO. Nicméně, mezi hladinami sérového CRP a MPO nebo VEGF nebyla přímá korelace. Naopak, hladiny MPO a VEGF byly v úzké korelaci mezi sebou během epizod zánětu, kdy byly zvýšeny hladiny a CRP a IL-6, které také spolu korelovaly. Výsledky naznačují, že vysoké plazmatické hladiny CRP nebo jiných proteinů akutní fáze zánětu přímo nekorelují s hladinami MPO a tedy akutní degranulací PMN u dlouhodobě hemodialyzovaných pacientů. Z těchto výsledků vyplývá, že periodické období zvýšené aktivity jaterních proteinů akutní fáze není v přímé souvislosti s aktivací PMN spojenou s jejich

degranulací, ke které dochází pravidelně po hemodialyzační proceduře. Lze předpokládat, že degranulované proteiny jako např. MPO se mohou ukládat v cévním sytému a chronickou aktivací endotelu pak přispívat k periodickému rozvoji chronického zánětu, jak bude uvedeno v následující kapitole.

Myeloperoxidáza

Druhá část práce je zaměřena na problematiku spojenou s významem enzymu MPO v regulaci funkcí PMN a regulaci zánětlivé odpovědi. MPO je hemová peroxidáza uložená v azurofilních granulích PMN (viz. souborné články (Arnhold and Flemmig 2010; Klebanoff 2005; Winterbourn et al. 2000). Během fagocytózy je uvolňována do vznikajících fagozómů, kde hraje roli v procesu produkce RKM během oxidativního vzplanutí (viz. výše). Kromě toho se MPO po aktivaci PMN také vylévá do extracelulárního prostoru, což bylo prokázáno v širokém spektru zánětlivých onemocnění. Od svého prvního popisu v roce 1941 Agnerem (Agner 1941), byla role MPO rozpoznávána zejména v obraně hostitele v procesu zabíjení fagocytovaných patogenů. Zároveň přibývá jasných důkazů o zapojení MPO v regulaci a patogenezi řady zánětlivých stavů (viz. souborné články (Nauseef 2001; Nicholls and Hazen 2005; Winterbourn et al. 2000)). Mnohé z těchto efektů jsou spojené s enzymatickým působení MPO. Přesto novější studie naznačují, že MPO také vykazuje efekty na buňky, které nejsou závislé na jejich katalytických vlastnostech (Klinke et al. 2011; Kolarova et al. 2013; Lau et al. 2005). MPO se skládá ze dvou identických protomerů, které jsou kovalentně spojeny jedním disulfidovým můstkem. Každá polovina se skládá z těžkého α řetězce (58,5 kDa) a lehkého β řetězce (14,5 kDa) a obsahuje hemovou skupinu. Zajímavou vlastností MPO je, že vzhledem k velkému množství argininových a lysinových zbytků se jedná o silně bazický (pI > 10) a tím vysoce kationaktivní protein při fyziologickém pH (Arnhold and Flemmig 2010; Klebanoff 2005).

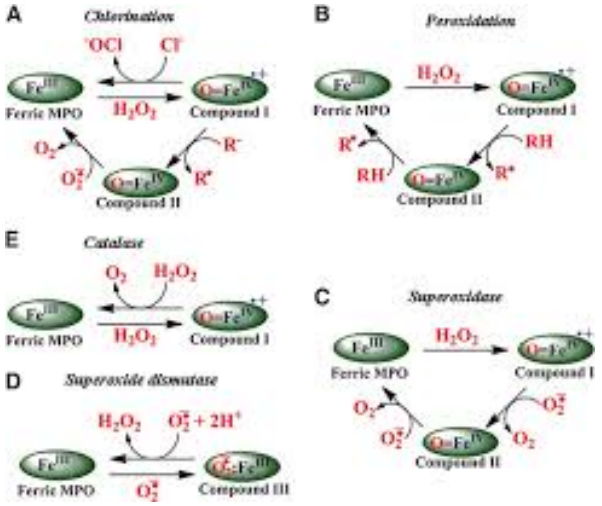
Exprese MPO je omezena na myeloidní linie hematopoetických buněk. Ve zralých lidských PMN je MPO velké množství a tvoří téměř 7 % celkové hmotnosti sušiny těchto buněk (Klebanoff 2005; Winterbourn et al. 2000). Kromě PMN se MPO nachází také v menším množství v monocytech. Pozoruhodné jsou mezidruhové rozdíly v expresi MPO. Například ptačí PMN neexprimují MPO vůbec (Rausch and Moore 1975). Dalším významným rozdílem je obsah MPO v myších PMN, které obsahují asi jen 15% MPO v porovnání s lidskými PMN (Rausch and Moore 1975). To je důležité z pohledu možného vysvětlení některých rozdílů zjištěných na experimentálních myších modelech v porovnání s klinickými výsledky u lidí, zejména v situaci kdy model myší deficientních na MPO je klíčovým a nejpoužívanějším nástrojem výzkumu zapojení MPO ve fyziologických a patofyziologických procesech.

Význam MPO pro zabíjení patogenů fagocyty je předmětem výzkumu více než čtyři desítky let a je stále předmětem probíhající debaty, zejména v kontextu ve velké míře klinicky nerozpoznatelného fenotypu MPO deficience u lidí. Přes jasné důkazy o snížené schopnosti zabíjení patogenů u MPO

deficientních PMN *in vitro* a vyšší citlivost MPO deficientních myší k některým patogenům, infekční komplikace u lidí vykazujících deficit MPO jsou obecně vzácné (Nauseef 2014). S rozvojem analytických metod v klinické imunologii, a rozpoznání relativně vysoké četnosti částečné nebo úplné MPO deficience, která se většinou klinicky fenotypově nijak neprojevuje, je význam MPO v imunitní obraně organismu člověka některými autory zpochybňován. To je v přímém kontrastu k pacientům trpícím chronickou granulomatózní chorobou, kdy porucha tvorby superoxidu NADPH oxidázou vede k výrazně zvýšené náchylnosti k život ohrožujícím infekcím. Paradox MPO deficience se vysvětluje záložními mechanismy, uplatňujícími se ve fagozómu, kompenzující tuto deficienci (Arnhold and Flemmig 2010; Klebanoff 2005; Nauseef 2014).

Enzymatické reakce MPO a modifikace biomolekul

Profil enzymatické aktivity MPO je velmi komplexní s množstvím reaktivních meziproduktů a množstvím reakčních partnerů (viz. komplexní souborné články (Klebanoff 2005; Nauseef 2014; Winterbourn et al. 2000)).



Obrázek č. 3: Schématický přehled katalytické aktivity MPO (Winterbourn and Kettle 2013)

Obecně lze reakce katalyzované MPO rozdělit do tzv. halogenačního a peroxidázového cyklu (obrázek č. 3). Primární reakcí společnou pro oba cykly je oxidace chloridových iontů MPO v přítomnosti peroxidu vodíku ve svém přirozeném stavu obsahujícím trojmocné železo [MPO-Fe (III)]. To vede k tvorbě krátkodobého redoxního meziproduktu označeného jako sloučenina I, která obsahuje oxyferryl hemu (Fe^{4+}=O) a porfyrinový π-kationový radikál [MPO-Fe (IV)]. V halogenačním cyklu, železitá MPO regeneruje redukcí sloučeniny I dvouelektronovou oxidací halogenidů (např. chloridu, bromidu, a jodidu) nebo pseudohalogenidů (thiokyananu), což vede k tvorbě (pseudo) halogenových kyselin. Kinetika této reakce je závislá na dostupnosti substrátu a pH, přičemž je výrazně zvýšena v kyselém prostředí. Za

fyziologických podmínek, chlorid a thiokyanát patří mezi klíčové substráty v halogenačním cyklu, což vede k tvorbě vysoce reaktivních meziproduktů, včetně HOCl. Jako alternativa k oxidaci halogenidů, MPO s hemovým železem v oxidačním stavu III může být regenerována v peroxidázovém cyklu oxidací velkého množství různých organických a anorganických substrátů, včetně aromatických aminokyselin, fenolových a indolových derivátů, askorbátu, či kyseliny močové. Avšak ne všechny reakce, které jsou možné *in vitro*, mohou probíhat *in vivo* nebo jsou z biologického hlediska relevantní.

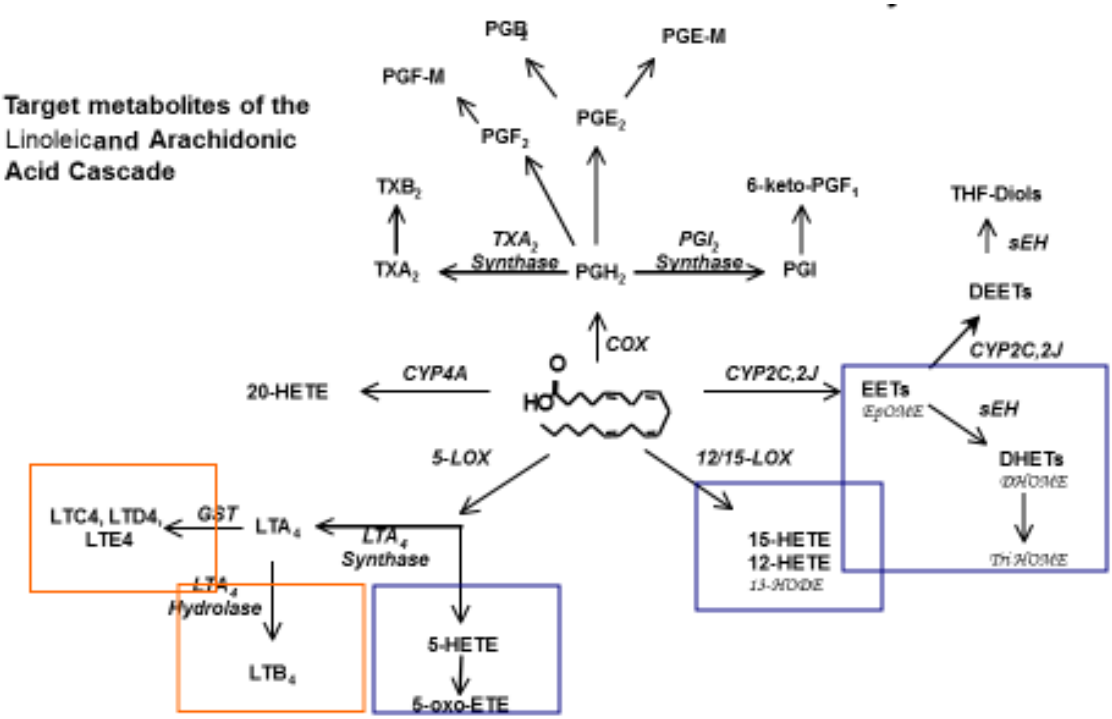
V posledních letech stále více důkazů podporuje význam MPO a jí katalyzovaných meziproduktů při zánětlivých procesech, které jsou nezávislé na roli MPO v přirozené imunitní obraně (viz komplexní souborné články (Klebanoff 2005; Nauseef 2014; Winterbourn et al. 2000)). HOCl, která je velmi silný oxidant, agresivně reaguje s množstvím molekul, jako jsou thioly a thioesterové skupiny, tyrosylové zbytky, aminy a amidy, sulfidy, volnými mastnými kyselinami a fenoly. Biologické cíle oxidací a chlorací HOCl a dalšího metabolitu vznikajícího MPO katalyzovanými reakcemi HOSCN zahrnují DNA, intracelulární signální proteiny, enzymy s hemovou skupinou, lipoproteiny a cholesterol, proteiny plazmy, glykoproteiny a mnoho dalších. Reakce HOCl a HOSCN s aminokyselinovými zbytky může způsobit změny terciární struktury cílového proteinu, a tím ovlivnit jeho strukturu a/nebo aktivitu. To vede k poškození tkáně změnou buněčné signalizace, růstu buněk, exprese proteinů, a následně buněčných funkcí. To je spojováno s patogenezí řady zánětlivých onemocnění (např. rozvoj aterosklerózy). V této souvislosti je možné zmínit tvorbu 3-chlorotyrosinu nebo 3,5-dichlorotyrosinu jako trvanlivý meziprodukt generovaný reakcí aminů s HOCl, který se nepoužívá jen jako klasický ukazatel MPO aktivity v tkáni, ale také přispívá k patologickým zánětlivým procesům.

Další významnou reakcí ovlivňující mezibuněčnou komunikaci a fyziologii řady buněk je katabolismus NO, který je dalším ze substrátů pro MPO-katalyzovaných oxidací. NO se oxiduje buď přímo nebo reaktivními meziprodukty vytvořenými v peroxidázovém cyklu sloučeninou I.

Další doposud velmi málo prozkoumanou oblastí enzymatické aktivity MPO je modulace biologicky aktivních metabolitů kyseliny arachidonové (AA) a kyseliny linolové (LA) (příloha č. 10) (Kubala et al. 2010). Jedním z charakteristických znaků zánětlivých procesů je peroxidace polynenasycených mastných kyselin AA a LA za vzniku bioaktivních derivátů těchto mastných kyselin, které mají významné regulační účinky během zánětu. Proto jsme zkoumali, jak MPO ovlivňuje plazmatické hladiny vybraných metabolitů AA a LA v průběhu systémového akutního zánětu, který byl indukován jednak u kontrolních myší a také myší deficientních na MPO. Ve srovnání s kontrolní skupinou, myši deficientní na MPO měly významně nižší hladiny LA epoxidů a odpovídajících diolů odvozených od LA a AA. Plazmatické hladiny hydroxy metabolitů AA a LA (např. kyseliny hydroxyeikosatetraenová a hydroxyoktadekadienová), byly také významně nižší u MPO deficientních myší. Naopak, MPO deficientní myši měly významně vyšší plazmatické hladiny cysteinylovaných leukotrienů, které jsou dobře známé svými prozánětlivými vlastnostmi. Význam MPO pro pozorovaný rozdíl v hladinách AA a LA metabolitů byl dále potvrzen *in vitro* experimenty s PMN izolovanými s krve. Tyto pokusy potvrdily, že PMN izolované z MPO

deficientních myší tvořily po stimulaci výrazně nižší množství AA a LA epoxidů, diolů a odvozených hydroxy metabolitů AA a LA než PMN izolované z kontrolních myší.

V této práci jsme tedy poprvé ukázali, že MPO v průběhu zánětlivých procesů může aktivně modulovat rovnováhu pro- a protizánětlivých lipidových mediátorů (obrázek č. 4) a tímto způsobem ovlivňovat průběh zánětlivých onemocnění.



Obrázek č. 4: Schématický přehled modulace biologicky aktivních lipidů u MPO deficientních myší (Kubala et al. 2010).

Význam MPO v indukci dysfunkce endoteliálních buněk a rozvoji kardiovaskulárních onemocnění

V následujících kapitolách jsou popsány výzkumné aktivity, ve kterých jsme se spolu s kolegy zaměřili na rozpoznání mechanismů, kterými MPO reguluje zánětlivé procesy v cévách a rozvoj endoteliální dysfunkce. Pozornost byla také věnována konkrétnímu významu MPO ve vybraných patologických stavech v rámci kardiovaskulárních onemocnění.

Vliv MPO na metabolismus oxidu dusnatého a funkce cév

Klíčovým objevem, pro pochopení významu MPO v rozvoji kardiovaskulárních onemocnění a cévní dysfunkce byla práce autorů Eiserich et al., kteří ukázali, že MPO katabolizuje oxid dusnatý (NO), a tím narušuje komunikaci mezi endoteliálními buňkami a hladkou svalovinou cév (Eiserich et al. 2002). Obecně snížená biologická dostupnost NO, který je důležitým regulátorem endotelové homeostázy, je považována za klíčový faktor endoteliální dysfunkce. *In vivo* byl vliv MPO na vasomotorické funkce potvrzen na prasečím modelu, kdy po systémové aplikaci MPO došlo ke snížení prokrvení myokardu a zvýšení plicní vaskulární rezistence (Rudolph et al. 2012). Jak bude podrobněji rozebráno dále, také u lidí existují studie poukazující na zřejmou inverzní korelaci plazmatických hladin MPO a relaxaci cév (viz. souborný článek (Nussbaum et al. 2013)).

NO byl identifikován jako substrát jednoelektronových reakcí MPO sloučenin I a II. Jelikož je NO klíčovou molekulou zprostředkovávající vazodilataci, MPO katalyzovaná oxidační degradace NO měla za následek poruchu acetylcholinem vyvolané relaxace aorty a tracheálních segmentů u potkanů a myší (Eiserich et al. 2002). Za fyziologických podmínek se NO může oxidovat také radikálovými meziprodukty malých molekul substrátů MPO, jako je askorbát nebo tyrosin (Eiserich et al. 2002). Kromě toho, MPO také může blokovat vazodilataci prostřednictvím oxidace L-argininu reakcí s HOCl (Zhang et al. 2001). Dále může mít MPO vliv přímo na syntázu NO, kdy oxidací kosubstrátů dochází k tzv. „rozpojení“ u tohoto enzymu, což má za následek produkci superoxidu namísto tvorby NO (Nussbaum et al. 2013).

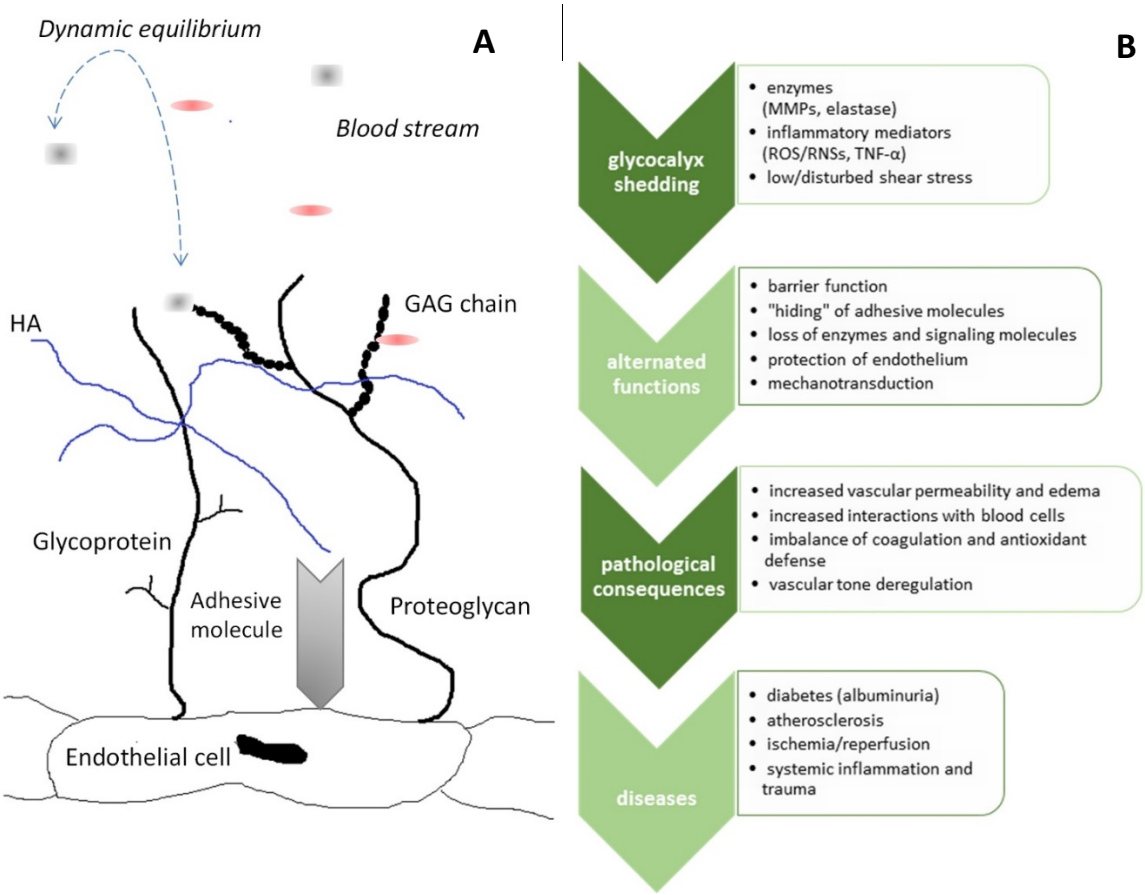
Dalším významným faktorem patologicky ovlivňujícím tvorbu NO endoteliálními buňkami je tvorba metabolitů L-argininu zejména asymetrického dimethylargininu (ADMA). Akumulace ADMA v organismu je spojena s rozvojem kardiovaskulárních chorob spojených s chronickým poklesem tvorby NO endoteliálními buňkami a indukcí endoteliální dysfunkce. Zajímavým faktem je, že během chronických zánětlivých stavů spojených s kardiovaskulárními nemocemi byla pozorována jak zvýšená plazmatická koncentrace ADMA, tak MPO. Avšak spojitost v patologickém působení těchto rozdílných látek nebyla popsána. Proto jsme se v naší další studii zaměřili na možné vzájemné ovlivnění zvýšené aktivity MPO a tvorby ADMA (příloha č. 11) (von Leitner et al. 2011). Bylo prokázáno, že ADMA indukuje aktivaci PMN jak *in vivo*, tak *in vitro*. V pokusech *in vitro,* PMN inkubované s ADMA vychytávaly tento metabolit L-argininu, což vedlo ke snížení produkce NO PMN. To mělo za následek, že PMN ovlivněné ADMA vykazovaly zvýšenou produkci RKM, zvýšenou degranulaci a zvýšenou adhezi na endoteliální buňky. To bylo také spojeno s uvolňováním MPO z PMN. Obdobně byl tento jev pozorován *in vivo* u zdravých dobrovolníků, kterým byla podávána ADMA infuzí, což vedlo k výraznému zvýšení plazmatické koncentrace MPO. Také dlouhodobá aplikace ADMA u myší vedla ke zvýšení plazmatické koncentrace MPO. Zajímavé zjištění bylo, že u myší, u kterých je díky genové manipulaci trvale zesílena exprese enzymu dimethylarginin dimethylaminohydroláza 1 (DDAH1) degradující ADMA, byl tento efekt snížen. Dále naše výsledky ukázaly, že HOCl deaktivuje hDDAH1 aktivitu *in vitro*, což má za následek akumulaci ADMA. Negativní význam MPO na degradaci ADMA byl potvrzen pomocí *in vivo* modelu, kdy u MPO deficientních myší nedocházelo během lipopolysacharidem indukovaného septického zánětu

k akumulaci ADMA v periferní cirkulaci. To poukázalo na existenci možného zpětného negativního mechanismu aktivace PMN na metabolismus ADMA.

Celkově lze tedy shrnout, že ADMA významně narušuje syntézu NO u PMN, což vede k jejich aktivaci. Tyto výsledky nejen ukazují na dosud nepopsaný aktivační efekt ADMA na PMN, ale také identifikují novou regulační funkci MPO, kdy během zánětlivých stavů může MPO ovlivňovat množství ADMA v organismu.

Interakce MPO s cévním endotelem

Výše uvedené studie, které ukázaly významný efekt MPO na indukci endoteliální dysfunkce a rozvoj cévních onemocnění vyvolaly komplexní zájem o výzkum mechanismů zodpovědných za ukládání enzymu MPO v cévách a interakci s endoteliálními buňkami. MPO má při fyziologickém pH vysoce kladný náboj. Proto jsme předpokládali, že se MPO bude vázat na silně negativně nabité povrchové vrstvy endoteliálních buněk uvnitř lumenu cév. Tato vrstva se nazývá "glykokalyx" a nese silně záporný náboj díky přítomnosti proteoglykanů s polysacharidovými řetězci složenými ze sulfatovaných a karboxylovaných disacharidů (Kolarova et al. 2014). Glykokalyxová vrstva endotelu je intravaskulární prostor, který vytváří bariéru mezi cirkulující krví a stěnami cév, jejíž strukturu a funkci jsme podrobně popsali v našem recentním souborném článku (obrázek č. 5) (příloha č. 12) (Kolarova et al. 2014).



Obrázek č. 5: Schématický přehled struktury glykokalyx (A) a patologických dopadů porušení glykokalyxové struktury (B) (Kolarova et al. 2014).

Glykokalyx hraje důležitou roli v mnoha fyziologických procesech v cévách, včetně regulace vaskulární permeability, přenosu smykového napětí a adheze a extravazace krevních buněk přes cévní stěnu. Různými autory byly vytvořeny modely a experimentální přístupy, které ukazují na změny ve struktuře a funkcích glykokalyx při různých zánětlivých stavech a jak tyto změny podporují zánětlivé procesy v cévách a přispívají k patogenezi řady chorob (Kolarova et al. 2014) (obrázek č. 5).

Výzkumy několika studií potvrdily, že dochází k intenzivní vazbě MPO k povrchu endoteliálních buněk jak *in vitro* v tkáňových kulturách (Baldus et al. 2001; Ballieux et al. 1994; Daphna et al. 1998; Kubala et al. 2013; Tiruppathi et al. 2004), tak *in vivo* (Baldus et al. 2006; Klinke et al. 2011). Hlavním vazebným partnerem pro vazbu MPO v glykokalyx byly detekovány heparansulfátové glykosaminoglykany (Baldus et al. 2001; Kubala et al. 2013). V experimentech jak v tkáňových kulturách *in vitro*, tak *in vivo* u pacientů s kardiovaskulárními chorobami bylo zjištěno, že v přítomnosti média nebo plazmy obsahující heparin či jiné negativně nabité glykosaminoglykany se MPO uvolní z povrchu endoteliálních buněk (Baldus et al. 2001; Kubala et al. 2013). Je to vysvětlováno jako důsledek kompetitivní vazby MPO s externě dodaným glykosaminoglykanem (heparinem) a glykokalyxových heparansulfátových glykosaminoglykanů (Baldus et al. 2001; Kubala et al. 2013). Tento předpoklad byl potvrzen v naší další studii, kde bylo ukázáno, že podání heparinu *in vivo* vede k uvolnění MPO z vazby na cévní endotel (příloha č. 13) (Baldus et al. 2006). U zdravých kontrol a u pacientů s výskytem stabilní ischemické choroby srdeční se sledovalo, zda podání heparinu mobilizuje MPO z cév a zda se toto uvolnění promítne do zvýšené biologické dostupnosti NO a zlepšené funkce cév. Zvýšení MPO v plazmě po podání heparinu bylo významně vyšší u pacientů s ischemickou chorobou srdeční oproti zdravým kontrolám. Podání heparinu zlepšilo biologickou dostupnost NO pro endoteliální buňky, jak prokázalo zlepšení acetylcholinem vyvolané dilatace cév a průtoku krve v předloktí. Intenzita heparinem indukovaného uvolnění MPO přímo korelovala se zlepšením funkce endotelu u pacientů. Celkově data prokázala, že glykosaminoglykany jako heparin, mohou díky uvolnění MPO z cévního endotelu působit protizánětlivě a zvyšovat vaskulární biologickou dostupnost NO.

Z předchozích studií však také vyplývá, že MPO se neváže pouze na luminální povrch endoteliálních buněk, ale je také transportována na bazolaterální stranu (Baldus et al. 2001; Kubala et al. 2013). Obecně, v *in vitro* tkáňové kultuře endoteliálních buněk se MPO velmi rychle, řádově v jednotkách minut, váže na povrch buněk a následně je transportována na bazolaterální stranu, kde se kolokalizuje s proteiny bazolaterální membrány (Baldus et al. 2001; Kubala et al. 2013). Mechanismus transcytózy však není plně objasněn. Jedna ze studií ukazuje na význam interakce MPO s albuminem a následnou transcytózou spojenou s kaveolami (Tiruppathi et al. 2004).

My jsme se v jedné z našich prací zaměřili na mechanismy vazby MPO na extracelulární matrix, a zda tato vazba moduluje enzymatickou aktivitu MPO (příloha č. 14) (Kubala et al. 2013). Byla charakterizována vazba MPO jednak na extracelulární matrix isolovanou z endoteliálních buněk aorty, buněk hladkého svalstva aorty a fibroblastů, a také z purifikovaných proteinů fibronektinu a kolagenu IV. Výsledky ukázaly, že se MPO váže jak ke kolagenu IV, tak fibronektinu a tato asociace je amplifikována po preinkubací těchto proteinů s glykosaminoglykany heparinem či chondroitin sulfátem. Dle očekávání, přebytek glykosaminoglykanů v roztoku během inkubace naopak inhiboval vazbu MPO na proteiny extracelulární matrix. Odpovídajícím způsobem byla vazba MPO potvrzena u extracelulární matrix izolovaných z různých typů buněk. Zajímavé výsledky přineslo testování oxidačního a chloračního potenciálu MPO po vazbě na proteiny extracelulární matrix. Jak oxidační, tak chlorační potenciál MPO byl nejen zachován po vazbě na kolagen IV a fibronektin, dokonce v některých typech reakcí bylo pozorováno i zesílení MPO aktivity v přítomnosti kolagenu IV a fibronektinu. Celkově tedy bylo prokázáno, že se MPO váže na proteiny extracelulární matrix na základě elektrostatických interakcí, a MPO chlorační a oxidační aktivita je zesílena při vazbě s těmito proteiny. V současnosti tedy zbývá lépe charakterizovat mechanismy průniku MPO do endoteliálních buněk a následná transcytóza MPO. Tento proces by totiž mohl být zajímavým cílem farmakologického působení s cílem snížit MPO indukovanou endoteliální dysfunkci.

Vliv MPO na rozvoj kardiovaskulárních onemocnění a funkce srdce

Význam chronického zánětu spojeného se zvýšenými hladinami mediátorů reflektující systémový zánětlivý stav jako např. CRP pro rozvoj kardiovaskulárních onemocnění je v současnosti jasně rozpoznán (viz. souborné články (Nicholls and Hazen 2005; Nussbaum et al. 2013)). Avšak mechanismy, kterými zánětlivé procesy přispívají k rozvoji kardiovaskulárních onemocnění, zůstávají nedostatečně charakterizovány.

Intenzivní zájem o spojitost mezi MPO a rozvojem kardiovaskulárních onemocnění vyvolaly studie, které dokumentovaly zvýšené hladiny MPO v periferní cirkulaci u pacientů s akutními stavy kardiovaskulárních onemocnění ve srovnání se zdravými kontrolami (Baldus et al. 2003; Brennan et al. 2003; Heslop et al. 2010; Mocatta et al. 2007). Je zajímavé, že sérové či plazmatické hladiny MPO u těchto pacientů, zejména u pacientů s akutní formou ischemické choroby srdeční, velmi dobře korelují s klinickým dopadem akutních koronárních příhod, především pokud jde o dlouhodobou kardiovaskulární mortalitu, závažnost kardiovaskulárního poškození, a de novo infarktu myokardu. Tyto a řada dalších studií jasně prokázaly, že významně zvýšené hladiny MPO v plasmě či séru u pacientů s akutními příznaky srdečního infarktu dokonce nabízí prognostické informace o nežádoucích sekundárních kardiovaskulárních příhodách (Baldus et al. 2003; Brennan et al. 2003; Heslop et al. 2010; Mocatta et al. 2007) . Také u pacientů se stabilizovanou formou kardiovaskulárních onemocnění někteří

autoři našli zvýšenou hladinu MPO. Výsledky jedné studie dokonce ukázaly, že plazmatické hladiny MPO nejen mohou predikovat nepříznivý výsledek a budoucí rizika u pacientů se stabilním nebo akutním koronárním onemocněním, ale také u zjevně zdravých jedinců (Meuwese et al. 2007). V některých studiích se ukázalo, že u pacientů s chronickou / stabilní ischemickou chorobou srdeční byly hladiny MPO spojeny s rostoucí diastolickou dysfunkci, což naznačuje vliv MPO na indukci srdečního selhání (Tang et al. 2006; Tang et al. 2011). Zajímavé je, že zvýšení plazmatické hladiny MPO u těchto pacientů predikovalo mortalitu a nutnost srdeční transplantace (Tang et al. 2009) Dále bylo prokázáno, že zvýšení plazmatických hladin MPO u pacientů se srdečním selháním je prokazatelné bez ohledu na etiologii onemocnění a koreluje s mortalitou u těchto pacientů (Reichlin et al. 2010; Rudolph et al. 2007).

Predikční hodnota hladiny MPO v periferní cirkulaci

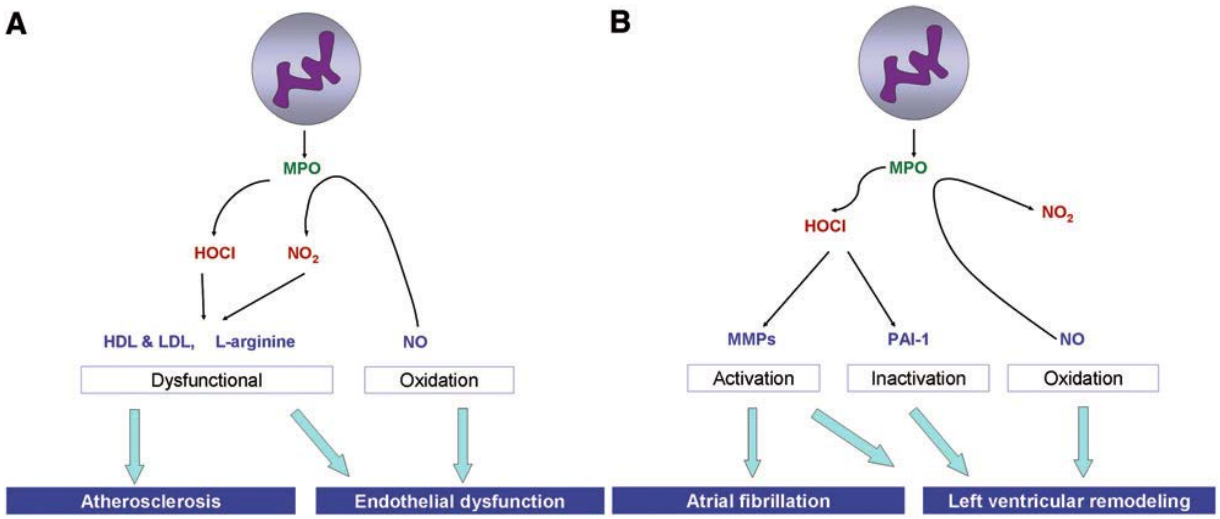
Avšak existují i studie, kde zvýšená hladina MPO v periferní cirkulaci nebyla u stabilizovaných pacientů s chronickou formou ischemické choroby srdeční detekována. Podle našich studií, u stabilizovaných pacientů s chronickou ischemickou chorobou srdeční na rozdíl od pacientů s akutní ischemickou chorobou srdeční, množství MPO v periferní cirkulaci nelze použít jako diagnostický nástroj, protože rozdílná hladina MPO v periferní cirkulaci mezi pacienty v porovnání se zdravými kontrolami nebyla prokázána (Baldus et al. 2006) (Kubala et al. 2008). V jedné z našich studii jsme porovnávali hladiny MPO v periferní cirkulaci u pacientů z různých etnických skupin se stabilní ischemickou chorobou srdeční, kteří podstoupili elektivní koronární angiografii (příloha č. 15) (Kubala et al. 2008). Výsledky ukázaly, že množství MPO se nijak výrazně nelišilo mezi pacienty s nebo bez ischemické choroby srdeční. Hladina MPO se nelišila u různých etnických skupin ani dle pohlaví, a pozitivně korelovala s hladinou CRP a fibrinogenu. Na základě těchto výsledků jsme konstatovali, že systémové uvolňování MPO není charakteristickým rysem symptomatické ischemické choroby srdeční. Chybějící korelaci mezi hladinami MPO a výskytem stabilní ischemické choroby srdeční byla potvrzena v naší další studii, kde, jak jsme očekávali na základě předchozí studie, výchozí hladina MPO v plazmě se nelišila mezi pacienty s nebo bez ischemické choroby srdeční prokázané angiografickým vyšetřením. Avšak jak je uvedeno výše, zvýšení MPO v plazmě po podání heparinu bylo významně vyšší u pacientů oproti kontrolám (příloha č. 13) (Baldus et al. 2006). Celkově lze uzavřít, že se detekce hladiny MPO v periferní cirkulaci jeví jako důležitý ukazatel stratifikace rizika iniciace a progrese kardiovaskulárních onemocnění. Avšak další rozsáhlé studie jsou nezbytné k posouzení skutečné diagnostické a prognostické hodnoty tohoto parametru v porovnání se současnými velmi citlivými testy detekujícími troponin. Dalším důležitým omezením pro klinickou využitelnost MPO jako biomarkeru u kardiovaskulárních onemocnění je nedostatek standardizovaných metod měření plazmatické hladiny MPO, které vykazují značně odlišné výsledky (Kubala et al. 2008). Kromě toho je nutné vzít v úvahu, že aktivace PMN spojená s uvolňováním MPO do oběhu je nespecifická událost a není omezena na kardiovaskulární příhody. Také aplikace

některých léčiv může ovlivňovat hladiny MPO v plazmě, včetně heparinu a bivalirudinu, jejichž podání moduluje hladinu cirkulující MPO (Baldus et al. 2006; Rudolph et al. 2008). To mohou být také důvody, proč se v některých studiích nepotvrdily prognostické informace plazmatických hladin MPO v predikci kardiovaskulárních onemocnění (viz. souborné články (Nicholls and Hazen 2005; Nussbaum et al. 2013).

Skutečnost, že vysoké hladiny MPO jsou prognostický ukazatel pro rozvoj kardiovaskulárních onemocnění u zdravých jedinců, vyvolává otázku, zda by MPO deficience u lidí mohla snižovat riziko vzniku kardiovaskulárních onemocnění. Proto jsme se v naší další studii zaměřili na studium významu polymorfismu v promotoru genu kódujícího MPO -463 G / (RS 2333227), který ovlivňuje intenzitu transkripce MPO. Alela G je spojena se zvýšenou expresi MPO. Cílem studie bylo zhodnotit, zda přítomnost tohoto MPO polymorfismu ovlivňuje výskyt srdečního onemocnění u pacientů s poruchou funkce levé srdeční komory (příloha č. 16) (Rudolph et al. 2009). Výsledky ukázaly, že GG genotyp byl spojen se snížením celkového přežití a tedy -463 G polymorfismus v promotoru genu kódujícího MPO je spojen s nepříznivým klinickým výsledkem u pacientů s poruchou funkce levé komory. Avšak zajímavé je, že jsme nepozorovali žádný vztah mezi polymorfismem MPO a množstvím MPO v plazmě. Význam MPO deficience pro snížení rizika kardiovaskulárních onemocnění je také dokumentována v práci autorů Kutter et al., kdy počet pacientů s kardiovaskulárními problémy, včetně infarktu myokardu, byl významně nižší u MPO-deficitních jedinců (Kutter et al. 2000). Avšak obě studie byly realizovány s limitovaným počtem pacientů a je potřeba tyto výsledky potvrdit dalšími studiemi zahrnujícími vyšší počet subjektů.

Mechanické spojení mezi MPO a patogenezí srdečních onemocnění

Celkově lze shrnout, že prognostický význam zvýšených hladin MPO v systémové cirkulaci nemusí nutně prokazovat zapojení samotné MPO v rozvoji srdečních onemocnění, ale může odrážet pouze zvýšenou aktivaci PMN během akutních stavů kardiovaskulárních onemocnění. Nicméně, celá řada výsledků, jako chybějící korelace mezi MPO a počtem leukocytů v periferní cirkulaci u těchto pacientů nebo přetrvávající predikční hodnota hladin MPO i po úpravě k dalším zánětlivým ukazatelům v prezentovaných klinických studiích a také experimentální výsledky *in vitro a in vivo* s využitím MPO deficientních zvířat, přímou roli MPO v patogenezi kardiovaskulárních onemocnění podporují. Řada autorů podporuje myšlenku, že se MPO nejen hromadí v plazmě pacientů s kardiovaskulárními onemocněními, ale je také mechanicky spojena s patogenezí tohoto onemocnění (obrázek 6.) (viz. souborný článek (Nussbaum et al. 2013)).



Obrázek č. 6: Příspěvek MPO k rozvoji kardiovaskulární a myokardiální dysfunkce (HDL, lipoprotein s vysokou hustotou; LDL, lipoprotein s nízkou hustotou; PAI-1, inhibitor aktivátoru plazminogenu) (Nussbaum et al. 2013).

Mezi hlavní navrhované mechanismy indukující změny myokardu patří indukce atriální fibrózy díky zvýšené aktivaci MMP radikály produkovanými MPO, jak bylo popsáno výše. To je spojeno také se zvýšenou syntézou a ukládáním kolagenu a jiných proteinů extracelulární matrix a následnou fibrózou tkáně. Bylo to prokázáno na myším modelu s využitím angiotensinu II, který indukuje chronický zánět a uvolňování MPO (Rudolph et al. 2010). Autoři této studie ukázali, že MPO podporuje aktivaci MMP-2 a -9 spojenou se zvýšením síňové fibrózy a zvyšuje pravděpodobnost fibrilací síní spojenou se zvýšenou tvorbou 3-chlorotyrosinu nebo nitrotyrosinu u kontrolních angiotensinem II ovlivněných myší oproti MPO deficientním angiotensinem II ovlivněným myším. Podobně jako u srdečních síní, MPO se také může podílet na patologické remodelaci levé komory. Tento proces je indukován zánětlivými pochody, které jsou důsledkem adaptivní změny funkce a tvaru levé komory, následkem zánětlivých procesů indukovaných infarktem myokardu nebo hypertenze (Nussbaum et al. 2013). Na modelu akutního infarktu myokardu bylo prokázáno, že u MPO deficientních myší byla zachována funkce levé komory, díky snížené infiltraci leukocytů, zmenšenému ukládání kolagenu, a snížení aktivity plasminu v myokardu (Askari et al. 2003). Dále Wong se spoluautory zjistili, že plazmatické hladiny MPO korelují s rozsahem ukládání vápníku v koronární arterii i u asymptomatických dospělých (Wong et al. 2009).

Tyto nálezy podporují názor, že MPO je kriticky zapojena do patogeneze srdečního selhání a farmakologická inhibice MPO aktivity představuje slibný potenciální cíl u onemocnění myokardu.

Interakce MPO s krevními buňkami

Současné poznatky ukazují, že MPO v průběhu zánětu v cévách může ovlivňovat nejen funkce cévního endotelu, ale také krevní buňky. Několik studií je věnováno sledování vlivu MPO na PMN, kdy přímo MPO nebo reaktivní metabolity katalyzované MPO mohou autokrinním nebo parakrinním způsobem ovlivňovat funkce PMN. Práce různých autorů ukazují, že specifické oxidanty katalyzované MPO působí na PMN stimulačně. Například stimulace PMN peroxidem vodíku nebo monochloraminem mělo za následek zvýšenou expresi β2 integrinů a následně zvýšenou extravazaci PMN *in vivo* (Suzuki et al. 1991). Obdobně působí oxidanty odvozené od MPO katalyzovaných reaktivních metabolitů, jako jsou například lipidy modifikované HOCl. Ty aktivují PMN a zvyšují jejich pro-adhezivní charakter zvýšením exprese adhezních molekul (Arnhold and Flemmig 2010; Undurti et al. 2009). V současnosti se objevují také studie, které naznačují i aktivační vliv MPO, který je na enzymatické aktivitě MPO nezávislý. Někteří autoři ukázali, že MPO je ligandem β2-integrinu Mac-1 na leukocytech a že po navázání MPO dochází k aktivaci NADPH oxidázy, nukleární translokaci NF-κB, degranulaci a zvýšené povrchové expresi CD11b (Johansson et al. 1997; Lau et al. 2005). Tyto účinky byly blokovány reakcí s protilátkou proti Mac-1, ale ne inhibicí enzymatické aktivity MPO inhibitorem 4-ABAH (4-aminobenzoic acid hydrazide). Podobný vliv na leukocyty byl popsán i pro jiné, vysoce kationické proteiny, jako je azurocidin nebo proteináza 3, které se uvolňují z aktivovaných PMN (viz. souborný článek (Nussbaum et al. 2013)). Zajímavé je, že zapojení β2 integrinu Mac-1 je uvažováno také u těchto proteinů, podobně jako pro MPO. Avšak specificita interakce MPO právě s integrinovým receptorem zůstává otázkou, zejména z pohledu velmi intenzivní interakce MPO s negativně nabitými glykosaminoglykany, které pokrývají celý povrch PMN.

Interakce MPO s leukocyty

V naší další komplexní studii jsme se tedy zaměřili na otázku, jak MPO ovlivňuje proces extravazace PMN nezávisle na specifické interakci s určitým receptorem, ale na základě modulace povrchového elektrostatického náboje PMN (příloha č. 17) (Klinke et al. 2011). Jak je uvedeno výše, extravazace PMN zahrnuje kaskádu událostí, které umožňují zachycení, adhezi a extravazaci leukocytů. Procesy, které jsou zprostředkovány receptory, včetně válcování, vázání, a diapedézy PMN, jsou v současnosti relativně dobře charakterizovány. Avšak mechanismy ovlivňující elektrostatické odpuzování mezi záporně nabitým povrchem PMN a glykokalyx endotelu zůstávají nepopsány. Lze současně spekulovat, že MPO může ovlivnit povrchový náboj PMN i endotelu svým kladným nábojem. Naše výsledky *in vitro* ukázaly, že MPO může indukovat významnou motilitu PMN, která je výhradně závislá na elektrostatické interakci s povrchem PMN. Následné pokusy *in vivo* s využitím různých zánětlivých modelů u myší ukázaly, že extravazace PMN byla výrazně vyšší v přítomnosti MPO a to jak v modelu jaterní ischemie a reperfuze po intraportální aplikaci MPO, tak i v kremastorovém svalu po intraarteriální aplikaci MPO nebo po indukci lokálního zánětu po aplikaci TNF-α. Tento efekt MPO, který

je založený na elektrostatické interakci mezi MPO a povrchem buněk, poprvé ukázal doposud nepopsanou funkci MPO. Ukázal také celkově na význam přitažlivosti řízené fyzikálními silami na mechanismus extravazace PMN.

V dalších studiích jsme se zabývali možností ovlivnění fyziologických funkcí dalších krevních buněk na základě interakce s MPO a to jak závislou na enzymatické aktivitě, tak závislou na modulaci elektrostatických interakcí těchto buněk s okolním prostředím.

Interakce MPO s trombocyty

Zaměřili jsme se na krevní destičky neboli trombocyty, které hrají významnou úlohu v cévním zánětu. Jejich zvýšená aktivace spojená se zvýšenou adherencí na endotel a s produkcí řady prozánětlivých mediátorů přispívá k patologickému procesu chronického zánětu. Avšak interakce MPO s trombocyty a modifikace jejich funkce jako mechanismu přispívajícímu k rozvoji vaskulárních zánětlivých onemocnění byla objasněna až v naší práci publikované v roce 2013 (příloha č. 18) (Kolarova et al. 2013). Získaná data jasně prokázala, že MPO je detekovatelná na izolovaných lidských destičkách, a že se dále může koncentračně závisle vázat na lidské i myší krevní destičky. Přítomnost MPO již u prekurzorů destiček, megakaryocytů, byla v souhlasu s literaturou vyvrácena. MPO se tedy váže na krevní destičky pravděpodobně až po jejich uvolnění do periferní cirkulace. Byla studována lokalizace MPO v trombocytech s cílem zjistit, zda dochází k podobnému efektu internalizace MPO jako u endoteliálních buněk. Trojrozměrná obrazová analýza imunocytochemických preparátů ukázala, že MPO je lokalizována jak na povrchu, tak uvnitř trombocytů. Avšak inhibice cytoskeletu nezabránila lokalizaci MPO uvnitř trojrozměrné struktury krevních destiček. Proto se domníváme, že MPO se dostane do vnitřní struktury destiček přes kanálkový systém, který volně komunikuje s vnějším prostředím, ve kterém se destičky nacházejí. Dále byla stanovena aktivita MPO vázané na destičky. Analýza potvrdila, že peroxidázová aktivita MPO byla zachována. Zajímavé bylo zjištění schopnosti MPO navázané na trombocyty katabolizovat NO, což bylo dokumentováno signifikantně sníženou produkci NO u MPO ovlivněných krevních destiček. Jelikož NO hraje důležitou regulační funkci v útlumu krevních destiček před nadměrnou aktivací, byl stanovován aktivační status trombocytů ovlivněných MPO. Aktivace krevních destiček MPO byla dokumentována zvýšením exprese povrchových receptorů krevních destiček P-selektinu a PECAM-1 a zvýšenou tvorbou RKM. Nicméně, aktivace byla pouze částečná, obdoba výše popsané částečné aktivace PMN spojené s dílčí degranulací a pouze velmi omezenému zvýšení tvorby RKM. Obdobně, MPO totiž neindukuje agregaci krevních destiček, ani neindukuje významné uvolnění obsahu granulí. Avšak tato částečná aktivace trombocytů byla spojena s jejich zvýšenou interakcí s PMN. Působení MPO na krevní destičky neovlivnilo jejich životaschopnost během krátké inkubace. Nicméně dlouhodobá inkubace po dobu 7 dnů ukazuje snížení životaschopnosti a počtů trombocytů. Zajímavé výsledky přinesly *in vivo* pokusy s využitím MPO deficientních myší. U těchto zvířat nebylo možné

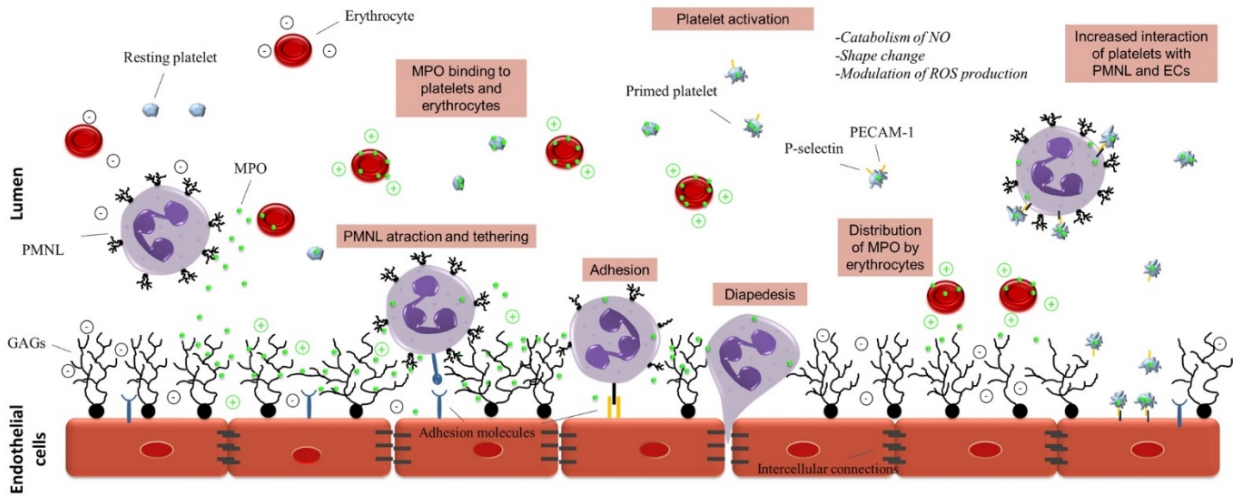
pozorovat sníženou schopnost agregace zprostředkovanou krevními destičkami. Ovšem intravenózní aplikace MPO indukovala výrazné zkrácení času potřebného k zastavení krvácení, což dále naznačuje, že krevní destičky v přítomnosti MPO byly již částečně aktivovány. Následující práce autorů Gorudko et al. potvrdila vazbu MPO na krevní destičky a aktivační vliv MPO na destičky, kdy MPO potencovala odpověď trombocytů na jiné aktivátory díky rychlejší odpovědi vápníkového signálování a reorganizací cytoskeletu (Gorudko et al. 2013). Celkově získaná data ukázala, že MPO může interagovat s krevními destičkami, což může vyvolat jejich částečnou aktivaci. Tímto mechanismem může MPO přispět k rozvoji chronických zánětlivých procesů v cévách. Potvrdily se tak předpoklady klíčové osobnosti ve výzkumu MPO, Dr. Klebanoffa, který spolu s kolegy již před více než 30 lety publikoval předběžná data naznačující aktivační potenciál MPO vůči krevním destičkám a jejich interakci s PMN (Clark and Klebanoff 1979; Clark and Klebanoff 1980).

Interakce MPO s erytrocyty

V závěrečné studii jsme se také zaměřili na erytrocyty, nejhojnější populaci krevních buněk (příloha č. 19) (Adam et al. 2014). Stejně jako ostatní krevní buňky, také erytrocyty mají na povrchu aniontový náboj, který je důležitý pro jejich správné fyziologické funkce. Analýza erytrocytů izolovaných z pacientů s akutním srdečním selháním, kterým byla detekována zvýšená hladina MPO v plazmě, ukázala významně vyšší množství MPO navázané na erytocytech oproti zdravým dobrovolníkům. Izolované erytrocyty z těchto pacientů byly inkubovány s glykosaminoglykanem heparinem což vedlo k uvolňování MPO z povrchu těchto eytrocytů. *Ex vivo* experimenty ukázaly koncentračně a časově závislou vazbu MPO na červené krvinky. Analýza průtokovou cytometrií kombinovaná s 3D konfokální analýzou prokázala přítomnost MPO pouze na povrchu erytrocytů. Zajímavé výsledky přinesla analýza aktivity MPO. Byla prokázána peroxidázová aktivita MPO i schopnost degradovat NO. Tímto mechanismem by mohly erytrocyty nesoucí MPO přispívat k indukci endoteliální dysfunkce v periferních cévách. Tento předpoklad potvrdily *ex vivo* pokusy, kde erytrocyty inkubované s MPO redukovaly acetylcholinem vyvolanou relaxaci aorty závislou na produkci NO. *In vivo*, u myší po infuzi erytrocytů inkubovaných s MPO došlo k indukci systémové vaskulární rezistence. Tyto výsledky naznačují, že erytrocyty mohou sloužit jako přenašeč MPO z místa akutní aktivace a degranulace PMN, kde dochází k uvolňování MPO a vazbě na erytrocyty, do malých cév v periferii, kde se MPO může z erytrocytů uvolnit a navázat na heparanové řetězce glykosaminoglykanů, které jsou nejabundantnější složkou glykokalyx endotelu. Tato spekulace byla potvrzena pokusy *in vivo*, kdy byla provedena infúze erytrocytů preinkubovaných s MPO, což vedlo ke zvýšení lokální koncentrace MPO v periferních tkáních včetně jater, sleziny, plic a srdeční tkáně. Celkově lze shrnout, že se MPO váže na membránu erytrocytů a na rozdíl od jiných buněk není internalizována. Erytrocyty tak mohou sloužit jako přenašeč MPO do

vzdálených míst v periferní cirkulaci, kde MPO následně ovlivňuje funkci endotelu a přispívá k systémové vaskulární rezistenci.

Celkově lze tedy říct, že MPO interaguje s povrchem krevních buněk a cévním endotelem, čímž významně zvyšuje možnost vzájemné interakce díky redukci repulse mezi těmito krevními komponentami díky původnímu negativnímu náboji (obrázek č. 6). Tím se zvyšuje pravděpodobnost interakce leukocytů s cévním endotelem a následnou extravazaci do periferní tkáně. Je zajímavé, že tyto efekty jsou nezávislé na katalytické aktivitě MPO a jsou naopak dány silně kationickým nábojem molekuly MPO a následnou změnou elektrostatického náboje povrchu krevních buněk a endotelu. Kromě tohoto vlivu na přímou interakci mezi buňkami byla prokázána také řada dalších efektů MPO spojených s její katalytickou aktivitou, zejména katabolismus NO přispívající k rozvoji a patologickým jevům kardiovaskulárních onemocnění.



Obrázek č. 6: Schématický přehled vazby MPO na komponenty cév a krevní buňky a naznačené dopady této vazby pro patologické procesy v cévě.

Závěr

PMN jsou považovány za první linii obrany organismu proti útoku patogenů, protože jsou prvními imunitní buňkami, které se akumulují v místě zánětu a jsou vybaveny arzenálem mikrobicidních mechanismů zajišťující odstranění patogenů. Je však třeba si uvědomit, že i když PMN jsou užitečné v imunitní odpovědi, jejich nadměrná aktivace může také vést k uvolnění potenciálně škodlivých molekul, které poškozují vlastní buňky a tkáně. Může také docházet ke vzniku antigenům indukujícím progres autoimunitních onemocnění. Proto, i když je zřejmé, že PMN jsou důležité v obraně organismu, musí být také považovány za potenciálně nebezpečné, a jako terapeutický cíl v léčbě zánětlivých onemocnění. Zásadní otázkou tedy je, jaký je potenciál pro terapeutické cílení funkcí PMN? Může být inhibice funkcí nebo naopak podpora funkcí a extravazace PMN použita běžně v klinické praxi? Jasná odpověď na tyto otázky není doposud dostatečně objasněna. I když naše znalosti o významu PMN při zánětlivých procesech se v posledních letech značně zvýšily, prospěšné a škodlivé příspěvky PMN do tohoto složitého procesu zůstávají sporné.

V našich studiích jsme přispěli k objasnění vztahu mezi částečnou a kompletní aktivací PMN polysacharidovými strukturami interagujícími s PPRs. Tato aktivace může přispívat k popsaným imunostimulačním efektům těchto polysacharidů *in vivo*, kdy jejich podávání zvyšuje obranyschopnost organismu proti infekcím. Naproti tomu negativní aktivace PMN spojená s rozvojem poškozujícího akutního a chronického zánětlivého procesu byla studována v našich pracích, kdy k intenzivní aktivaci PMN docházelo v reakci na rozsáhlé operační výkony spojené s transplantacemi orgánů či s využitím mimotělních systémů pro filtraci a okysličování krve, ať už u pacientů během otevřených operací srdce nebo hemodialýzy. V našich studiích jsme ukázali, že protichůdné pozitivní a negativní funkce PMN během zánětlivé odpovědi jsou regulovány řadou faktorů, včetně typu stimulu a generování prozánětlivých cytokinů a chemoatraktantů, které regulují intenzitu extravazace PMN, interakce PMN s jinými imunitními buňkami, které modulují naopak funkce PMN, včetně regulace odstranění aktivovaných PMN a indukci hojení. Tyto studie jasně ukázaly, že inhibice aktivace PMN u těchto patologických situací by mohla být jako velmi účinný cíl podpůrných terapií těchto procesů. Lepší pochopení této komplexní souhry může poskytnout nové cesty pro manipulaci funkce PMN v léčbě patologických zánětů.

V dalších studiích jsme se zaměřili na problematiku MPO jako jednoho z nejabundatnějších enzymů PMN uvolňovaného během aktivace PMN. Tvorba RKM katalyzovaná MPO přispívá nejen k imunitní obraně, ale může mít značný dopad na podporu zánětlivých procesů. MPO může přispívat k poškození tkáně, ke kterým dochází během zánětlivých stavů, včetně patologických procesů kardiovaskulárního systému. Naše studie ukázaly, že nejen schopnost MPO katalyzovat tvorbu RKM a dusíkových meziproduktů, ale také její efekty nezávislé na katalytické aktivitě, které ovlivňují aktivační stav PMN, endoteliálních buněk i krevních destiček. Bylo prokázáno, že MPO zvyšuje extravazaci PMN během zánětu do periferních tkání. Celkově bylo popsáno několik scénářů podporujících prozánětlivou

roli MPO. Naše *in vivo* studie spolu se studiemi jiných autorů ukázala, že MPO se může účastnit řady událostí zapojených do iniciace, propagace i následným komplikacím v patologii kardiovaskulárních onemocnění. Díky tomu MPO v současnosti představuje atraktivní cíl pro rozvoj prognostických biomarkerů a terapeutických zásahů nejen v léčbě kardiovaskulárních nemocí, ale i dalších onemocnění spojených s patologickými procesy indukovanými zánětem.

Obecně, léčba chronického zánětlivého onemocnění má za odstranit přetrvávající zánět a obnovit funkce tkání. Současné farmakologické strategie jsou založeny na inhibici endogenních faktorů, které se podílejí na normální fyziologii, a proto mohou vyvolat nerovnováhu homeostázy a nežádoucích vedlejších účinků. Ačkoli PMN a jimi uvolňované enzymy včetně MPO jsou obecně rozpoznávány jako mediátory zodpovědné za poranění přilehlých zdravých tkání v blízkosti místa poškození tkáně, je stále více zřejmé, že PMN a jimi uvolňované enzymy včetně MPO hrají rozhodující roli při spuštění hojících procesů a útlumu zánětu. Pochopení toho, jak a proč PMN a jejich mediátory ovlivňují jiné typy buněk je proto velmi důležitá. Další výzkum musí objasnit, jak se PMN chovají v komplexu překrývajících se signálů, které jsou jak ve sterilním zánětlivé prostředí, tak v přítomnosti infekčních mikroorganismů. Širokospektrá farmaka, jako jsou glukokortikoidy a nesteroidní protizánětlivé látky, jsou dnes nejrozšířenější protizánětlivé léčiva buď nemají vliv na funkce PMN, nebo mohou ovlivnit činnosti PMN potenciálně škodlivým způsobem. Pochopením mechanismů, které řídí chování PMN v místech sterilního poškození, může pomoci identifikovat nové možné terapeutické cíle, které by umožnily tlumit zánětlivé reakce, aniž by se snížil potenciál PMN indukovat procesy hojení, které jsou odpovědné za vrácení poškozené tkáně k homeostáze. Tyto znalosti by mohly být použity k řešení komplexních zánětlivých patologických stavů, kterých se PMN účastní jako v případech ischemie, infarktu myokardu, traumatech či toxinem indukovaného poškození jater. Další studie rovněž určí, zda je možné modulovat zánět v reakci na sterilní poranění, aniž by zasahoval do reakce hostitele vůči patogenům. Doufejme, že v nadcházejících letech bude další vývoj v této oblasti.

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The effect of (1→3)-β-D-glucans, carboxymethylglucan and schizophyllan on human leukocytes in vitro

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Abstract

(1→3)-β-D-glucans are known as potent inductors of humoral and cell-mediated immunity in humans and animals. (1→3)-β-D-glucans isolated from various sources differ in their chemical structure and physical parameters and consequently in their immunomodulatory potential. In this study the immunomodulatory activity of two (1→3)-β-D-glucans schizophyllan (SPG) and carboxymethylglucan (CMG) was determined and compared on human blood leukocytes in vitro. Both SPG and CMG activated blood phagocytes and lymphocytes as demonstrated by increased whole blood production of reactive oxygen species, by increased production of pro-inflammatory cytokines IL-6, IL-8, and TNF-α, by increased surface expression of CD69 on lymphocytes, and by altered expression of CD11b and CD62L on polymorphonuclear leukocytes and monocytes. SPG demonstrated a significantly higher potential to stimulate blood phagocytes and production of selected pro-inflammatory cytokines than CMG. The higher potency of SPG to stimulate human blood phagocytes in vitro could be caused by factors such as higher branching frequencies or neutral polymer charge of SPG or different conformation in solution if compared with CMG.

Keywords: Carboxymethylglucan; Schizophyllan; Lymphocytes; Phagocytes

1. Introduction

Glucans are (1→3)-β-linked glucose polymers that are produced as cell wall constituents of fungi, algae, lichens and plants. These glucose polymers can exist as a non-branched (1→3)-β-linked backbone or as a (1→3)-β-linked backbone with (1→6)-β-branches.[1] (1→3)-β-D-glucans exhibit various immunomodulating activities such as decreasing infectious complications and inhibiting tumour growth.[2–5] The enhancement or potentiation of host defence mechanisms has been suggested as a possible means of (1→3)-β-D-glucans tumour growth inhibition rather than direct cytotoxic effects on tumour cells.[5,6]

Schizophyllan (SPG) is one of the (1→3)-β-D-glucans, which is known to have immunomodulating potential and antitumour activity.[2,3,5] SPG is now clinically used as an immunopotentiator against some types of cancer or leukocytopenia. Another (1→3)-β-D-glucan, carboxymethylglucan (CMG), was also proved to be a potent immunomodulator and an agent enhancing hematopoiesis.[7–9]

In contrast to the proved considerable immunostimulatory effect of (1→3)-β-D-glucans, the cellular and molecular mechanisms by which (1→3)-β-D-glucans affect immunological functions have yet not been clearly defined.[10] The first step in the modulation of cellular activity by (1→3)-β-D-glucans seems to be binding to specific cell surface receptor on polymorphonuclear leukocytes (PMNL), macrophages or lymphocytes.[1,11] However, the structural variability of polysaccharides obtained from various natural sources profoundly influences their biological activity.[10,12] Many authors

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suggest that parameters, such as primary structure—degree of branching, solution conformation, solubility, molecular weight and/or polymer charge may play a role in determination of (1→3)-β-D-glucans activity and strongly modulate immune functions.[1,3,5,11,13] However, the relationships between the structure of (1→3)-β-D-glucans and their stimulatory activities are still controversial and investigations of immunological potential of (1→3)-β-D-glucans with different structures are needed.[4,10,14–16]

In the present study, we investigated and compared the immunomodulatory effects of two different polysaccharides in vitro: SPG isolated from *Schizophyllum communae* and CMG prepared from *Saccharomyces cerevisiae* cell wall.

2. Results

2.1. Blood phagocyte production of reactive oxygen species (ROS)

Both SPG and CMG induced an increase in spontaneous chemiluminescence (CL) as well as *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated CL in comparison with untreated control (Table 1). However, SPG enhanced both spontaneous and FMLP-activated CL response significantly more than CMG. Phorbol myristate acetate (PMA)-activated CL was significantly increased only by SPG in comparison with untreated control (Table 1).

2.2. Expression of CD11b and CD62L on PMNL and monocytes

SPG and CMG significantly increased the expression of CD11b and decreased the expression of CD62L on both PMNL and monocytes when compared with control (Fig. 1). However, SPG increased the expression of CD11b to a significantly higher extent than CMG.

Table 1
The effect of SPG and CMG on the production of ROS by phagocytes measured as a whole blood CL

	Spontaneous CL [RLU *s]	PMA activated CL [RLU *s]	FMLP activated CL [RLU *s]
Control	57.4±0.4	866.9±179.7	97.4±23.3
SPG	140.9±3.5 *	1056.4±122.7 *	231.0±33.3 *
CMG	94.4±13.1 *,+	934.6±79.7	187.7±19.8 *,+

The data represent mean±S.E.M. ($n=10$).
* Statistically significant differences against control.
+ Statistically significant differences between SPG and CMG effects.

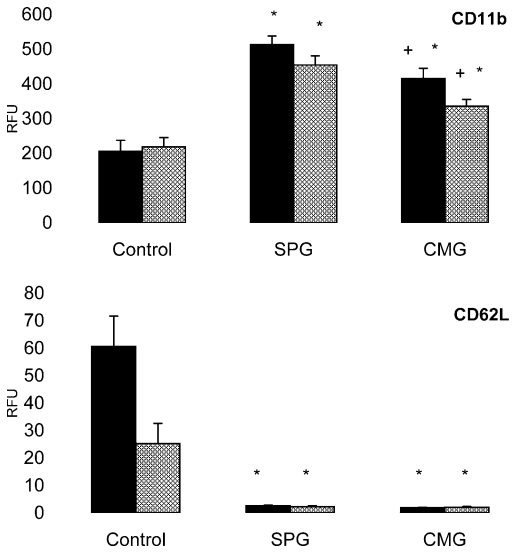

Fig. 1. Expression of CD11b and CD62L on PMNL (black bars) and monocytes (grey bars) incubated with SPG or CMG (100 μg/mL both) or with HBSS as a control for 3 h prior of surface antigen determination. Data are expressed as RFU (mean±S.E.M.; $n=10$). Asterisks indicate statistically significant differences ($P<0.05$) compared with controls. Crosses indicate statistically significant differences ($P<0.05$) comparing the effects of SPG and CMG.

2.3. Expression of surface molecule CD69 on lymphocytes

Both SPG and CMG significantly increased the expression of CD69 on blood lymphocytes when compared with control without significant differences between SPG and CMG (Fig. 2).

2.4. Production of IL-6, IL-8, and TNF-α

Both SPG and CMG significantly increased leukocyte production of IL-6 and IL-8 when compared with

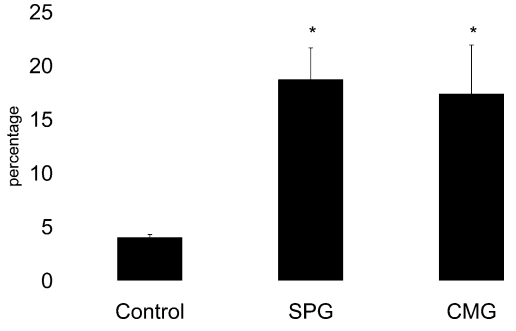

Fig. 2. Expression of surface molecule CD69 on lymphocytes incubated with SPG or CMG (100 μg/mL both) or with HBSS as a control for 3 h prior of surface antigen determination. Data are expressed as % of positive lymphocytes (mean± S.E.M.; $n=10$). Asterisks indicate statistically significant differences ($P<0.05$) compared with controls.

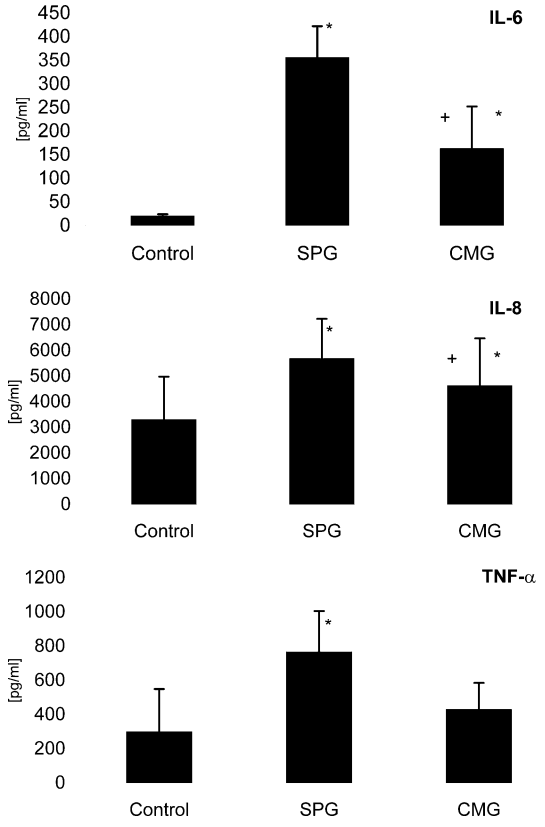

Fig. 3. Production of IL-6 (A), IL-8 (B) and TNF-α (C) by isolated leukocytes incubated with SPG and CMG (100 μg/mL) and HBSS as a control after 18 h. Data are expressed as concentration of cytokines in supernatant at the end of incubation (mean ± S.E.M.; $n = 10$). Asterisks indicate statistically significant differences ($P < 0.05$) compared with controls. Crosses indicate statistically significant differences ($P < 0.05$) comparing the effects of SPG and CMG.

control (Fig. 3a and 3b). However, the production of IL-6 induced by SPG was significantly higher than the increase induced by CMG. TNF-α production was significantly increased only by SPG when compared with control (Fig. 3c).

3. Discussion

It has been claimed that the most probable mode of (1→3)-β-D-glucans action is through the activation of macrophages, dendritic cells, endothelial cells, neutrophils and monocytes. Activation of lymphocytes and NK cells also plays a key role in (1→3)-β-D-glucan action.[2,10] Polysaccharides cannot penetrate cells due to their large molecular mass, so the first step in the modulation of cellular activity by (1→3)-β-D-glucans is binding to B-cell, T-cell, PMNL and macrophage receptors. However (1→3)-β-D-glucan receptors are not precisely described and they may include multiple glucan binding sites on macrophages, neutrophils and NK-cells.[1,3,11] Complement receptor CR3 (CD11b/CD18), expressed on the surface of neutrophils, monocytes, macrophages and NK-cells has been identified as the key receptor of β-glucans.[17] However, the existence of other non-CR3 receptors has also been reported.[1,18] Binding of (1→3)-β-D-glucans to their receptors stimulates intracellular signalling pathways, which culminate in the activation, translocation and nuclear binding of immunoregulatory and pro-inflammatory transcriptional activator proteins.[18]

Production of free radicals during oxidative burst of blood phagocytes plays one of the major roles in antimicrobial, anti-tumour, and inflammatory response of the human body and is a sensitive marker of phagocyte activation.[19] Further, activation of blood phagocytes, both PMNL and monocytes, is also associated with a rapid increase in CD11b surface expression and a rapid decrease in CD62L surface expression.[20] Lymphocyte activation is linked to a fast rise in CD69 surface expression. Determination of CD69 surface expression is a very early marker of lymphocyte activation and correlates well with the [^{3}H]thymidine incorporation assay.[21] In our study, both tested (1→3)-β-D-glucans activated blood phagocytes (PMNL and monocytes) and also lymphocytes as demonstrated by increased whole blood ROS production, by increased production of selected pro-inflammatory cytokines, and by the quantitative changes of selected surface antigen expression. Production of selected pro-inflammatory cytokines IL-6, IL-8 and TNF-α by human blood leukocytes represents an important factor indicative of an immune system response and is a sensitive marker of leukocyte activation.[22] The capability of SPG to stimulate the synthesis of pro-inflammatory cytokines by various cell lines has already been described by other authors.[10,11,16,23,24]. In our study, the SPG tested demonstrated a significantly higher potential for blood phagocytes stimulation and production of selected pro-inflammatory cytokines in comparison with CMG. In contrast to our findings, Vetvicka et al. did not find regulation of neutrophil CD11b expression or superoxide production by SPG.[6] They suggested that soluble β-glucans prime neutrophils without causing non-specific pro-inflammatory action of neutrophils. However, in contrast to our study they used solubilised zymosan.

The higher potency of SPG than CMG to stimulate human blood phagocytes in vitro could be caused by several factors. A number of reports suggest that the bioactivity of (1→3)-β-D-glucans is related to the degree of side chain branching.[1,3,5] The ratio of (1→3)/(1→6)-linkages and the architecture of β-D-glucans networks varies significantly depending on species.[11] We observed that SPG with the higher branching frequency (1/3) exhibited more intensive biological activity than CMG with lower frequency of branching (1/8). In agreement, Williams et al. showed that SPG exhibited a fivefold

increase in affinity for human macrophage (1→3)-β-D-glucan receptor compared to non-branched glucan phosphate.[4] Other authors also concluded that the higher branching frequency might enhance the affinity of (1→3)-β-D-glucans to leukocytes and extend their biological activity.[1,5] However, significant differences between binding of SPG and scleroglucan were observed even though their branching frequency was very similar.[1]

According to various authors biological activity of these polysaccharides is also dependent on their size, so polymers with the greater molecular weight exhibit higher binding affinities and biological activity than lower molecular weight (1→3)-β-D-glucans.[1,3,5] In contrast, Kulicke et al. reported that low molecular mass glucans (around 550,000) increased TNF-α release and superoxide anion release by human blood monocytes more intensively than high molecular mass glucans (over 2,000,000).[14] We observed higher stimulation activity to blood phagocytes with SPG than with CMG regardless of their similar molecular weight.

The observed higher potential of SPG than CMG can be due to their different conformations, triple helix respectively random coil. Solution conformation has been suggested as an important factor for the binding of glucans to their receptors and therefore for their biological activity.[1,3,5,10,14,16] Generally, glucans can exist as a random coil, as a single polymer strand with a helical conformation (single helix) or as a stable complex of three polymer strands forming a triple helix.[4,5,14,15] Contradictory data exist concerning the effect of the specific molecular structure on biological activity of (1→3)-β-D-glucans. There are data describing both lentinan and SPG are active only when they exist in a single helical structure.[25] Similarly the single helix and random structures of curdlan were more active then double- or triple-stranded helices[10] and the single helical conformer of SPG had a higher ability than triple helical conformer of SPG to produce nitric oxide and pro-inflammatory cytokines.[15,16,26] In contrast, Kulicke et al. observed that helical structure was not essential or advantageous for induction of immunological activity.[14] Tsuzuki et al. did not observe dependence of hematopoietic response and IL-6 production in mice in vivo on single or triple helix conformation of SPG.[23] Neither did Suzuki observe a difference in IL-8 production by human leukocytes or in platelets activation in vitro induced by single or triple helix conformation of SPG.[11]

However, interestingly only high molecular weight (1→3)-β-D-glucans appear to form triple helical structures, therefore the molecular weight of tested polysaccharides could play a role in these contradictory observations.[25]

Another important factor influencing the potency of (1→3)-β-D-glucans to bind and consequently stimulate leukocytes is the presence of charged species.[1,5,10] SPG belongs to neutral polysaccharides whereas CMG is a polyelectrolyte glucan. In agreement with our results Mueller et al. showed that neutral polysaccharides exhibit a higher affinity binding than polyelectrolyte glucans.[1] However, in this study these neutral polysaccharides also exhibited higher branching frequency. Kataoka et al. showed that carboxymethylated curdlan was inactive when compared with untreated curdlan.[10]

In conclusion, we have proven that the tested (1→3)-β-D-glucans SPG and CMG exhibited immunostimulatory activity on human blood leukocytes in vitro. The observed higher potency of SPG than CMG to stimulate human blood phagocytes in vitro could be caused by different chemical structure and physical properties of tested (1→3)-β-D-glucans as was discussed above.

4. Experimental

4.1. Reagents

SPG and CMG were prepared in the laboratories of CPN (Czech Republic). SPG was produced as an extracellular product by *Schizophyllum communae* cultured under standard cultivation conditions. All post-fermentation processes were done under a pH between 5 and 6 and room temperature. CMG was isolated from cell walls of *Saccharomyces cerevisiae* by alkali digestion. Obtained β-D-glucans were treated by monochloracetic acid to substitute free carboxyl groups. This modification of chemical structure allowed the extraction of CMG. The presence of endotoxin was tested by Limulus amebocyte lysate (LAL) coatest (Chromogenix, US) and all tested samples did not have the content of endotoxins higher than 500 IU/mg. Basic chemical properties of tested SPG and CMG are summarised in Table 2.

PMA, FMLP, RPMI-1640 medium and gentamycin sulphate were obtained from Sigma-Aldrich (USA). Luminol was obtained from Molecular Probes (USA), Dextran-T500 from Pharmacia (Sweden) and Telebrix N 300 from Leciva (Czech Republic). Heat-inactivated human AB serum was obtained from a local hospital. Lysing solution Cal-Lyse, fluorescein isothiocyanate (FITC)-labelled anti-human CD11b murine monoclonal antibody, phycoerythrin labelled anti-human CD62L murine monoclonal antibody, FITC labelled anti-hu-

Table 2
Basic chemical characteristics of tested (1–3)-β-D-glucans

	SPG	CMG
Degree of branching	1/3	1/8
Polymer charge	neutral	negative
Solution conformation	triple helix	random coil

man CD69 murine monoclonal antibody and appropriate control isotype murine antibodies were purchased from Caltag Laboratories (USA). Phycoerythrin–cyanin 5.1 labelled anti-human CD14 murine monoclonal antibody was purchased from Beckman Coulter (USA). Enzyme-linked immunosorbent assays (ELISA) Modul Sets for human IL-6, IL-8 and TNF-α were obtained from BenderMedSystems (Austria). All other chemicals were purchased in the highest grade p.a. from local distributors or Sigma-Aldrich (USA).

4.2. Blood sampling and leukocyte isolation

Heparinised (50 IU/mL) blood samples were obtained from the cubital vein of ten healthy volunteers after overnight fasting. The number of leukocytes in the blood and their relative differentiation counts were determined using Coulter counter STKS (Coulter, England) and stained blood smears, respectively. Isolation of leukocytes was performed as described previously.[27] Blood was layered in ratio 1:1 over the separation mixture (4% Dextran-T500 in saline and 60% telebrix N 300 in saline in ratio 3.7:1; final density 1.08 g/cm^3). Erythrocytes were removed after 1 h sedimentation at room temperature and leukocytes with plasma were obtained. Then the leukocytes were washed in RPMI-1640 (200 g, 5 min) and resuspended to reach final density 1×10^5 mL in RPMI-1640 supplemented with 10% heat inactivated human AB serum.

4.3. Experimental protocol

Whole blood samples were incubated with SPG and CMG (100 μg/mL) at 37 °C for 1 h prior to the determination of oxidative burst by CL or for 3 h prior determination of expression of surface antigens on PMNL by flow cytometry. The tested concentration of polysaccharides and incubation times were selected based on our previous unpublished results and they are in agreement with literature as the most efficient concentration for in vitro test.[10,11]

Isolated leukocytes were incubated with SPG and CMG (100 μg/mL) in RPMI-1640 medium supplemented with 10% of heat inactivated human AB serum and gentamycin sulphate (45 mg) at 37 °C for 18 h. This time period was chosen based on results of Suzuki et al.[11] Hanks balanced salt solution, pH 7.4 (HBSS) was used instead of polysaccharides as a control. Concentrations of selected cytokines were determined in supernatant at the end of incubation.

4.4. Measurement of oxidative burst of blood phagocytes

Luminol-enhanced CL of whole blood phagocytes was measured using microplate luminometer LM-01T (Immunotech, Czech Republic) as described previously.[28] Briefly, the reaction mixture consisted of 10 μl whole blood, 1 mM luminol (stock solution of 10 mM luminol in 0.2 M borate buffer) and one of the activators (PMA-0.5 μM or FMLP-3 μM). Stock solutions of 10 mM activators were prepared in Me_2SO. Final concentration of Me_2SO did not exceed 0.1%, which was proved not to effect CL reaction. The total reaction volume of 200 μL was adjusted with Hanks balanced salt solution, pH 7.4 (HBSS). The assays were run in duplicates. Spontaneous CL measurements in samples containing 10 μL of whole blood and other substances except any activator were included in each assay. The CL emission expressed as relative light units (RLU) was recorded continuously for 90 min at 37 °C. The integral value of the CL reaction, which represents the total ROS production by blood phagocytes, was corrected to the number of PMNL.

4.5. Determination of the expression of cell surface molecules

The measurements were performed according to the manufacturer's protocol using unfixed whole blood (Caltag Laboratories, USA) with minor modifications.[20] Briefly, 100 μL of blood were incubated with anti-CD11b and anti-CD62L or anti-CD14 and anti-CD69 monoclonal antibodies at room temperature for 15 min. 100μL of blood incubated with FITC- or PE-conjugated murine immunoglobulins of the same isotype were used as the negative controls. Then the samples were fixed by Cal-lyse and the red blood cells were lysed by distilled water in the case of whole blood. The remaining cells were resuspended in PBS, placed on ice and analysed within 2 h. At least 10,000 PMNL, 1000 monocytes or 10,000 lymphocytes selected on the basis of their typical scattering characteristics and CD14 antigen expression were analysed by flow cytometer FACSCalibur (Becton Dickinson, USA). The geometric mean of relative fluorescence units (RFU) was quantified and corrected to the background fluorescence of the isotype control in the case of CD11b and CD62L determination or percentage of CD69 positive lymphocytes were determined.

4.6. Cytokine determination

The determination of cytokines in supernatant was performed according to the manufacturer's protocol for IL-6 Modul Set, IL-8 Modul Set, and TNFα Modul Set (BenderMedSystems, Austria).

4.7. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM) of ten experiments. Results were analysed by the Student t-test for dependent samples and significances were verified by the non-parametric Wil-

coxon test using software Statistica for Windows 5.0 (Statsoft, USA).

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Soluble glucomannan isolated from *Candida utilis* primes blood phagocytes

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ABSTRACT

It is well documented that the polysaccharide glucomannan (GM), an abundant constituent of the fungal cell wall, in the form of particulate induces strong activation of phagocytes, however, the effects of soluble GM are not known. Activation of phagocyte anti-microbial mechanisms is a crucial part of the innate host defense against invading pathogens. However, under uncontrolled inflammatory conditions they contribute to damage of surrounding tissues. Thus, to prevent these deleterious effects, the activation of phagocytes is a tightly regulated process. Therefore, in this study we analyzed the effect of soluble GM on some neutrophil functions such as reactive oxygen species production, degranulation, and receptor mobilization at the plasma membrane. Soluble GM at the tested concentrations did not stimulate oxidative burst of phagocytes directly but significantly potentiated oxidative burst in response to opsonized zymosan particles. GM induced significant phosphorylation of p47phox subunit of NADPH oxidase on Ser345. This priming effect of GM was accompanied by time and concentration dependent degranulation characterized by increased surface expression of receptors stored in neutrophil granules (CD10, CD11b, CD14, CD35, and CD66b). Degranulation was further confirmed by increase of elastase activity in media. Thus, it could be suggested that soluble GM induces priming of phagocytes connected with their degranulation, the increase of surface receptor expression, and potentiation of oxidative burst response to opsonized particles through the activation of NADPH oxidase.

1. Introduction

Polysaccharides such as glucans and glucomannans (GMs), which are major structural components of fungal cell walls, are known to be potent activators of leukocytes including human blood phagocytes.[1–4] These polysaccharides have been shown to stimulate various facets of immune responsiveness in humans, including anti-infective activities against fungal, bacterial, viral, and protozoal infections. Many studies suggest that the activity and immunostimulatory potential of microbial polysaccharides depend on structural parameters such as primary structure, degree of branching, solution conformation, solubility, and molecular weight.[1–5] Thus, polysaccharides obtained from various microbial sources and by diverse isolation procedures significantly differ in their biological activity. The widely exploited insoluble particles isolated from *Saccharomyces cerevisiae* called zymosan, composed of β-glucans and GM, induce intensive oxidative burst of phagocytes accompanied by intensive degranulation.[1–5] Interestingly, in contrast to numerous studies describing effects of particular GM (e.g., zymosan particles) on leukocytes, only limited information exists about the effects of soluble forms of β-glucans and particularly GM on phagocyte activation, and specifically induction of respiratory burst of neutrophils. To the best of our knowledge there are no reports about the effects of soluble GM on the physiological response of human blood phagocytes.

Phagocytes, including polymorphonuclear leukocytes (PMNLs) and monocytes, play a key role in host defense against invading pathogens and play a crucial role in inflammatory processes.[6,7] In response to a variety of stimuli, phagocytes degranulate their secretory vesicles and release large quantities of reactive oxygen species (ROS) in a phenomenon described as the respiratory burst.[6,8] Oxidative burst is primarily characterized by the production of superoxide anion, which gives rise to other forms of ROS.[6] The production of superoxide anion during respiratory burst is

Abbreviations: CL, luminol-enhanced chemiluminescence; GM, glucomannan; GM-CSF, granulocyte–macrophage colony-stimulating factor; HBSS, Hank's buffered salt solution; NADPH, nicotinamide adenine dinucleotide phosphate; OD, optical density; OZP, opsonized zymosan particles; PGG-glucan, poly-β1-glucotriosyl-β1-3-pyranose glucan; PMNL, polymorphonuclear leukocytes; PBS, phosphate buffer solution; RLU, relative light units; RFU, relative fluorescence unit; ROS, reactive oxygen species; SEM, standard error of mean; TNF-α, tumor necrosis factor α.

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dependent on the activation of NADPH oxidase, a membrane bound multicomponent enzyme. Upon NADPH oxidase activation, its components, p47phox, p67phox, p40phox, and p22phox, become phosphorylated and translocate with other components to the membrane where they associate with the membrane-bound component (flavocytochrome b558) to assemble the catalytically active oxidase.[8–10]

Because oxidants produced by NADPH oxidase are highly toxic not only for infectious agents but also for neighboring host tissues, tight regulation of the enzyme complex is necessary to control their production.[6] Interestingly, some agents do not directly induce strong activation of phagocytes, but instead induce the so-called 'priming' of phagocytes accompanied with only limited degranulation, however, with significant potentiation of oxidative burst in response to consequent stimulation with other activators.[7–9,11] Thus, these priming agents including pro-inflammatory cytokines granulocyte–macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor α (TNF-α) are known to induce a weak ROS production by neutrophils but they strongly enhance ROS formation on exposure of phagocytes to a second, activating stimulus.[8–10,12–14] Priming of phagocytes is accompanied by limited activation of NADPH oxidase, translocation of its cytosolic components to membrane and their assembly. Interestingly, the importance of phosphorylation status of NADPH oxidase subunits in phagocyte priming was suggested by various authors.[8,13–15] Recently, a phosphorylation of Ser345 on p47phox NADPH oxidase subunit was shown to be directly related to GM-CSF- and TNF-α-induced priming of oxidative burst.[12]

Primed neutrophils are characterized not only by increased ROS production in response to secondary stimuli but also by increased degranulation of phagocyte granules and increased surface expression of receptors including complement receptors 1 and 3 (CR1 and CR3), fMetLeuPhe (fMLP) receptors, and alkaline phoshatase.[7,10,5,16]

Herein, effects of soluble GM isolated from *Candida utilis* on human blood phagocyte respiratory burst together with a degranulation of phagocytes and an activation of NADPH oxidase subunit p47phox were evaluated. The soluble form of GM-induced degranulation of phagocytes and phosphorylation of p47phox, however, without significant induction of respiratory burst. The demonstration that GM augmented oxidative burst separately from direct induction of overwhelming ROS production by phagocytes significantly contribute to characterization of mechanism of GM action.

2. Results

2.1. Soluble GM did not induce significant oxidative burst of phagocytes, however, but rather augmented oxidative burst of phagocytes activated by OZP

To evaluate a potential of soluble GM to activate human phagocytes, determination of ROS production was employed. Interestingly, soluble GM in any tested concentrations did not stimulate ROS production as was determined by whole blood CL (Fig. 1A). Further, pre-incubation of whole blood with GM (0.1, 0.5, and 1 mg/mL) for 1 h and consequent determination of ROS production by CL did not reveal any increase of CL by GM either (data not shown). In contrast, OZP-activated ROS production determined by whole blood CL was dose-dependently potentiated by pre-incubation of blood with GM for 1 h (Fig. 1B).

2.2. Soluble GM induces degranulation of human phagocytes

Release of neutrophil granules is a characteristic feature of phagocyte priming and activation. The inner side of vesicle membranes expresses antigens and receptors, which, upon a phagocyte activation and degranulation, appear on the surface of the phagocyte. Thus, determination of increases of surface expression of antigens presented in granules was used as a marker of degranulation of particular vesicles. Interestingly, GM increased relative expression of CD10, CD14, and CD35, all representatives for secretory vesicles (Fig. 2A–C). Relative expression of CD11b, which was described to be present in membranes of secretory, gelatinase, and specific granules, was also significantly increased by GM with a notable dose dependent effect (Fig. 2D). Finally, GM increased dose dependently relative to the expression of CD66b, a receptor presented in specific granules (Fig. 2E). The significance of soluble GM-induced degranulation of PMNL was further confirmed by the observed GM-induced release of elastase from PMNL. Elastase enzymatic activity was significantly higher in supernatant of isolated PMNL incubated in the presence of GM (1 mg/mL) for 2 h and 6 h (control sample 0.109 ± 0.003 vs GM sample 0.173 ± 0.019 after 2 h and control 0.116 ± 0.021 vs GM sample 0.209 ± 0.041 after 6 h, both $p < 0.05$).

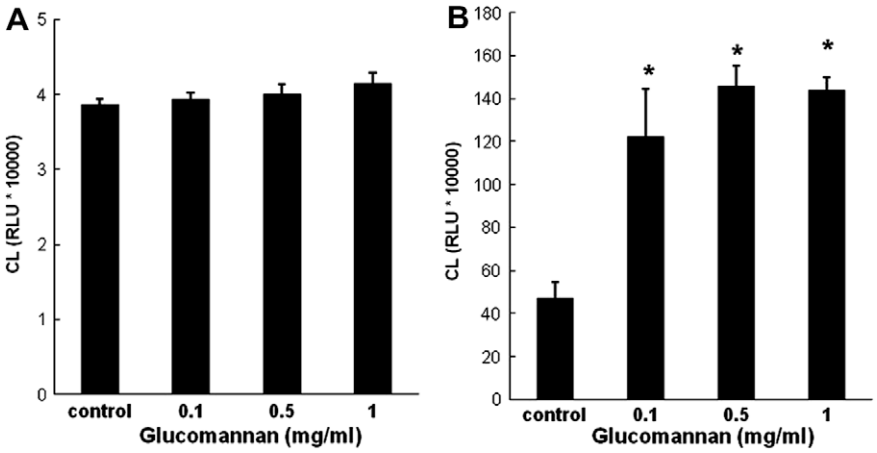

Figure 1. GM did not directly stimulate ROS production by phagocytes, however, potentiated the OZP stimulated production of ROS. (A) CL detection of ROS production by whole blood incubated with GM (0.1, 0.5, or 1 mg/mL) during the time course of the measurement (90 min). (B) CL detection of OZP stimulated ROS production by whole blood pre-incubated with GM (0.1, 0.5, and 1 mg/mL) for 1 h. Data are expressed as mean ± SEM ($n = 8$). Asterisks indicate statistically significant differences ($P < 0.05$) compared to control without GM.

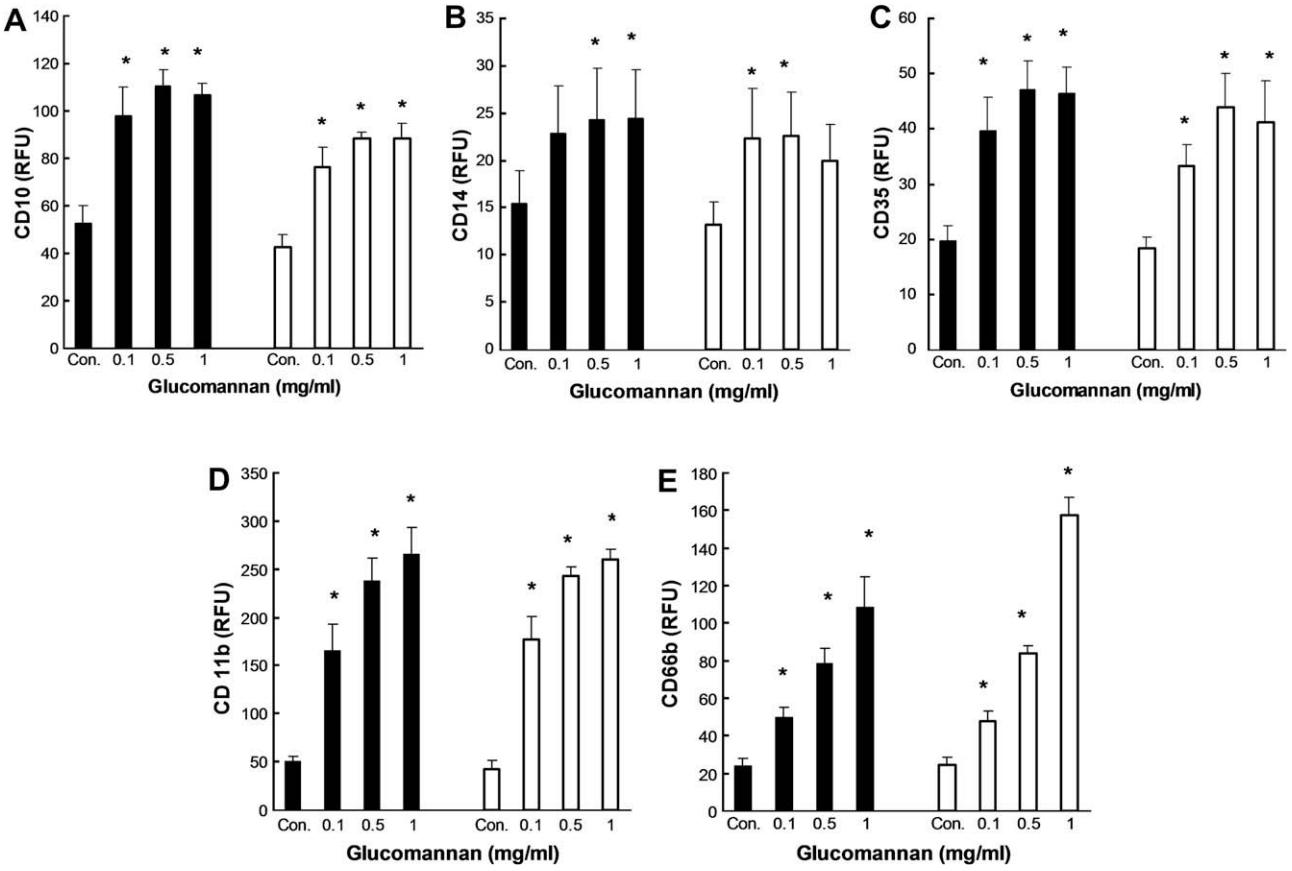

Figure 2. GM increased cell surface expression of receptors primarily expressed on inner surface of phagocyte vesicles. Incubation of whole blood with GM (0.1, 0.5, and 1 mg/mL) for 30 min (full bars) and 120 min (open bars) increased surface expression of CD66b (A), CD10 (B), CD35 (C), CD11b (D), and CD14 (E) determined by flow cytometer. Data are expressed as mean ± SEM (n = 5). Asterisks indicate statistically significant differences (P <0.05) compared to control without GM.

2.3. GM induced phosphorylation of Ser 345 of p47 phox NADH subunit

Another typical feature of neutrophil priming is the activation of NADPH oxidase subunits, particularly phosphorylation of NADPH oxidase subunit p47phox on Ser 345. GM induced significant phosphorylation of p47phox in leukocytes as was determined in leukocytes isolated from fixed whole blood samples incubated for different times with GM (1 mg/mL) (Fig. 3A). Similarly, GM induced significant phosphorylation of p47phox in isolated PMNL incubated with GM for 15 min as depicted in Figure 3B.

2.4. GM induced activation of Rac2

To explore the activation status of NADPH oxidase, an activation of small GTPase Rac2 crucial for NADPH oxidase activity was analyzed. GM induced significant Rac2 activation in isolated PMNL incubated with GM (1 mg/mL) for 10 min (Fig. 4). Thus, these data suggest induction of NADPH oxidase activation status by soluble GM in human PMNL.

3. Discussion

Soluble GM isolated from *C. utilis* induced degranulation of human blood phagocytes, however, without significant stimulation of phagocyte ROS production. Although, GM potentiated ROS production induced by secondary applied activator OZP. This priming effect of GM was connected with phosphorylation of NADPH oxidase subunit p47phox.

Numerous reports exist on the general role of polysaccharides on activation of innate immunity.[1–5] However, only limited information exists on the effects of soluble forms of β-glucan polysaccharides on phagocytes and to our knowledge there are no reports regarding biological effects of soluble GM. Present observations have expanded critical linkages between the activity of soluble forms of polysaccharides with the β-glucan backbone and phagocyte activity by revealing that soluble GM (a) induces increase PMNL surface expression of receptors crucial for response to pathogens and contact with endothelium, (b) does not directly induce ROS production, (c) significantly potentiates ROS production by blood phagocytes in response to opsonized particles.

The importance of polysaccharide chemical and physical properties on their biological activity has been suggested by many authors.[1–5] In general, a particular or high molecular weight glucan was mostly revealed to activate leukocytes directly, stimulating their phagocytic, cytotoxic and anti-microbial activities, including oxidative burst, as well as the production of pro-inflammatory mediators, cytokines, and chemokines. In contrast, the effects of low molecular weight and soluble β-glucans are less clear. Data presented herein suggest soluble β-glucan fails to activate full phagocyte antimicrobial response directly, but may modulate the response to another challenge. In agreement with our results, the soluble form of β-glucan poly-β1-glucotriosyl-β1-3-pyranose glucan isolated from *S. cerevisiae* (PGG-glucan) enhanced ROS production by PMNL accompanied by increased antimicrobial activity of PMNL, and the activation of nuclear transcription factors particularly nuclear factor κB and NF-interleukin 6-like factor.[17,18] Further, Reichner and co-workers re-

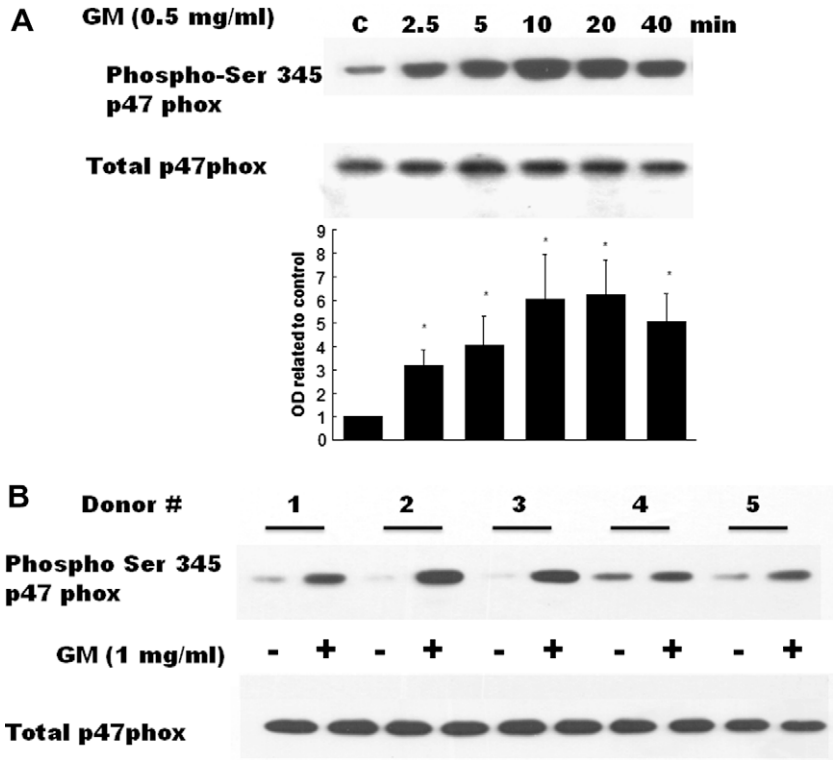

Figure 3. GM induced phosphorylation of NADPH oxidase subunit p47phox on serine 345. (A) Typical WB analysis of phosphorylated (Ser 345) and total p47phox in isolated leukocytes from whole blood incubated with GM (1 mg/mL) for different time. Relative optical density (OD) is related to control samples without GM. Data are expressed as mean ± SEM (n = 5). Asterisks indicate statistically significant differences (P <0.05) compared to control without GM. (B) WB analysis of phosphorylated (Ser 345) and total p47phox in PMNL isolated from 5 donors incubated with GM (1 mg/mL) for 15 min.

ported the ability of this PGG-glucan to increase the chemotactic capacity of human neutrophils in vitro,[19] and to enhance migration of neutrophils into a site of inflammation and improved antimicrobial function mouse model in vivo.[20] Větvička et al. found that soluble β-glucan from yeasts generated a primed state of complement receptor type 3 on neutrophils connected with increased ability of neutrophils to destroy opsonized target cells.[21,22] Furthermore, soluble β-glucan from *S. cerevisiae* primed production of cytokines by human phagocyte in response to endotoxin.[23] Interestingly, in contrast to our data and a generally observed function of glucans and GM as agents potentiated phagocyte functions, Drabikova et al. reported significant down-regulation of both spontaneous and phorbol myristate acetate activated ROS production by human blood phagocytes in vitro.[24] Furthermore, phagocytes isolated from rats with induced adjuvant arthritis treated by GM revealed significantly lower production of ROS then untreated rats. These effects were suggested to be mediated by antioxidant properties of soluble GM.[24]

The priming response is associated with events such as degranulation and up-regulation of receptors. Changes in surface antigen expression seen in pre-activated phagocytes are due to regulated degranulation, wherein most of the secretory granules and smaller amounts of the gelatinase and specific granules are mobilized to cause the observed changes in the cell surface molecules.[7,11,16] In agreement to data reported herein, soluble β-glucan from *S. cerevisiae* induced degranulation of human blood PMNL.[23] In contrast, in other studies, soluble β-glucan isolated from yeast did not up-regulate neutrophil CR3 expression[21] and PGG-glucan did not increase surface expression either of CR3 and receptors for fMLP and Fcγ.[17]

Several cellular receptors for β-glucan have been suggested including CR3, lactosylceramide, scavenger receptors, murine Dectin-1, and human β-glucan receptor.[1–5,17,21,22,25] In this study, the involvement of a particular receptor responsible for phagocyte response to soluble GM was not characterized. However, based on current literature, it can be speculated that majority of the mentioned receptors recognizing β-glucans expressed by human blood phagocytes can recognize soluble GM from *C. utilis*.

In conclusion, the present study shows that soluble GM is recognized by and interacts with blood PMNL, the key cells of innate immune system in humans, and that soluble GM can up-regulate the function of these phagocytes. Although the effective potentiation of the innate immune system has been studied for a number of years, well-defined pharmacological interventions to improve the function of phagocytes is still lacking.[20] This is because the favorable aspects of phagocyte activation to improve their microbicidal functions are often accompanied by the overproduction of pro-inflammatory mediators. The data reported herein significantly contributes to the important areas of convergence, which have been noted for soluble polysaccharides and potentiation of phagocyte functions. Knowledge gained from this study will help to determine potential uses of soluble GM isolated from *C. utilis* in the clinic.

4. Experimental

4.1. GM preparation

GM was isolated from *C. utilis* at the laboratories of CPN spol. s.r.o.[26] Briefly, GM bound to the cell wall was isolated by sodium hydroxide hydrolysis (2% NaOH, 25 min at 95 °C). HCl was used to adjust pH to 5.5. After the extraction, suspension was cooled and cell debris was discarded. GM was precipitated by isopropanol. The precipitate was dissolved in water (40 °C) and was filtered several times through paper filters and active charcoal filters. Finally

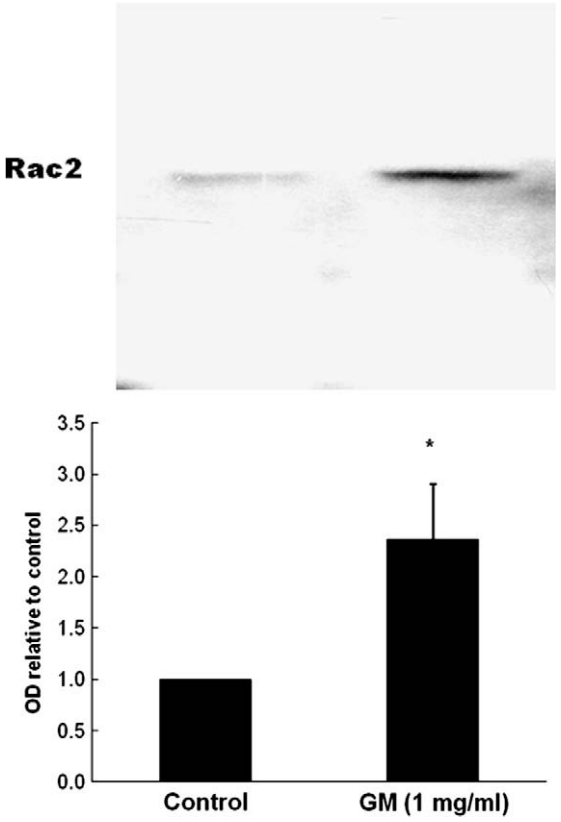

Figure 4. GM induced activation of Rac2. Typical WB analysis of activated Rac2 isolated from PMNL incubated with GM (1 mg/mL) for 10 min. Relative optical density (OD) is related to control samples without GM. Data are expressed as mean ± SEM ($n = 3$). Asterisk indicates statistically significant differences ($P < 0.05$) compared to control without GM.

GM was precipitated from water solution by isopropanol, the precipitate was dissolved in absolute isopropanol and dried under vacuum. GM had a molar mass between 60 and 70 kDa and a narrow molecular distribution (polydispersity index between 1.15 and 1.24) determined by size exclusion chromatography coupled with differential refractive index, ultraviolet, and light scattering detection. The ratio of mannan versus glucan was 2.73:1 in GM isolated from *C. utilis*. The monosaccharide analysis with hydrolyzed samples (2 M trifluoroacetic acid, 100 °C for 4 h) was performed by ion-exchange high performance chromatography (Shimadzu, Japan). Separation was accomplished using water as a mobile phase on Polymer IEX Ca-form (250 × 8 mm I.D., 8 μm) (Watrex, USA) column heated at 80 °C coupled with differential refractive index detection RID-10A (Shimadzu, Japan).[27] The presence of endotoxins was tested using PyroGene® Recombinant Factor C Endotoxin Detection System (Cambrex, USA) which did not detect any significant amount of endotoxin (less than 0.01 EU per mL). For experiments GM was dissolved in PBS or 0.9 % NaCl.

4.2. Human blood sample preparation and PMNL isolation

Heparinized (50 IU/mL) blood samples were obtained from the cubital vein of healthy volunteers with given informed consent. The number of leukocytes in the blood and their relative differentiation counts was determined using Coulter counter STKS (Beckman Coulter, England), and stained blood smears, respectively. PMNLs were isolated as described previously.[28] Blood was layered in a ratio of 1:1 over separation mixture consisting of 4% Dextran-T500 (Amersham Biosciences AB, Sweden) in saline and 60% telebrix N 300 (Leciva, Czech Republic) in saline in a ratio of 3.7:1 and its final density was 1.08 g/cm^3. Erythrocytes were removed after 1 h of sedimentation at room temperature and leukocytes with plasma were obtained. Then, leukocytes were under-layered with Lymphoprep (Fresenius Kabi Norge, Norway) and PMNLs were separated from lymphocytes according to manufacture's instruction by centrifugation 400*g* for 40 min at room temperature. The supernatant with lymphocytes was discarded and PMNLs were washed in phosphate buffer solution (PBS) pH 7.4 and resuspended to reach a final density 1×10^6 cells/mL in Hank's buffered salt solution (HBSS) pH 7.4 containing glucose and divalent cations.

4.3. Chemiluminometric determination of ROS production in whole blood

Luminol-enhanced chemiluminescence (CL) was measured using microplate luminometer LM-01T (Immunotech, Czech Republic).[29–31] Briefly, the reaction mixture consisted of 10 μL of whole blood in total volume 200 μL of HBSS. Just before the start of the measurement 1 mM luminol (stock solution of 10 mM luminol in 0.2 M borate buffer, Molecular Probes, USA) was added together with GM (0.1, 0.5 and 1 mg/mL) and opsonized zymosan particles (OZP—0.1 mg/mL, Sigma–Aldrich, USA). Further, CL of samples pre-incubated with GM (0.1, 0.5 and 1 mg/mL) for 1 h prior to the addition of other reagents was evaluated. Untreated samples without GM and samples without activators (spontaneous ROS production) were evaluated as controls. The samples were run in duplicates and the CL emission expressed as relative light units (RLUs) was recorded continuously for 90 min at 37 °C.

4.4. PMNL surface expression of receptors

Whole blood was diluted 1:1 with pre-warmed HBSS and incubated with GM in the final concentration of 0.1, 0.5 and 1 mg/mL at 37 °C for 30 and 120 min.[32] For control samples, PBS was used instead of GM. Further, samples were incubated with mouse anti-CD66-FITC, mouse anti-CD68-PE (BD Biosciences, USA), mouse anti-CD10-PE (clone SN5c/L4-1A1), mouse anti-CD35-FITC (clone E11), mouse anti-CD11b-FITC (clone ICRF44) (all 1:50, Ancell Corporation, USA), anti-CD14-PC5.1 (Immunotech, France), or appropriate isotype controls (BD Biosciences and Ancell Corporation, USA) for 15 min at room temperature followed by fixation of samples by 3.7% formaldehyde in PBS for 15 min at room temperature. Red blood cells were lysed using distilled water for consequent 20 min at rt. After centrifugation (300*g*, 7 min, rt) the remaining cells were resuspended in cold PBS and kept on ice until assessment of fluorescence by flow cytometer FACSCalibur (BD Biosciences). Ten thousand blood granulocytes selected on the basis of their typical scattering characteristics were analyzed. The geometric mean of relative fluorescence unit (RFU) was quantified.

4.5. Determination of elastase release

Elastase-dependent activity in supernatants of samples incubated with tested compounds was determined by measuring the rate of degradation of specific substrate *N*-succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide. Supernatants (100 μL) were mixed with 100 μL TRIS–HCl buffer (pH 8) containing 1.2 mM *N*-succinyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide in a 96-well microtitre plate and were incubated in dark for 30 min at 37 °C. The change of optical density (OD) was monitored at 405 nm on platelet reader Spectra Rainbow reader (Tecan, Austria). Rates are expressed as a change in OD at 405 nm/30 min.

4.6. Detection of p47 phox and its phosphorylated form

Whole blood or isolated PMNLs were incubated with GM in the final concentration of 0.1, 0.5 and 1 mg/mL for various times at 37 °C. Samples without GM were used as a control sample. After the incubation the blood samples were fixed by 3.7% formaldehyde dissolved in PBS for 15 min at room temperature. Red blood cells were lysed using distilled water for consequent 15 min. The isolated leukocytes were washed in PBS. Fixed isolated leukocytes or PMNLs just at the end of incubation without fixation in formaldehyde were lysed in 300 μL of lysis buffer (50 mM TRIS–HCl pH 7.5, 20% glycerol, 1% sodium dodecyl sulfate, and protease inhibitor cocktail (Complete, Roche, Germany) and phosphatase inhibitor cocktail (Phosphostop, Roche). Next, 60 μL of loading buffer (100 mM TRIS–HCl pH 6.8, 2% sodium dodecyl sulfate, 0.02% bromphenol blue, 20% glycerol, 2% b-mercaptoethanol) were added. Samples were boiled for 10 min and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% gels using established procedures. Proteins separated by one-dimensional electrophoresis were electro-transferred using a Mini Trans Blot System (Bio-Rad Laboratories, USA) onto PVDF membranes (Millipore Corp.; Bedford, MA). Membranes were blocked in TBS (20 mM TRIS–HCl pH 7.6, 150 mM NaCl) containing 0.1% Tween 20 and 5% non-fat milk for 1 h. The blots were then washed three times with TBS–Tween, and incubated with anti-phospho-Ser345 p47 phox antibody[12] or anti-p47phox (total p47phox—mouse anti-p47phox from Santa Cruz Biochemicals, USA) antibody (both 1:3000) overnight at 4 °C. The membranes were washed three times with TBS–Tween and then incubated with horseradish peroxidase-labeled anti-rabbit antibody (1:4000) (ECL Anti-Rabbit IgG, HRP-Linked Whole Ab, Amersham, USA) or anti-mouse antibody (1:1000) (Anti-mouse IgG, HRP-Linked Whole Ab, R&D systems, USA) for 1 h. The secondary antibodies were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, USA) and detected autoradiography using on radiographic films (Agfa, Belgium). The levels of each protein were quantified by scanning densitometry using the ImageJ™ (NIH, USA) and the individual band density value was expressed in arbitrary units.

4.7. Detection of Rac2 activation

Isolated PMNLs were incubated with GM (final concentration of 1 mg/mL) for 10 min at rt. The suspension was aliquoted, PMNLs were washed, snap-frozen, and stored at −70 °C. Rac2 activation was subsequently determined using Rac2 activation assay kits (Upstate Biotechnology, USA) according to the manufacturer's protocol. Shortly, the PMNLs were lysed on ice, precleared and cell lysates were incubated with 10 μg of PAK-1 PBD agarose to bind Rac-GTP and the proteins bound to PAK-1 PBD were separated by SDS–PAGE, transferred to PVDF membrane and probed with anti-Rac2 (1:10,000 dilution). Proteins were visualized and analyzed similarly as described above.

4.8. Statistical analysis

Data were analyzed by Student's two-tailed *t* test using Statistica software (StatSoft, Tulsa, OK) for comparing the differences among the groups. All data are reported as means ± standard error of mean (SEM). A *p* value of less than 0.05 was considered significant.

Acknowledgments

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IL-10 Does Not Affect Oxidative Burst and Expression of Selected Surface Antigen on Human Blood Phagocytes in vitro

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Summary

Cytokines play a major role in the control of inflammatory responses, participate in the regulation of blood phagocyte activities and as such are used for immunomodulatory therapy. In the present study, the influence of IL-10 on human blood phagocyte activity in the presence/absence of IL-6, IL-8 and TNF-α was tested *in vitro*. Our research analyzed the effects of cytokines on the production of reactive oxygen species measured by chemiluminescence and flow cytometry, and on the expression of surface molecules (CD11b, CD15, CD62L, CD31) measured by flow cytometry. IL-10 had no inhibitory effect on reactive oxygen species production and the expression of any examined adhesion molecule by resting or stimulated blood phagocytes within 3 h of incubation. Conversely, TNF-α, IL-6, and IL-8 increased reactive oxygen species production and the expression of CD11b and CD15 on both neutrophils and monocytes and decreased the expression of CD62L. These priming effects of the tested pro-inflammatory cytokines were not affected by IL-10. The obtained results suggest that IL-10 does not directly control blood phagocyte activation. These results also provide better information about the contribution of IL-6, IL-8 and TNF-α to the regulation of blood phagocyte-mediated inflammatory processes.

Key words
Interleukins • TNF-α • Leukocytes • Oxidative burst • Surface molecules

Introduction

Polymorphonuclear leukocytes (PMNL) and monocytes are the most important blood phagocytes that play a key role in the non-specific immune defense of the body. Phagocytosis is accompanied by an oxidative burst, a process in which a significant increase in the production of reactive oxygen species (ROS) occurs (Drábiková *et al.* 2000). However, an overproduction of ROS evokes tissue inflammation and injury (Land *et al.* 1994, Lojek *et al.* 1998, Huber *et al.* 2000, Hamar *et al.* 2003). Cytokines may play a critical role in triggering this systemic inflammatory response. A sharp increase in the plasma concentration of both pro- and anti-inflammatory cytokines was observed during inflammatory pathological states including septic shock (Huber *et al.* 2000), organ transplantation (Lang *et al.* 1996, Wan *et al.* 1997, Kubala *et al.* 2001, 2002) and many others.

IL-10 is referred to as a cytokine with anti-inflammatory activity. It is produced by the CD4+/TH1

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and CD4+/TH2 subsets of lymphocytes, B lymphocytes and macrophages/monocytes (Wakkach *et al.* 2000). IL-10 inhibits the activation and proliferation of T lymphocytes, downregulates the production of pro-inflammatory cytokines and the expression of various cell surface antigens associated with the induction of inflammation (Wakkach *et al.* 2000). IL-10 exerts direct anti-inflammatory and immunosuppressive effects on various cell types (Raychaudhuri *et al.* 2000, Roilides *et al.* 2000). Previously, IL-10 was reported as a potent inhibitor of phagocyte H_2O_2 production (Bogdan *et al.* 1991), nitric oxide synthesis (Gazzinelli *et al.* 1992) and microbicidal activity (Silva *et al.* 1992). These activities of IL-10, together with its capacity to reduce the production of pro-inflammatory cytokines and chemokines, indicate that IL-10 is a potent immunosuppressant *in vitro* (de Vries 1995, Yano *et al.* 1995). Therefore, IL-10 appeared to be a promising agent for the therapeutic treatment of acute inflammations (Huber *et al.* 2000). However, its direct effect on phagocytes is still a matter of discussion. Conversely, cytokines such as IL-6, IL-8 and TNF-α are potent pro-inflammatory mediators with a wide range of biological activities (Pirenne *et al.* 1994). They have been shown to activate blood phagocyte chemotaxis, the expression of various adhesion molecules with consequent adhesion to endothelial cells, infiltration into tissues and the release of ROS, proteolytic enzymes and other bioactive substances (Pirenne *et al.* 1994).

The present study was designed to determine the effect of IL-10 on blood phagocyte activity: ROS production and the expression of surface antigens involved in phagocyte adhesion and phagocytosis, namely the complement receptor 3 (CD11b), Lewis-X (CD15), L-selectin (CD62L) and platelet-endothelial cell adhesion molecule (CD31). The combined effects of IL-10 and pro-inflammatory cytokines (IL-6, IL-8, TNF-α) were also tested at various times of incubation.

Material and Methods

Reagents

Recombinant human (rh) IL-10, IL-6, IL-8, and TNF-α were obtained from R&D Systems (USA). Phorbol myristate acetate (PMA), zymosan A from *saccharomyces cerevisiae* and luminol were obtained from Sigma-Aldrich (USA). Lysing solution Cal-Lyse, fluorescein isothiocyanate (FITC)-labeled anti-human CD62L murine monoclonal antibody, FITC-labeled anti-human CD15 murine monoclonal antibody, phycoerythrin (PE)-labeled anti-human CD11b murine monoclonal antibody, PE-labeled anti-human CD31 murine monoclonal antibody and appropriate control isotype murine antibodies were purchased from Caltag Laboratories (USA). Phycoerythrincyanin 5.1 (PC5)-labeled anti-human CD14 murine monoclonal antibody was purchased from Immunotech (USA). Dihydrorhodamine-123 (DHR-123) was purchased from Molecular Probes (USA). All other chemicals were purchased in the highest grades from local distributors.

Blood sampling and cytokine reconstitution

Heparinized blood samples (50 IU/ml) were obtained from five healthy volunteers having given informed consent. Blood was diluted ten times with Hanks balanced salt solution (HBSS). The recombinant human cytokines were reconstituted to a concentration of 10 μg/ml in HBSS containing 5 % bovine serum albumin. These cytokine solutions were further diluted with HBSS to obtain stock solutions. The final concentration of bovine serum albumin did not exceed 0.001 %.

Experimental protocol

Four experimental protocols were used in this study. Protocol A – diluted blood samples were incubated with various concentrations (400, 800, 1200 and 1600 pg/ml) of IL-10, IL-6, IL-8 or TNF-α at 37 °C for 15 min or 2 h prior to the measurement of ROS production by luminol-enhanced chemiluminescence (CL). Protocol B – blood samples were incubated with 1200 pg/ml of IL-10, IL-6, IL-8 or TNF-α at 37 °C for 2 h prior to the determination of selected surface antigens and intracellular ROS production using a flow cytometer. Protocols C and D were used to test the effects of IL-10 on the pro-inflammatory cytokine induced changes in both total and intracellular ROS production as well as surface antigen expression. Protocol C – blood samples were pre-incubated with IL-10 (1200 pg/ml) for 1 h prior to 15 min or 2 h of incubation with pro-inflammatory cytokines (1200 pg/ml). Protocol D – blood samples were simultaneously incubated with IL-10 (1200 pg/ml) and pro-inflammatory cytokines (1200 pg/ml). HBSS was used instead of cytokines as a control. The viability of leukocytes was determined by trypan blue exclusion after a hypotonic lysis of a part of each sample at the end of the incubations. The leukocyte viability of any sample did not decrease below 95 %.

Measurement of blood phagocyte oxidative burst

Two different methods for ROS detection were used to obtain comprehensive information about the oxidative burst of phagocytes.

Chemiluminescence determination of ROS

Chemiluminescence of the diluted blood was measured using a microplate luminometer LM-01T (Immunotech, Czech Republic). The principle of the method is based on luminol interaction with phagocyte-derived free radicals, which results in large measurable amounts of light. Briefly, the reaction mixture consisted of 100 µl of diluted blood, 1 mM luminol (stock solution of 10 mM luminol in 0.2 M borate buffer) and one of the activators (PMA – 0.4 µM or OZP – 0.25 mg/ml). The total reaction volume of 130 µl was adjusted with HBSS. The assays were run in duplicates. Spontaneous CL measurements in samples containing 100 µl of diluted blood and all other substances, but none of the activators, were included in each assay. The CL emission was followed for 90 min at 37 ^{o}C. The integral value of the CL reaction, which represents the total ROS production by blood phagocytes was evaluated.

Flow cytometric determination of ROS

Diluted blood samples (100 µl) were incubated with 10 µM DHR-123 (stock solution 10 mM DHR in dimethyl sulphoxide) and an anti-CD14 monoclonal antibody (2 µl). The tubes were shaken gently and incubated in the dark at 37 ^{o}C for 20 min. Then either PMA (0.4 µM) or OZP (0.25 mg/ml), or HBSS was added and the cell suspensions were incubated in the dark at 37 ^{o}C for further 20 min. At the end of the incubation period samples were fixed with Cal-lyse. The red blood cells were lysed by distilled water. The remaining cells were resuspended in PBS, placed on ice and analyzed within 2 h on the flow cytometer similarly to the analysis of surface antigens.

Determination of the cell surface expression of adhesion molecules

The measurements were performed according to the manufacturer's protocol (Caltag Laboratories, USA) for unfixed whole blood. Briefly, 100 µl of diluted blood was incubated in plastic tubes (Falcon, USA) with anti-CD11b, anti-CD15, anti-CD62L and anti-CD31 monoclonal antibodies (two antibodies per vial, 5 µl of each). Blood (100 µl) was incubated with FITC- or PE-conjugated murine immunoglobulins of the same isotype were used as the negative controls. Each tube also contained anti-CD14 monoclonal antibody (2 µl) for the discrimination of monocytes. Samples were incubated at room temperature for 15 min and then fixed with Cal-lyse. The red blood cells were lysed with distilled water. The remaining cells were resuspended in PBS, placed on ice and analyzed within 2 h by a flow cytometer FACSCalibur (Becton Dickinson, USA). Blood granulocytes and monocytes were selected on the basis of their typical scattering characteristics and their different expression of CD14 antigen. The median fluorescence intensity (MFI) was determined and corrected for unspecific staining by subtracting the fluorescence of cells stained with the control antibody (isotype control).

Statistical analysis

The results were analyzed by Student t-test for independent or dependent samples and significances were verified by the non-parametric Mann-Whitney U-test or Wilcoxon test using Statistica for Windows 5.0 (Statsoft, USA). The differences of $p<0.05$ were regarded as statistically significant. All values are reported as the means of five different experiments ± S.E.M.

Results

Influence of cytokines on spontaneous ROS production measured by chemiluminescence

No significant differences in the spontaneous ROS production were found among control samples and samples incubated with IL-10, IL-6 or IL-8 at any of the concentrations used either after 15 min or 2 h of incubation (Fig. 1). TNF-α significantly increased the spontaneous ROS production when compared with the controls at all concentrations used after 15 min of incubation (Fig. 1A). The effect of TNF-α after 2 h of incubation was not significant (Fig. 1B).

Influence of cytokines on activated ROS production measured by chemiluminescence

After 15 min of incubation, PMA-activated ROS production was significantly increased by IL-6 at a concentration of 1600 pg/ml and by TNF-α at all tested concentrations when compared with control samples (Fig. 1A). However, IL-6 at concentrations of 800, 1200 and 1600 pg/ml, IL-8 at a concentration of 1600 pg/ml and TNF-α at all tested concentrations significantly increased PMA-activated ROS production after 2 h of incubation (Fig. 1B). OZP-activated ROS production was

significantly increased by IL-6 at a concentration of 1600 pg/ml, by IL-8 at concentrations of 1200 and 1600 pg/ml and by TNF-α at all tested concentrations when compared with the controls after 15 min of incubation (Fig. 1A). On the other hand, OZP-activated ROS production was significantly enhanced by all the tested concentrations of IL-6, IL-8 and TNF-α with the exception of IL-6 and IL-8 at concentrations of 400 pg/ml after 2 h of incubation. IL-10 at concentrations of 1200 and 1600 pg/ml significantly increased OZP-activated ROS production after 2 h of incubation (Fig. 1B).

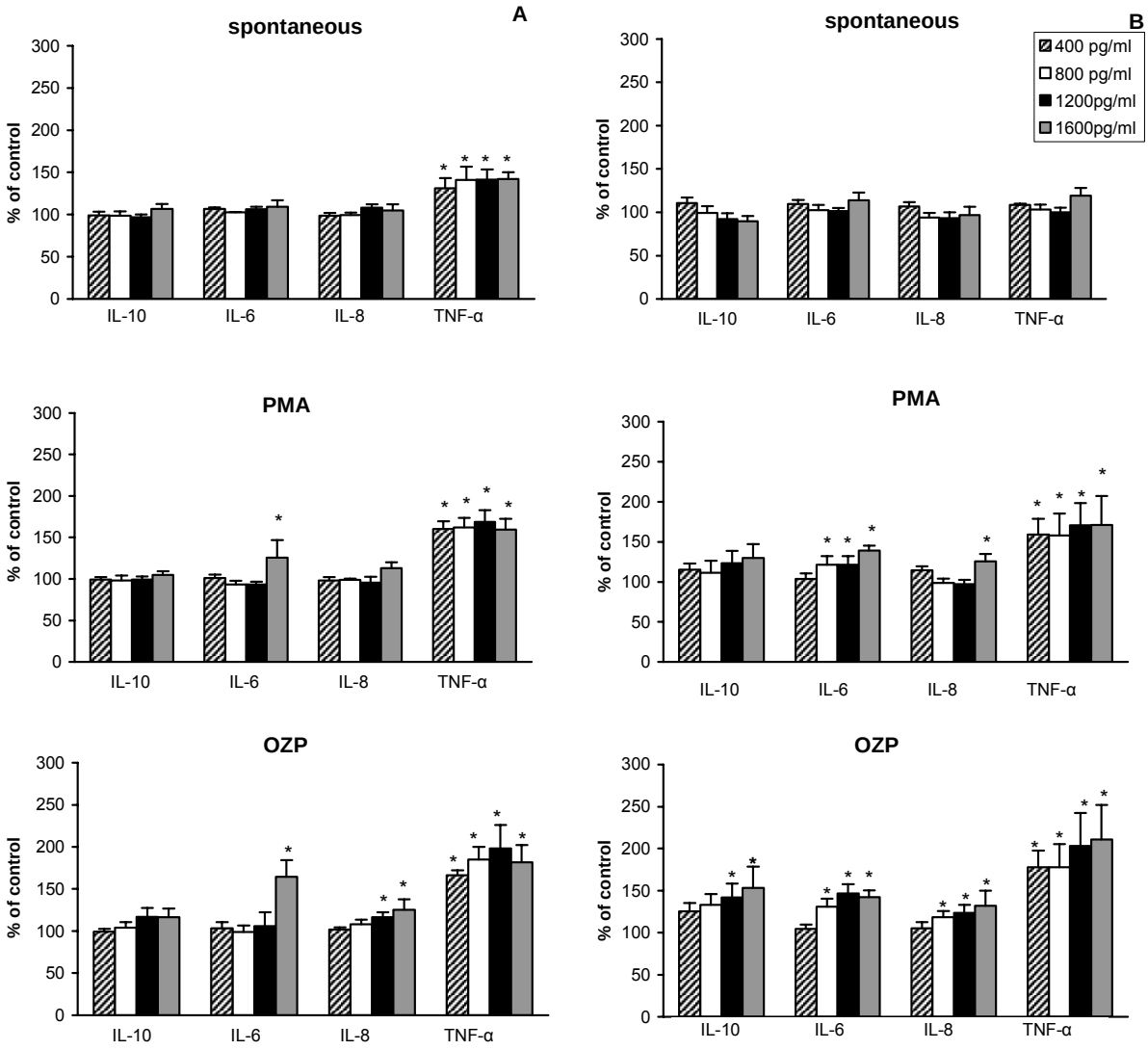

Fig. 1. Effects of IL-10, IL-6, IL-8 and TNF-α at concentrations of 400, 800, 1200 and 1600 pg/ml on spontaneous and PMA- or OZP-activated ROS production by blood phagocytes after 15 min (A) and 2 h (B) of incubation. Results are expressed as percentages of controls. The asterisk indicates significant ($p<0.05$) difference vs. control incubated in the absence of cytokine (n=5).

Influence of cytokines on ROS production measured by DHR-123

IL-10 and IL-8 did not significantly affect either spontaneous or activated intracellular ROS production by neutrophils or monocytes. Incubation of blood samples with IL-6 significantly increased spontaneous and OZP-activated ROS production by neutrophils and PMA-activated ROS production by monocytes (Table 1). TNF-α increased significantly spontaneous and PMA- or OZP-activated ROS production by both neutrophils and monocytes when compared with the controls.

Influence of cytokines on the expression of adhesion molecules

IL-10 did not significantly affect the expression of any of the studied surface molecules (CD11b, CD15,

CD62L and CD31) (Fig. 2). IL-6 and TNF-α induced significant increases in CD11b and CD15 expression and a decrease in CD62L expression on both neutrophils and monocytes when compared with control samples. IL-8 significantly increased the expression of CD11b on neutrophils and CD15 on both neutrophils and monocytes. IL-8 did not affect the expression of CD62L. The expression of CD31 was not significantly affected by any of the tested cytokines.

Table 1. The influence of IL-10 (1200 pg/ml) on spontaneous and PMA- or OZP-activated ROS production by neutrophils and monocytes measured by DHR-123. Blood samples were simultaneously incubated with IL-10 and with IL-6, IL-8 or TNF-α (1200 pg/ml) for 2 h or preincubated with IL-10 for 1 h before a 2 h incubation with IL-6, IL-8 or TNF-α (1200 pg/ml).

		NEUTROPHILS			MONOCYTES		
		Individual cytokines	**Simultaneous incubation with IL-10**	**Pre-incubation with IL-10**	**Individual cytokines**	**Simultaneous incubation with IL-10**	**Pre-incubation with IL-10**
Spontaneous	*IL-10*	101±6	–	–	96±9	–	–
	IL-6	125±11*	114±14	116±4*	119±8*	117±8	118±3*
	IL-8	101±8	108±11	98±3	94±11	103±4	104±7
	TNF-α	135±18*	137±14*	150±27*	145±19*	155±25*	151±18*
	–	–	–	99±11	–	–	97±5
PMA-activated	*IL-10*	109±13	–	–	103±5	–	–
	IL-6	112±4	126±18*	110±7	127±11*	128±7*	117±5*
	IL-8	102±6	114±25	111±7	96±7	104±6	104±4
	TNF-α	122±18*	133±21*	129±14*	119±6*	121±9*	128±18*
	–	–	–	110±12	–	–	89±17
OZP-activated	*IL-10*	114±26	–	–	103±5	–	–
	IL-6	124±16*	118±6*	129±10*	113±5*	107±10	109±15
	IL-8	114±16	115±12	112±12	103±7	110±13	108±4
	TNF-α	126±14 *	120±15*	122±11*	119±6*	125±14*	126±14*
	–	–	–	96±11	–	–	91±8

Results are expressed as percentages of controls incubated without any cytokine. The asterisk indicates significant ($p<0.05$) difference between controls and samples incubated simultaneously with IL-10 or preincubated with IL-10 for 1 h (n=5).

Influence of simultaneous incubation of IL-10 with individual pro-inflammatory cytokines

Simultaneous incubation of blood with IL-10 along with IL-6 or TNF-α did not significantly influence either spontaneous or PMA- or OZP-activated ROS production measured by CL when compared with analogous samples incubated without IL-10 at both 15 min and 2 h time intervals (Table 2). A simultaneous incubation of blood with IL-10 and IL-8 did not influence ROS production when compared with analogous samples incubated without IL-10 with the exception of spontaneous (significant increase) and OZP-activated (significant decrease) ROS production after 2 h of incubation (Table 2).

Simultaneous incubation of blood samples with IL-10 (1200 pg/ml) and any of the pro-inflammatory cytokines did not significantly affect either spontaneous or PMA- or OZP-activated ROS production by neutrophils or monocytes measured by DHR-123 when compared with analogous samples incubated without IL-10 (Table 1).

Simultaneous incubation of blood with IL-10 (1200 pg/ml) and any of the pro-inflammatory cytokines did not significantly affect the expression of any evaluated surface molecules on either neutrophils or monocytes when compared with analogous samples without IL-10 (data not shown).

Influence of preincubation with IL-10 on the priming effect of pro-inflammatory cytokines

Preincubation of blood with IL-10 for 1 h before 15 min or 2 h incubation with individual pro-

inflammatory cytokines did not have any influence on either spontaneous or activated ROS production measured by CL when compared with analogous samples without IL-10 pre-incubation (Table 2). Similarly, preincubation of blood with IL-10 for 1 h before incubation with individual pro-inflammatory cytokines did not significantly affect either spontaneous or PMA- or OZP-activated intracellular ROS production by neutrophils or monocytes as measured by DHR-123, when compared with analogous samples without IL-10 preincubation (Table 1). The expression of any evaluated surface molecules either on neutrophils or monocytes was not significantly affected when compared with analogous samples without IL-10 preincubation (data not shown).

Table 2. The influence of IL-10 (1200 pg/ml) on spontaneous and PMA- or OZP-activated ROS production by blood phagocytes measured by luminol-enhanced chemiluminescence. Blood samples were simultaneously incubated with IL-10 and with IL-6, IL-8 or TNF-α (1200 pg/ml) for 15 min or 2 h or preincubated with IL-10 for 1 h before 15 min or 2 h of incubation with IL-6, IL-8 or TNF-α (1200 pg/ml).

		Without IL-10	Simultaneous incubation with IL-10	Pre-incubation with IL-10	Without IL-10	Simultaneous incubation with IL-10	Pre-incubation with IL-10
		15 min incubation			2 h incubation		
Spontaneous	*IL-6*	106±3	99±6	105±3	101±3	91±11	111±17
	IL-8	107±4	98±2	99±1	93±6	111±6†	86±15
	TNF-α	141±1*	137±14*	166±25*	100±5	95±8	112±18
	–	–	–	101±1	–	–	94± 8
PMA-activated	*IL-6*	93±3	91±1*	103±4	121±10*	129±13*	117±15
	IL-8	95±7	94±8	118±3*	96±5	99±4	107±15
	TNF-α	168±14*	148±13*	170±6*	170±27*	174±31*	168±41 *
	–	–	–	102±7	–	–	96±9
OZP-activated	*IL-6*	105±16	100±5	110±6	146±10*	150±21*	139±18 *
	IL-8	116±5*	102±4	118±4*	123±9*	92±6†	121±16 *
	TNF-α	197±28*	192±22*	177±19*	203±39*	204±57*	206±54*
	–	–	–	116±5*	–	–	90±9

Results are expressed as percentages of controls incubated without any cytokine. The cross indicates significant ($p<0.05$) difference between samples without IL-10 and analogous samples simultaneously incubated or preincubated with IL-10 (n=5). For other symbols and explanations see Table 1.

Discussion

IL-10 is classified as an anti-inflammatory cytokine (Vicioso *et al.* 1998). However, its effect on phagocytes, which belong to the key components of the inflammatory response, is still a matter of discussion. In the present study, the influence of IL-10 on human blood phagocytes was tested by monitoring ROS production and the expression of several adhesion molecules on neutrophils and monocytes in the presence and in the absence of selected pro-inflammatory cytokines. In our experimental conditions, IL-10 did not have any inhibitory effect on ROS production by blood phagocytes. On the contrary, IL-10 at concentrations of 1200 and 1600 pg/ml significantly enhanced the activated ROS production after 2 h of incubation which agrees with our previous observations (unpublished data). Bussolati *et al.* (1997) found that IL-10 directly stimulated an early production of superoxide by monocytes and to a slight extent by PMNL, but inhibited the subsequent phagocytosis and the ability of these cells to respond to fMLP. Roilides *et al.* (2000) found that IL-10 (2-100 ng/ml) did not have any significant effect on the activated oxidative burst of PMNL after 1 h of incubation. Our results also show that the expression of neutrophil and monocyte surface antigens CD11b, CD15, CD31 and CD62L (Kubala *et al.* 2002ab) is not modified by IL-10. Similarly Vicioso *et al.* (1998) did not observe any effect of IL-10 (10 ng/ml) on CD11b/CD18 expression on PMNL and monocytes after 30 min of incubation. In

contrast, some authors have shown that IL-10 downregulated the expression of CD11b (Laichalk *et al.* 1996), chemotaxis (Vicioso *et al.* 1998) and the ability of phagocytosis (Laichalk *et al.* 1996).

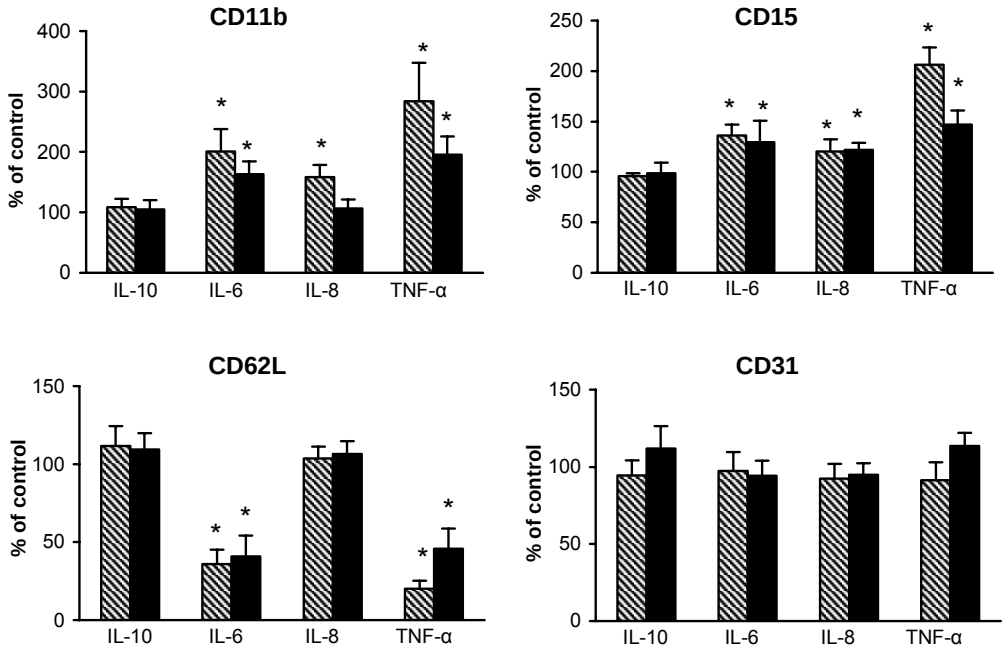

Fig. 2. Effects of IL-10, IL-6, IL-8 and TNF-α at a concentration of 1200 pg/ml on the expression of surface molecules (CD11b, CD15, CD62L and CD31) on neutrophils (hatched bars) and monocytes (full bars) after 2 h of incubation. Results are expressed as percentages of controls. The asterisk indicates significant ($p<0.05$) difference vs. control incubated in the absence of cytokine (n=5).

Furthermore, the effects of IL-10 in the presence of individual pro-inflammatory cytokines were analyzed in our study. Each of the tested pro-inflammatory cytokines alone induced a strong increase in phagocyte ROS production. The influence of TNF-α was the most effective at all the concentrations used as early as after 15 min of incubation. Other authors also found that IL-6, IL-8 and TNF-α enhanced the cytotoxicity and primed the oxidative burst of PMNL (Borish *et al.* 1989, Elbim *et al.* 1994, Elbim and Gougerot-Pocidalo 1996, Niwa *et al.* 1996, Vondráček 1997). Khwaja *et al.* (1992) observed a fast response of blood phagocytes to TNF-α stimulation after 10-20 min. Finally, in agreement with the literature (Baggiolini *et al.* 1994, Asman *et al.* 1996), IL-6, IL-8 and TNF-α increased the expression of CD11b and CD15 and decreased the expression of CD62L on both neutrophils and monocytes. Surprisingly, IL-10 did not have any influence on the priming effect of IL-6, IL-8, TNF-α on phagocyte ROS production and the expression of adhesion molecules during simultaneous incubation. Our results are in accord with Reglier-Pouplet *et al.* (1998) who showed that IL-10 did not have a direct effect on production of H_2O_2 and did not modulate priming effect of TNF-α on the response to fMLP. The only exception was that IL-10 abolished the stimulating effect of IL-8 on OZP-activated ROS production. It was also observed that preincubation with IL-10 did not affect the changes induced by IL-6, IL-8 or TNF-α. Ωε also confirmed that IL-10 did not have an inhibitory effect on phagocyte activity *in vitro* at the tested concentrations. The influence of IL-10 on the activation of blood phagocytes *in vitro* could be affected by several factors. The effects of cytokines are dose-dependent so that the selection of tested concentrations is very important for *in vitro* experiments (Laichalk *et al.* 1996, Capsoni *et al.* 1997). The concentrations of cytokines used in our study were selected on the basis of previous studies (Kubala *et al.* 2001, 2002ab) and other clinical studies on patients in various pathophysiological situations when the highest plasma level of any tested cytokine did not exceed 2 ng/ml (Lang *et al.* 1996). When high concentrations (10-100 ng/ml) of IL-10 were used for *in vitro* experiments, IL-10 downregulated the generation of ROS by blood phagocytes (Bogdan *et al.* 1991, Laichalk *et al.* 1996),

slightly augmented phagocytosis by monocytes and decreased phagocytosis by neutrophils (Buchwald *et al.* 1999). ICAM-1 expression on monocytes was markedly enhanced by IL-10 in the concentration range of 1 to 100 ng/ml, while the expression on neutrophils was not affected (Buchwald *et al.* 1999). Surface expression of IL-10 receptor on blood phagocytes is another important factor affecting the influence of IL-10 on phagocyte activity. It was reported that expression of IL-10 receptors on the surface of resting blood granulocytes was low and was not sufficient to induce a response of these cells to IL-10 (Elbim *et al.* 2001). These authors observed that a preincubation of cells with TNF-α for 10-40 min induced an increase of the expression of IL-10 receptor and consequently induced the responsiveness of cells to IL-10 which was expressed by a lower production of superoxide.

Another explanation for the absence of anti-inflammatory activity of IL-10 could be a short incubation of diluted blood with cytokines in our study. Bogdan *et al.* (1991) found that IL-10 reduced the generation of ROS when macrophages were preincubated with IL-10 for 48 h and then stimulated by PMA. Similarly, the incubation of monocytes or PMNL with IL-10 for 24 h before stimulating these cells with fMLP decreased superoxide production (Bussolati *et al.* 1997). Incubation of neutrophils with IL-10 for 18 h down-regulated their capacity to produce a superoxide anion, but conversely, shorter incubation times with IL-10, from 30 min to 5 h, were ineffective (Capsoni *et al.* 1997). It seems that the anti-inflammatory properties of IL-10 could be observed mainly after long incubation times of blood phagocytes with IL-10. However, a massive increase of IL-10 occurs *in vivo* at the same time as a massive increase of pro-inflammatory cytokines which cause phagocyte activation at time intervals up to several hours. Therefore, if an increase of IL-10 is protective against damage caused by activated phagocytes, as has been suggested by several authors (Kubala *et al.* 2001, Wan *et al.* 1997), it has to affect the phagocytes' activity at these time intervals. Therefore, the incubation times in our experiments did not exceed three hours.

Most studies have been performed on PMNL or monocytes isolated from blood by various procedures, which may modulate ROS production (Číž and Lojek 1997), surface receptor expression (Zahler *et al.* 1997), and thus alter the cell response. Therefore, in this study we measured the activation of blood phagocytes in diluted blood in order to avoid PMNL and monocyte activation and to simulate the *in vivo* situation more closely.

In conclusion, pro-inflammatory effects of IL-6, IL-8 and TNF-α expressed as an increased ROS production and the enhanced expression of adhesion molecules by neutrophils and monocytes were observed *in vitro*. These effects were dependent on cytokine concentrations and the time of incubation of blood with cytokines. On contrary, our results show that IL-10 does not have any direct anti-inflammatory activity towards blood phagocytes. The properties of IL-10 described in our study should be taken into consideration in therapeutic strategies where some anti-inflammatory benefit of IL-10 is expected.

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Reprint requests

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SHORT COMMUNICATION

PERI- AND POST-OPERATIVE COURSE OF CYTOKINES AND THE METABOLIC ACTIVITY OF NEUTROPHILS IN HUMAN LIVER TRANSPLANTATION

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Peri- and post-operative (up to day 7 after surgery) neutrophil chemiluminescence and the plasma concentrations of interleukin 6 (IL-6), IL-8, IL-10 and tumour necrosis factor α (TNF-α) were evaluated in the blood of patients undergoing liver transplantation. IL-6, IL-8 and IL-10 levels increased during early reperfusion and then returned to normal mostly within the first post-operative day. TNF-α was increased during the whole period observed. Spontaneous as well as activated neutrophil chemiluminescence was depressed in early reperfusion and remained low during the whole period followed. Samples collected during early reperfusion provided positive correlation for IL-6 vs IL-8 as well as for IL-6 and IL-8 vs chemiluminescence. The data were also evaluated with respect to the outcome of transplantation. Since IL-8, IL-10 and TNF-α levels increased significantly during the first post-operative week, mainly in a group of patients who developed serious complications within the first month after surgery, we proved a connection between peri- and early post-operative induction of cytokine release and the outcome of liver allograft transplantation.

Liver transplantation (LTx) is associated with systemic inflammatory response, which is an extremely complex phenomenon related to the release of various cytokines[1] and changes in the metabolic activity of neutrophils, which have been implicated in the development of graft injury for being an important source of free radicals.[2] Our study was designed to obtain better information on cytokine release [interleukin 6 (IL-6), IL-8, IL-10 and tumour necrosis factor α (TNF-α)] and neutrophil mobilization during the peri- and early post-operative period in liver graft recipients. The relation of the monitored parameters to the outcome of transplantation was also assessed.

RESULTS

Pre-operational plasma levels of cytokines (11.9 ± 10.6; 25.3 ± 21.5; 12.4 ± 3.8 and 2.5 ± 1.0 pg/ml for IL-6, IL-8 IL-10 and TNF-α, respectively), the mean total leukocyte number (6700 ± 400 per ml), and both spontaneous (1.7 ± 0.4 mV × s × 10^3) and activated (167.5 ± 38.2 mV × s × 10^3) chemiluminescence (CL) in LTx patients were not significantly different from the values of healthy controls.

IL-6 and IL-10 were extremely elevated during the early reperfusion period (first 4 h) when compared with the initial value (Fig. 1). IL-8 was significantly elevated only 4 h after reperfusion. TNF-α was significantly increased during the whole recorded period.

Spontaneous CL was significantly lower than its pre-operational level in all sampling intervals (Fig. 2).

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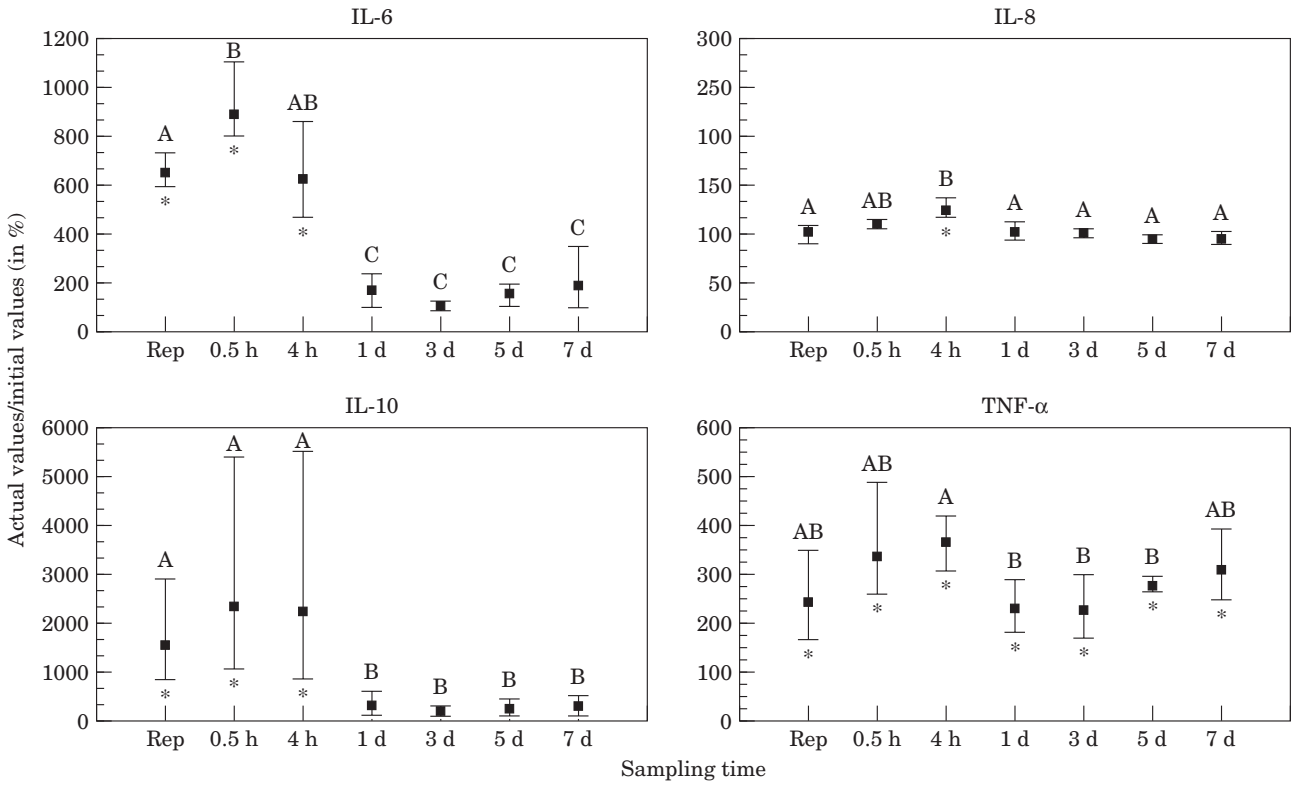

Figure 1. Time profiles of IL-6, IL-8, IL-10 and TNF-α levels.

The ordinate represents actual values at different sampling times (Rep=reperfusion, h=hours and d=days after reperfusion) related to the initial values determined before the transplantation and expressed in %. Data are expressed as a mean ± 95% limits of confidence. Within one parameter, the results from subsequent sampling times marked by the same capital letter are not significantly different (one way ANOVA; $P<0.05$). Asterisks mark sampling times when the values of a specified parameter were significantly higher or lower than pre-operational values (one sample *t*-test; $P<0.05$).

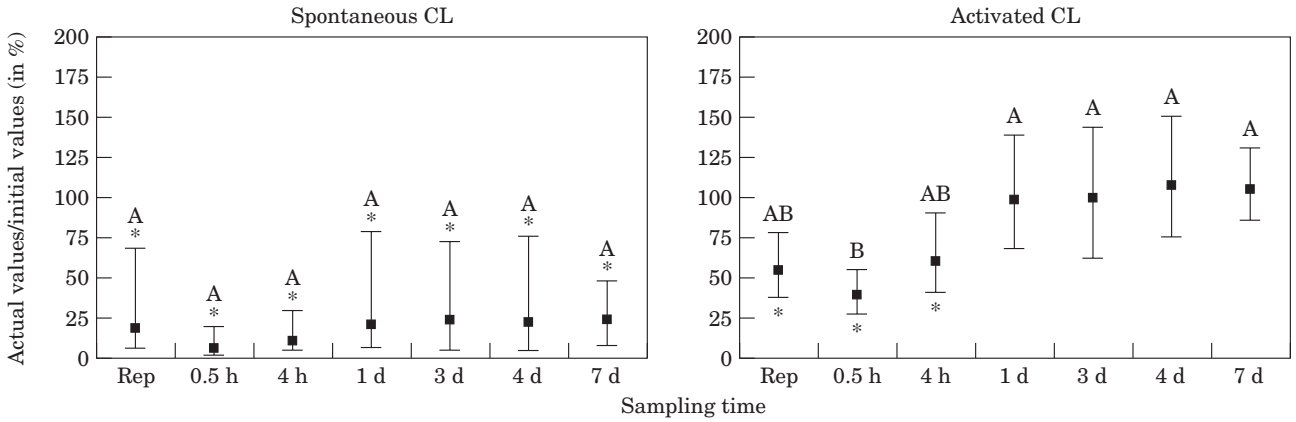

Figure 2. Time profiles of spontaneous and starch activated chemiluminescence activity of neutrophils in whole blood.

Symbols and explanations are as given for Figure 1.

Activated CL was significantly lower only during the early reperfusion period.

When compared with the pre-operational values, the total numbers of leukocytes decreased significantly ($P<0.05$) 30 min after reperfusion (60.6 ± 15.2%), but they were rising in later sampling times. A significant increase in this parameter was observed from the first day (132.3 ± 25.5%) up to the seventh day (151.4 ± 27.9%) after transplantation. While the total numbers of neutrophils did not differ from the pre-operational values at any sampling time, the relative numbers of not fully maturated neutrophils (bars) were increased significantly ($P<0.05$) at the time of reperfusion (295 ± 45%), 30 min (473 ± 108%) and 4 h

TABLE 1. Interrelationships among IL-6 and IL-8 and spontaneous and activated CL

	IL-6	IL-8
Spontaneous CL		
Reperfusion	+ $P=0.03$	$P=0.45$
0.5 h	+ $P<0.01$	+ $P<0.01$
4 h	+ $P<0.01$	+ $P<0.01$
Activated CL		
Reperfusion	$P=0.47$	$P=0.24$
0.5 h	+ $P<0.01$	+ $P=0.02$
4 h	+ $P<0.01$	+ $P<0.01$

+Indicate positive correlation with the shown significance values of Spearman's correlation coefficients.

(485 ± 115%) after reperfusion, and on the first day (253 ± 86%) and the third day (265 ± 73%) after transplantation when compared with the initial value.

Correlation analysis detected strong positive correlation between IL-6 and IL-8 in samples collected before transplantation ($r= 0.784$; $P<0.001$), at the time of reperfusion ($r=0.905$; $P<0.001$), 30 min ($r=0.710$; $P<0.01$) and 4 h ($r=0.753$; $P<0.001$) after reperfusion. Similarly, high positive correlation was also found between IL-6 or IL-8 and spontaneous or activated CL (Table 1).

Based on the outcome of the transplantation in the first month after surgery, the patients were additionally divided into three groups: group I (no complications), group II (mild complications), and group III (serious complications). There was a significant increase in the levels of IL-8, IL-10 and TNF-α in group III ($n=5$), which mostly started at the time of reperfusion, intensified during 0.5–4 h, and remained rather obvious on days 5–7 after reperfusion, when the increases were quite significant in comparison both with the pre-operational levels and with those recorded for groups I ($n=7$) and II ($n=4$) (Fig. 3). The concentrations of IL-6 were also increased in group III, but changes were less significant (data not shown).

DISCUSSION

The massive release of the analysed cytokines with a consequent normalization occurring within the first day after LTx is consistent with the findings of other authors.[3–9] On the other hand, the depressed phagocyte-derived radical production is surprising. Microvasculature infiltration by activated neutrophils manifested as an increased relative number of bars could be a possible reason for this phenomenon. Other possible reasons could be increased IL-10 concentrations or immunosuppressive therapy. However, the inhibitory effects of both IL-10 and immunosuppressives are still a matter of discussion.[2,10,11]

Positive correlation between IL-6, IL-8 and blood CL activity proved a stimulatory effect of these cytokines on phagocyte metabolic activity. Concerning IL-10, we failed to find an inhibitory effect of its high plasma levels on the release of IL-8 or IL-6. Le Moine *et al.*[6] found even a positive correlation between IL-8 and IL-10 plasma levels.

The increased concentrations of the observed cytokines during transplantation and especially their remaining elevated levels were found to be related to a negative outcome of LTx. Other authors have also found higher TNF-α, IL-6 or IL-8 release during and after LTx in patients with primary graft non-function or dysfunction, viral and bacterial infections or early rejections.[3,5,8,9,12] However, opposite findings also exist.[6,7] IL-10 has been proposed to inhibit allogeneic response and the initiation of graft rejection in LTx patients.[6] In contrast, we observed an association between increased IL-10 levels and a poor outcome of LTx.

In conclusion, it seems that sequential peri-operative and post-operative monitoring of cytokine concentrations in the peripheral blood can provide useful information on the subsequent further clinical status of the liver recipients and the potential risk of complications. However, since surgical stress itself induces systemic cytokine release, the question of whether peri-operative cytokine monitoring can predict the outcome of transplantation should be considered carefully.

MATERIALS AND METHODS

A group of 16 patients who underwent orthotopic liver transplantation (eight males and eight females, 36 ± 17 years) was included in the study. Healthy volunteers (10 males and 10 females, 44 ± 10 years) were used as controls. Written informed consent was obtained from each subject. The immunosuppressive regimen consisted of cyclosporine A, azathioprine, methylprednisone or prednisone, and antithymocyte globulin.

Heparinized peripheral blood samples were taken before surgery, at the beginning of reperfusion, 30 min and 4 h after reperfusion and then on days 1, 3, 5, and 7 after the operation. Total number of leukocytes and their relative differentiation counts were determined. Plasma cytokine levels were determined using ELISA kits Quantikine™ IL-6 and IL-10 (R&D Systems, Minneapolis, USA), and Cytoscreen™ IL-8 and TNF-α (BioSource International, Camarillo, USA). Phagocyte-derived free radical generation was measured by luminol-enhanced CL as described previously.[13]

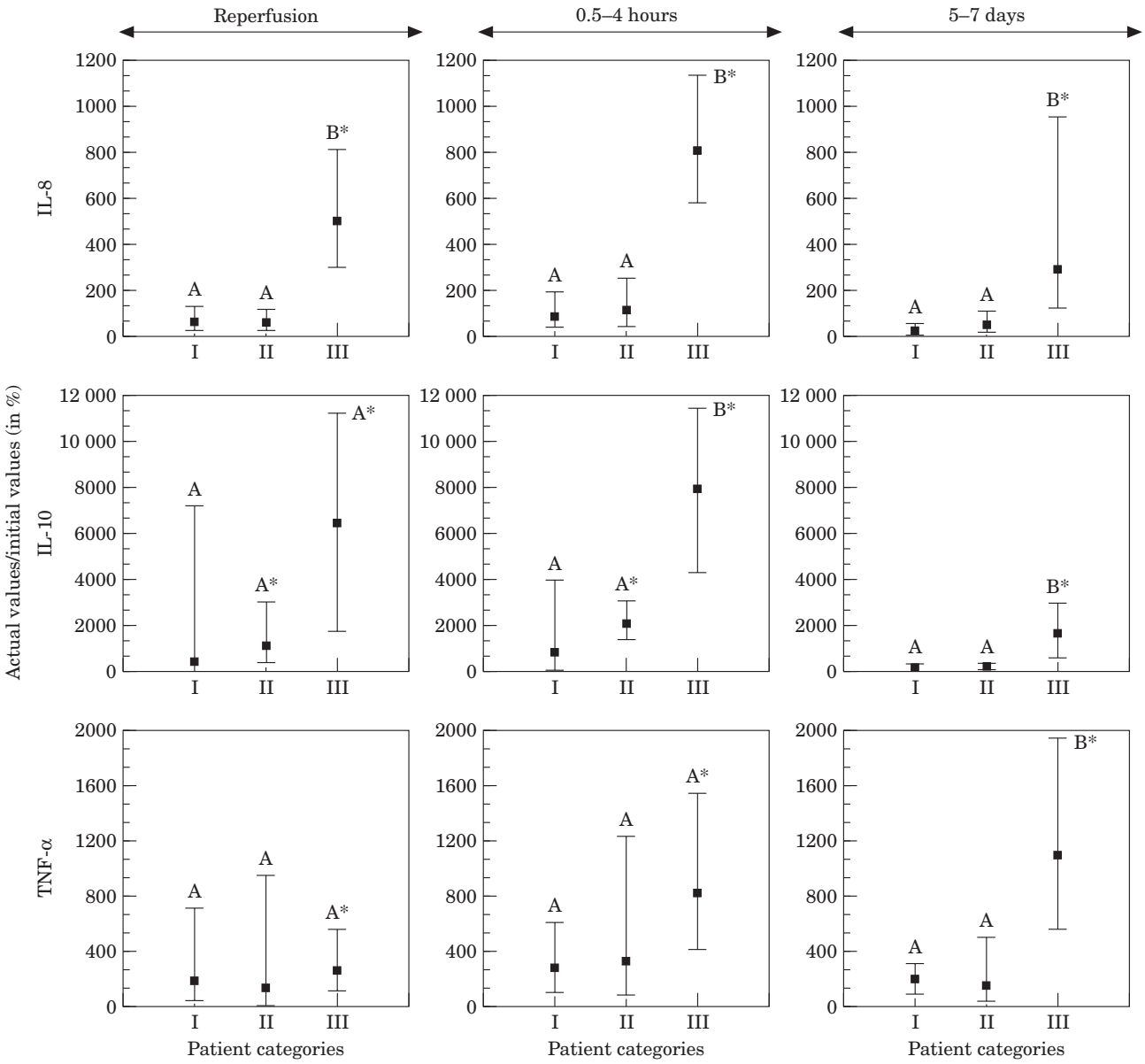

Figure 3. Time profiles of IL-8, IL-10 and TNF-α in three groups of liver graft recipients: group I (no complications), group II (mild complications), and group III (serious complications).

Symbols and explanations are as given for Figure 1.

Data in the text are expressed as the mean ± standard error of the mean (SEM). One-way ANOVA models and Student's *t*-tests were used for data analyses. Interrelationships among variables were assessed using Spearman's rank correlation coefficients and its value (*r*) and a level of its significance (*P*) are expressed.

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Blood Phagocyte Activation During Open Heart Surgery With Cardiopulmonary Bypass

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Summary

Open heart surgery with a cardiopulmonary bypass (CPB) is associated with a systemic inflammatory response which significantly contributes to adverse postoperative complications. The purpose of this study was to characterize the activation of blood phagocytes during open heart surgery with CPB. Blood samples were collected during and up to 24 h after surgery. The production of reactive oxygen species (ROS) in whole blood, the expression of surface molecules by blood phagocytes and complement activity in the plasma were determined. A cDNA microarray analysis of leukocyte RNA profile of genes was performed related to the inflammatory response. Activation of the complement was already observed at the beginning of CPB. This was followed by an increase in the neutrophil number and in both spontaneous and opsonized zymosan-activated ROS production after the onset of reperfusion. The activation of blood phagocytes was affirmed by changes in surface receptors involved in the adhesion and migration of leukocytes (CD11b, CD62L and CD31). Gene arrays also confirmed the activation of leukocytes 4 h after reperfusion. In conclusion, open heart surgery with a cardiopulmonary bypass was found to be associated with a rapid and pronounced activation of blood phagocytes and complement activation which was partly independent at the onset of CPB.

Key words

Phagocytes • Complement • Surface receptors • Reactive oxygen species • Gene expression

Introduction

Open heart surgery with a cardiopulmonary bypass (CPB) is associated with systemic inflammation, which significantly contributes to adverse postoperative complications (Dernek *et al.* 1999). The induction of systemic inflammation is caused by several factors including exposure of blood to the extracorporeal circuit, post-ischemic reperfusion of the heart and lungs as well as surgical trauma (Dernek *et al.* 1999). Mediators released during an inflammatory response, such as pro-inflammatory cytokines and complement activation products, promote polymorphonuclear cell adhesion to the endothelial surface, evoke their infiltration in tissues and cause the release of reactive oxygen species (ROS) and enzymes (Lojek *et al.* 1992, Starkopf *et al.* 1997, Boyle *et al.* 1997, Defraigne *et al.* 2000).

The recruitment of white blood cells into tissues

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requires a stepwise interaction between adhesion molecules on the surface of leukocytes and their corresponding receptors on the luminal surface of inflamed endothelium (Vinten-Johansen 2004). Various adhesion molecules are involved in this process: (1) selectins, which mediate the initial attachment and the rolling of leukocytes along the vessel wall under shear stress changes, (2) β2 integrins, which mediate firm adhesion of leukocytes to endothelium, and (3) adhesion molecules of immunoglobulin superfamily expressed on the endothelial side which, in conjunction with the β2 integrins, regulate a firm adhesion and transendothelial migration of activated phagocytes (Asimakopoulos *et al.* 2000). This study investigated the expression of CD62L (L-selectin), CD11b/CD18 (Mac-1) and CD31 (PECAM-1) on polymorphonuclear leukocytes (PMNL) and monocytes during open heart surgery. These surface antigens together with the production of ROS were selected as markers of phagocyte activation.

The activation of complement pathways significantly promotes phagocyte adhesion and migration while contributing to the systemic inflammation connected to open heart surgery (Gu *et al.* 1998, Struber *et al.* 1999). Therefore the complement activity in the plasma was also determined. To further describe the process of blood leukocyte activation during open heart surgery with CPB, the alterations in gene expression associated with a systemic inflammatory response were examined using cDNA microarray analysis.

The aim of this study was to clarify the mechanisms of activation of blood phagocytes during open heart surgery and their contribution to the induction of systemic inflammatory response induced by CPB.

Methods

Patients

A group of 30 patients who had had open heart surgery was examined in the study. Heart surgery was performed for ischemic heart disease (n=19), aortic valve replacement (n=5) and mitral valve replacement (n=6). Cold crystalloid cardioplegic solution (St. Thomas Hospital solution) was used in a total volume of 1000-1500 ml depending on the crossclamping time. Three patients (males: 56, 69 and 69 years) with ischemic heart disease (IHD) without peri- and postoperative complications were chosen for gene expression analysis using the cDNA microarray technique. The study was approved by the local ethics committee.

Sampling

Heparinized peripheral blood samples were taken from the patients before surgery, at the beginning of ischemia, at the start and after 30 min, 4 h and 24 h of reperfusion. Cell counts and the oxidative burst of phagocytes were determined immediately. Total number of leukocytes in the blood and their relative differentiation counts were determined using a Coulter counter STKS (Coulter, England) and in stained blood smears, respectively. Whole blood (12.5 ml) was collected according to the manufacturer's protocol (PAXgene^{TM} Blood RNA tubes, PreAnalytiX, Germany), before and 4 h after the operation for cDNA microarray analysis. Plasma samples were frozen and stored at –20 ^{o}C.

Oxidative burst of phagocytes

Luminol-enhanced chemiluminescence (CL) of whole blood phagocytes was measured using Luminometer LMT-01 (Immunotech, Czech Republic) as described previously (Pečivová *et al.* 2004). The principle of the method is based on luminol interaction with the phagocyte-derived ROS which results in large light emission. Opsonized zymosan particle (OZP)-activated CL was measured for 60 min at 37 ^{o}C. The assays were run in duplicates. Spontaneous CL measurements in samples containing all substances except the activator were included in each assay. The integral value of the CL reaction, which represents total ROS production by blood phagocytes, was corrected for 10^3 neutrophil granulocytes.

Determination of the expression of adhesion molecules

The measurements were performed according to the manufacturer's protocol (Caltag Laboratories, USA) using unfixed whole blood with minor modifications as described previously (Gallová *et al.* 2004). Briefly, blood samples were incubated with anti-CD11b, anti-CD62L, and anti-CD31 monoclonal antibodies. Phycoerythrin- or fluorescein isothiocyanate-conjugated murine immunoglobulins of the same isotype were used as the negative controls. Ten thousand PMNL selected on the basis of their typical scattering characteristics were analyzed by a flow cytometer FACSCalibur (Becton Dickinson, USA) and the median of relative fluorescence was determined.

Determination of complement activity

Complement activity was determined by a bioassay using an *E. coli* JM109 strain carrying luciferase

plasmid pCSS962 (a gift of Prof. E.M. Lilius, University of Turku, Finland). Cultivation of bacteria and the determination of complement activity was described previously (Virta *et al.* 1998, Nikoskelainen *et al.* 2002). Briefly, the bacteria cultivated overnight to the log phase in L broth (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl; pH 7.0) and containing appropriate antibiotics, were collected by centrifugation (1500 x *g*, 10 min), washed with Hanks' balanced salt solution (HBSS), resuspended in HBSS, and diluted to a concentration of 10^7 cells/ml with HBSS. Complement reactions were carried out by mixing the plasma with bacteria suspension at a ratio of 1:5. Reaction mixtures were incubated for 90 min at 37 °C without shaking.

The reactions were stopped by placing the samples on ice for 10 min prior to luminometric analysis. The reaction mixture was then mixed with 50 µl of luciferin solution (1 mmol/l D-luciferin in 100 mmol/l sodium citrate, pH 5.0), and luminescence was monitored with a luminometer LMT-01 for 10 min at 37 °C. The assays were run in duplicates. The luminescence data were converted to a percentage of the reference sample containing human serum without any detectable complement activity.

cDNA microarrays

Pure total RNA was isolated from whole blood by PAXgene^{TM} Blood RNA Kit (PreAnalytiX, Germany). The quality of total RNA was examined by agarose gel electrophoresis. The quantity of total RNA was determined spectrophotometrically at 260 nm using NanoDrop spectrophotometer (NanoDrop Technologies, USA). The labeling of cDNA of the samples and subsequent hybridization to the arrays were done according to the manufacturer's manual (SIRS-Lab, Germany). Briefly, 20 µg of total RNA of leukocytes was used for analysis. mRNA was annealed to an anchored Oligo-dT18V primer and used to generate cDNA utilizing the Superscript II reverse transcription**.** For labeling, the reaction was spiked with Cy5-dUTP (4 h after operation) or Cy3-dUTP (before operation) fluorescence-tagged nucleotides and then analyzed using a Lab-arraytor Human-500 (SIRS-Lab, Germany). Fluorescence detection of Cy-3 and Cy-5 fluorophores was performed using a glass slide scanner GenePix4000B (Axon Instruments, USA). Relative rates of gene expression were determined with "AIDA Array Evaluation" software.

Statistical analysis

A standard two-sample t-test was applied for inspection of differences among variants using Statistica for Windows 5.0 (StatSoft. Inc., USA). Data are expressed as means and standard errors of the mean.

Results

Total leukocyte number was significantly increased from the start of reperfusion till 24 h after operation. The sharp increase in total number of leukocytes was associated with a relative increase in neutrophils (both bars and segments) (Table 1). The changes in leukocyte numbers correspond with the activation of blood phagocytes. A significant increase in both spontaneous and OZP-activated production of ROS from the start of reperfusion to 24 h after operation was also observed (Table 2).

Table 1. Changes in the numbers of blood leukocyte

	Before operation	**Initiation of CPB**	**End of CPB**	**30 min after CPB**	**4 hours after CPB**	**24 hours after CPB**
Total leukocyte [x10^3]	4.9±0.3	5.1±0.4	7.8±0.6 *	10.3±0.9 *	11.3±0.7 *	11.3±0.7 *
Neutrophil granulocytes - segmented [%]	59.0±2.2	58.5±1.6	62.7±1.2 *	64.9±1.4 *	69.6±1.2 *	70.9±1.6 *
Neutrophil granulocytes - bars [%]	5.9 ±0.7	6.3±0.9	13.6±1.4 *	14.8±1.0 *	14.1±1.5 *	15.0±1.1 *

Total leukocyte counts and percentage of segments and bars (not fully maturated neutrophils) at different sampling times ([before operation, before the initiation of CPB, at the time of reperfusion (the end of CPB), 30 min, 4 h and 24 h after reperfusion (after the end of CPB)]. Asterisks mark the sampling times when the values differed significantly from the pre-operational values ($p<0.05$).

Table 2. Changes in the ROS production by blood phagocytes

	Before operation	Initiation of CPB	End of CPB	30 min after CPB	4 hours after CPB	24 hours after CPB
Spontaneous CL	18±3	26±5	60±11 *	54±9 *	43±6 *	112±17 *
OZP-activated CL	647±70	641±60	1622±201 *	2120±239 *	2010±182 *	2162±174 *

Spontaneous and OZP activated chemiluminescence of whole blood at different sampling times [before operation, before the initiation of CPB, at the time of reperfusion (the end of CPB), 30 min, 4 h and 24 h after reperfusion (after the end of CPB)], expressed as RLU*s*10^3 cells. Asterisks mark the sampling times when the values differed significantly from the pre-operational values ($p<0.05$).

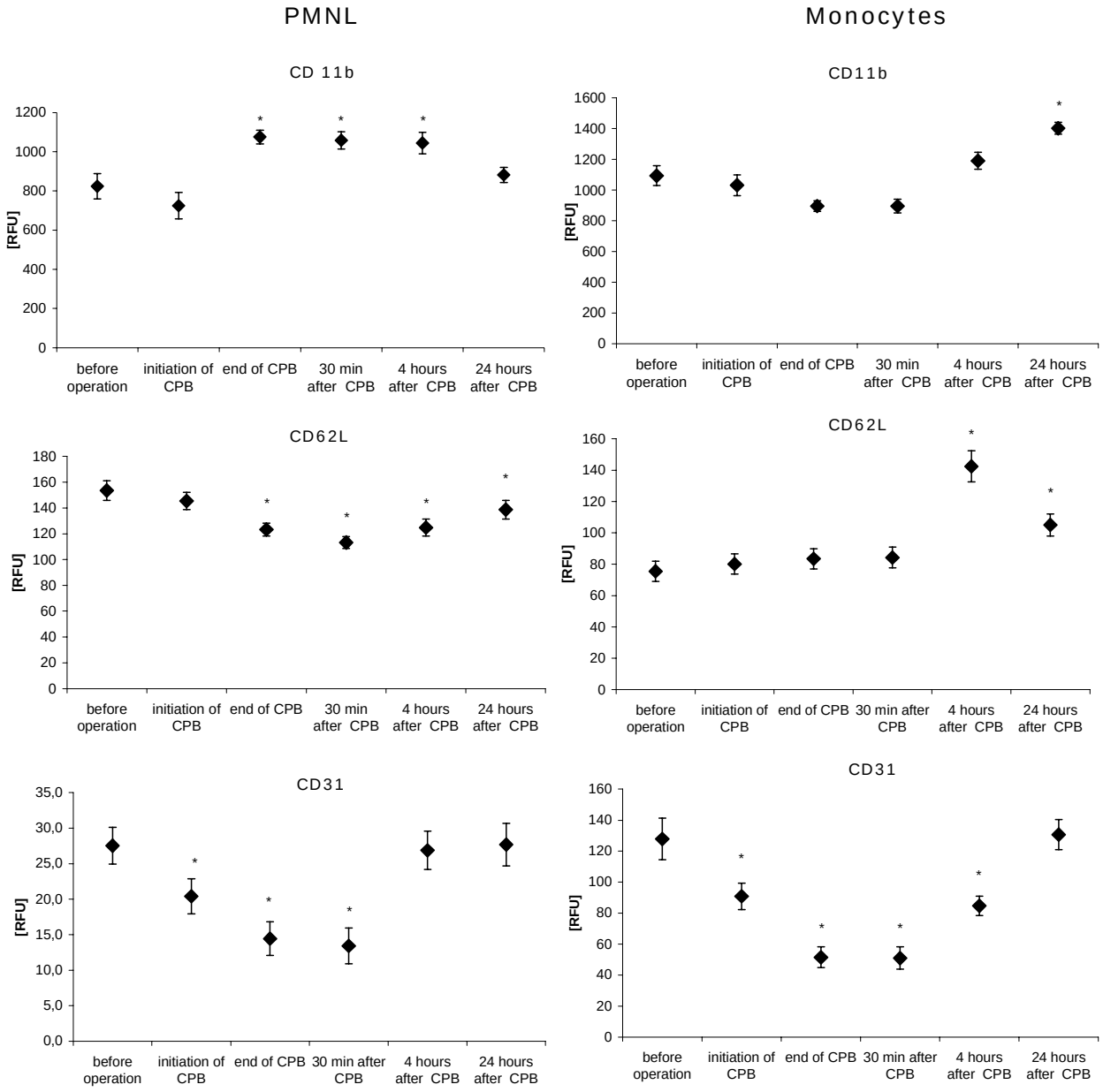

Fig. 1. Changes in the expression of leukocyte adhesion receptors. The expression of CD11b, CD62L and CD31 on blood phagocytes – PMNL and monocytes at different sampling times [before operation, before the initiation of CPB, at the time of reperfusion (the end of CPB), 30 min, 4 h and 24 h after reperfusion (after the end of CPB)]. Asterisks mark the sampling times when the values of a specified parameter differed significantly from the pre-operational values ($p<0.05$).

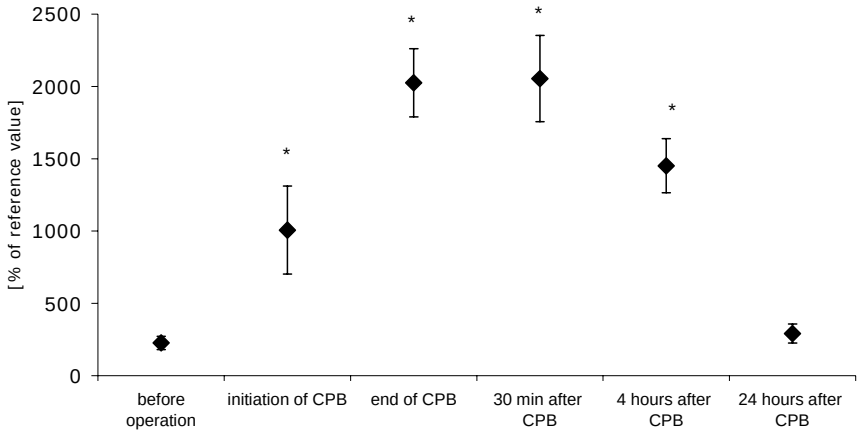

Fig. 2. Changes in the plasma complement activity. The activity of complement in plasma at different sampling times [before operation, before the initiation of CPB, at the time of reperfusion (the end of CPB), 30 min, 4 h and 24 h after reperfusion (after the end of CPB)]. Asterisks mark the sampling times when the values differed significantly from the pre-operational values ($p<0.05$).

Table 3. Changes in the blood leukocyte gene expressions - list of genes up-regulated 4 h after reperfusion.

	HUGO	NameMean	fold change
Cytokine receptors	IFNAR1	interferon (alpha, beta and omega) receptor 1	2.8
	IL1R2	interleukin 1 receptor, type II	3.8
	IL4R	interleukin 4 receptor	3.7
Cytokine-induced molecules	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	5
Chemokines	CX3CL1	chemokine (C-X3-C motif) ligand 1	4.9
	S100A12	S100 calcium-binding protein A8 (calgranulin A)	3.7
	S100A8	S100 calcium-binding protein A12 (calgranulin C)	13.3
CD and adhesion molecules	CD14	CD14 antigen	3
	DPP4	dipeptidylpeptidase IV (CD26, adenosine deaminase complexing protein 2)	5
	ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	3.3
Cell cycle and apoptosis	BCL2A1	BCL2-related protein A1	7.5
	IER3	immediate early response	3.5
Signal transduction	HPRT1	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan syndrome)	4.6
	IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	2.9
	MAPK14	mitogen-activated protein kinase 14	4.2
	MKNK1	MAP kinase-interacting serine/threonine kinase 1	3
Eicosanoid signaling pathway	ALOX5AP	arachidonate 5-lipoxygenase-activating protein	3.9

HUGO`s (The Human Genome Organization) gene abbreviations were used. The table summarizes data from three cDNA microarray experiments.

The activation of PMNL from the end of CPB was confirmed by significant changes in the expression of selected surface receptors. The upregulation of CD11b on PMNL surface from the end of CPB to 4 h after reperfusion was accompanied by a downregulation of the CD62L antigen. The downregulation of the CD31 antigen, a process that is also connected to phagocyte activation, had already started at the start of reperfusion and lasted up to 3 h after reperfusion (Fig. 1). In contrast, monocytes, the second major group of blood phagocytes, have been activated to a lesser extent compared to PMNL during open heart surgery. A significant increase in the CD11b antigen expression on monocytes was not observed until 24 h after the surgery (Fig. 1). The expression of CD62L on monocytes was increased 4 h and 24 h after reperfusion. On the other hand, a significant decrease in the CD31 expression (from the start reperfusion up to 4 h after reperfusion) was found on monocytes.

The increased number of blood phagocytes, along with their activation, was preceded by a significant increase in complement activity. The complement activity in the plasma significantly increased at the beginning of CPB and reached its maximum at the time of reperfusion and 30 min after reperfusion (Fig. 2).

Gene array analysis allowed us to investigate mRNA expression of 340 genes, among them membrane ligands and their receptors, kinases, phosphatases, molecules induced by inflammatory stress, transcription factors and transcription modulators. It was found that the expressions of 17 genes (ALOX5AP, BCL2A1, CD14, CX3CL1, DPP4, HPRT1, IER3, IFNAR1, IL1R2, IL4R, IL6ST, ITGA5, MAPK14, MKNK1, S100A12, S100A8, TNFAIP6) were upregulated significantly (2.5-fold or more) in all three patients (Table 3). No decrease was found in the expression of any gene.

Discussion

Several inflammatory pathways become activated during open heart surgery with cardio-pulmonary bypass and subsequent generalization of inflammatory response occurs. The systemic inflammatory response is assumed to be one of the major factors responsible for the diffuse tissue damage in the lung, myocardium, kidney and brain as described in patients undergoing open heart surgery (Baue *et al.* 1998, Wan and Yim 2001). Blood phagocyte activation is one of the causative mechanisms of this processes and leads to the increased production of ROS by phagocytes and the consequent oxidative stress, further adhesion and migration of phagocytes to tissues after ischemia/reperfusion.(Baue *et al.* 1998, Kotani *et al.* 2000, Čížová *et al.* 2004)

In our study, the significant increase in leukocyte numbers was observed from the start of reperfusion up to 24 h after surgery. The sharp increase in the total number of leukocytes was associated with a relative increase in both forms of neutrophils – bars and segments. The increase in circulating leukocyte count during and after operation in patients undergoing CPB has also been observed by other investigators (Ashraf *et al.* 1998, Gu *et al.* 1998, Kotani *et al.* 2000, Rothenburger *et al.* 2002). This systemic leukocytosis is thought to be caused by a combined effect of complement activation and the release of proinflammatory cytokines resulting in the mobilization of leukocytes from the marginating pools and bone marrow.

The changes in leukocyte numbers corresponded with the activation of blood phagocytes as was determined by various methods. One of them was the determination of spontaneous as well as OZP-activated blood phagocyte ROS production which increased remarkably. The increased ROS production induces lipid peroxidation which was described in patients undergoing open heart surgery with CPB (Lojek *et al.* 1992, Davies *et al.* 1993, Partrick *et al.* 1999, Kubala *et al.* 2002). The activation of blood phagocytes during open heart surgery was also supported by the determination of selected surface receptors involved in the adhesion and migration of leukocytes. CD62L antigen, which is constitutively expressed on non-activated leukocytes, is responsible for leukocyte rolling and margination and is rapidly downregulated by chemotactic stimulation (Venturi *et al.* 2003). The subsequent firm adhesion of leukocytes to activated endothelium is mediated by the upregulation of the β2-integrin complex, particularly CD11b/CD18 (Alonso *et al.* 1999, Asimakopoulos *et al.* 2000, Rinder *et al.* 2003). Downregulation of CD62L and upregulation of CD11b are indicators of neutrophil activation (Ilton *et al.* 1999, Gallova *et al.* 2004). Similarly, CD31 is implicated in the transendothelial migration of leukocytes, angiogenesis and integrin activation (Buckley *et al.* 1996). We observed a significant upregulation of CD11b expression on the surface of PMNL in early post-operative intervals. The downregulation of CD62L and CD31 expressions on PMNL was observed during the same time period. Upregulation of CD11b/CD18 and

downregulation of CD62L expressions on PMNL during CPB were also observed by other authors, but there are also contrary reports which did not observe any changes in CD62L expression (Galinanes *et al.* 1996, Le Deist *et al.* 1995, 1996). A significant decrease in CD31 expression, which also confirms the complex activation of PMNL during open heart surgery, was found on monocytes similar to that on PMNL. The expression of CD11b on monocytes also increased, but only after 24 h of reperfusion. Similarly to our findings, no significant increase in the expression of CD11b on monocytes during open heart surgery was reported by Rinder *et al.* (2003). While neutrophils demonstrated a downregulation of CD62L expression, its expression on monocytes was increased. These findings suggest that when compared to PMNL, monocytes are activated to a lesser extent during and immediately after the operation. We can consequently speculate that the immune response to surgical trauma is mediated particularly by PMNL.

Complement activation is one of the most important factors for leukocyte activation and leukocytosis during open heart surgery and it is considered to be a "trigger" of CPB-induced inflammatory response (Struber *et al.* 1999). A significant increase in plasma complement activity was determined early after the beginning of the operation (at the time of the start of CPB). In contrast, significant changes in the number of circulating leukocytes and activation of blood phagocytes were observed later during surgery. This supports the idea that the complement is the key initiator of the immune response during open heart surgery. The main suggested pathway of complement activation is the exposure of blood to the artificial surface of the CPB circuit (Gu *et al.* 1998, Cartier 2003). Nevertheless, in our study, a significant increase in complement activity was determined prior to the contact of blood with the artificial surface in the CPB circuit. From these results it could be concluded that the extensive surgical procedure itself activates the complement even before CPB begins. This is an important finding especially in view of the current trend in heart surgery where CPB is not used to avoid the induction of inflammatory responses (Cartier 2003). Our data suggest that significant complement activation could be expected even during heart surgery without the use of CPB.

Leukocyte activation is a complex process that is accompanied by changes in the expression of a wide range of genes participating in activation and regulation of cells. Studying the variation in leukocyte gene expression can therefore provide essential information about this sophisticated process. We have used gene expression profiling with cDNA microarrays to investigate the spectrum of genes related to the systemic inflammatory response. Of the 340 genes evaluated, the expression levels of 17 genes were increased while no genes were repressed 4 h after surgery. The over-expressed genes belong to the groups of cytokine receptors, cytokine-induced molecules, chemokines, adhesion molecules, cell cycle and apoptosis related molecules, signal transductors, and eicosanoid signaling pathway related molecules.

The mRNA expression of some proinflammatory cytokines appears to be of particular interest. In a previous study we reported a significant increase in plasma levels of the proinflammatory cytokine IL-6 during heart transplantation and open heart surgery with CPB, where the maximal level was observed 4 h after CPB (Kubala *et al.* 2002). Interestingly, a cDNA micro-array assay showed that mRNA of this cytokine was not upregulated at this time. Therefore, we would suggest that IL-6 present in the blood 4 h and later is not of leukocyte origin, but could be produced by cells from organs such as the liver, lung and heart which undergo ischemia or partial ischemia with consequent reperfusion. This conclusion concurs with Wan *et al*. (1997) who suggested that the myocardium is an important source of IL-6. The absence of any overexpressed cytokines in leukocytes 4 h after CPB was in accordance with the good clinical outcome in all three patients.

In conclusion, open heart surgery with cardiopulmonary bypass was associated with a rapid and pronounced activation of blood phagocytes. The findings suggest that the complement is one of the key "triggers" of the inflammatory response in patients undergoing open heart surgery and its activation begins before the onset of cardiopulmonary bypass. Consequently, phagocytes produce high amounts of reactive oxygen species and express the surface molecules required for adhesion and the recruitment of leukocytes in the vascular endothelium. On the basis of our results, we can speculate that the immune response to surgical trauma is mediated in particular by activated polymorphonuclear leukocytes.

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CSP

Perioperative and postoperative course of cytokines and the metabolic activity of neutrophils in human cardiac operations and heart transplantation

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See related editorial on page 1071.

Objectives: The purpose of this study was to compare systemic inflammatory responses after heart transplantation and nontransplant cardiac operations, both involving cardiopulmonary bypass with a focus on the role of polymorphonuclear leukocytes.

Methods: Lipid peroxidation, blood phagocyte radical production, and interleukin 6, 8, and 10 plasma concentrations during surgical intervention and on the first and seventh postoperative days were evaluated in patients undergoing heart transplantation (n = 24) and in patients not undergoing transplantation (n = 30).

Results: Levels of interleukin 6, 8, and 10 increased in both groups of patients during early reperfusion. They normalized within the first postoperative day in the transplant group, whereas the nontransplant group's interleukin 6 and 8 levels remained increased on the seventh day after the operation. Interleukin 10 plasma levels were higher in the heart transplant group during reperfusion. Lipid peroxidation was increased after the operation in both groups of patients. Phagocyte activity was enhanced at reperfusion and at all other sampling times only in the nontransplant group. On the other hand, phagocyte activity oscillated around the preoperative level during heart transplantation, or it was even decreased.

Conclusion: Both cardiac operations involving heart transplantation and those without transplantation are associated with increased oxidative stress and an enhanced production of proinflammatory and anti-inflammatory cytokines. Differences in interleukin 10 production and phagocyte activity could be caused mainly by the immunosuppressive therapy in heart transplant operations.

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Cardiac surgery involving cardiopulmonary bypass (CPB) is associated with systemic inflammatory response, which is considered to affect the clinical outcome of operations and could be caused by several factors, including the exposure of blood to the extracorporeal circuit, postischemic reperfusion of the heart and lungs, and surgical trauma.[1-3] Mediators released during the inflammatory response promote polymorphonuclear cell adhesion to the endothelial surface and evoke their infiltration in tissues and the release of reactive oxygen species (ROS) and enzymes.[4,5] There are some important differences between the early phase of heart transplantation (HTx) and nontransplant cardiac operations (non-HTx). Organ

TABLE 1. Clinical data on the HTx and non-HTx groups

	HTx group	Non-HTx group
Age (y)	46.0 (44.5; 54.5)	63.0* (55.0; 72.0)
Sex ratio (male/female)	21:3	15:15
CPB time (min)	103.0 (88.5; 125.0)	66.5† (58.0; 85.0)
Ischemic time (min)	164 (147; 202)	36* (31; 52)

Data are expressed as medians and lower and upper quartiles, except the sex ratio, which is expressed as the number of patients. *Asterisks* mark significant differences between the HTx and non-HTx groups (Mann-Whitney *U* test; *$P < .001$; †$.001 \leq P < .01$). The term *ischemic time* denotes the graft ischemic time in the HTx group or the aortic crossclamping time in the non-HTx group.

grafts are subjected to periods of both cold and warm ischemia during storage and implantation, which extend the duration of heart ischemia.[6]

Cytokines, such as interleukin 6 (IL-6) and IL-8, which are known to be produced during cardiac operations, belong to potent proinflammatory mediators with a wide range of biologic activities.[7,8] Their effects on neutrophils could exacerbate both local and remote tissue damage.[5,9] The extent of their increase can be correlated with the severity of the operation, and both cytokines have been suggested as markers with prognostic value for the outcome of the operation.[3,6,7,10,11]

Conversely, the anti-inflammatory cytokine IL-10, which is also produced during human organ transplantation, has been reported to downregulate the activity and release of proinflammatory cytokines, including IL-6 and IL-8.[12] Through these mechanisms, IL-10 can contribute to the downregulation of the postreperfusion injury development and systemic inflammatory response.[8]

The aim of this study was to better clarify interrelationships among the plasma levels of IL-6, IL-8, and IL-10; blood phagocyte ROS production; and oxidative stress-induced damage in patients undergoing HTx and non-HTx. The parameters from both groups of patients were compared.

Materials and Methods

Patients

The study was approved by the local ethics committee, and verbal informed consent was obtained from each subject. Only patients undergoing HTx or non-HTx without complications during the first postoperative week were selected, and their data were evaluated. Indications for HTx were dilated cardiomyopathy (n = 18) and ischemic heart disease (n = 6). Indications for non-HTx were ischemic heart disease (n = 19), aortic valve replacement (n = 5), and mitral valve replacement (n = 6). The groups are described in Table 1. Cold crystalloid cardioplegia (St Thomas Hospital solution) was used for cardiac preservation in both groups of patients. In the HTx group the heart was perfused with 1000 mL of cardioplegic solution before donor cardiectomy. In the non-HTx group, the total volume of cardiplegic solution used was 1300 mL (median) with lower and upper quartiles of 1000 and 1500 mL, respectively, depending on the crossclamping time. The postoperative care was similar in both groups of patients, except for the immunosuppressive therapy.

Immunosuppressive Protocol in the HTx Group

Generally, the immunosuppressive regimen consisted of azathioprine (1.5 mg/kg before transplantation and 3 $mg \cdot kg^{-1} \cdot d^{-1}$ on days 1-2 after the operation reduced to 2 $mg \cdot kg^{-1} \cdot d^{-1}$ on days 3-7), methylprednisolone (500 mg before the operation and then 300 mg/d for the first 3 postoperative days), cyclosporine A (INN: ciclosporin A; 0.5 $mg \cdot kg^{-1} \cdot d^{-1}$ on days 1-2 after the operation and then maintained at 600-800 ng/mL blood on days 3-7), and prednisone (0.6 $mg \cdot kg^{-1} \cdot d^{-1}$ on days 3-7).

Sampling and Determination of Cytokine and Lipid Peroxide Levels

Heparinized peripheral blood samples were taken from the patients before the operation; at the time of reperfusion; 30 minutes, 4 hours, and 24 hours after reperfusion; and on the seventh day after the operation. Total and differential counts, hematocrit levels, and the oxidative burst of phagocytes were determined immediately. Plasma samples for cytokine and lipid peroxidation detection were frozen and stored at −30°C. Plasma levels of cytokines were determined by using commercially available enzyme-linked immunosorbent assay kits from R&D Systems (Quantikine IL-6, IL-8, and IL-10). The detection limit for cytokine levels was 5 pg/mL. The concentration of thiobarbituric acid reactive substances, which is the index of lipid peroxidation, was determined spectrophotometrically.[13]

Oxidative Burst of Phagocytes

Luminol-enhanced chemiluminescence of whole-blood phagocytes was measured with a Luminometer LM-01T (Immunotech), as described previously.[4] The principle of the method is based on luminol interaction with the phagocyte-derived free radicals, which results in large measurable amounts of light. Spontaneous and opsonized zymosan particle (OZP)-activated chemiluminescence was measured for 60 minutes at 37°C. The integral value of the chemiluminescence reaction, which represents the total ROS production by the blood phagocytes, was corrected for 10^3 neutrophil granulocytes.

Statistical Analysis

Data were not normally distributed, and therefore nonparametric statistical methods were used (Statistica for Windows 5.0, StatSoft, Inc). Data are expressed as medians and lower and upper quartiles. At each time point, the Mann-Whitney *U* test was applied to compare the groups of patients. The Wilcoxon test was used for the inspection of differences between preoperative values and different time points within each group. Interrelationships among variables were assessed by using the Pearson correlation test.

Results

Time Profiles of Cytokine Levels

Plasma levels of IL-6, IL-8, and IL-10 were increased in patients undergoing HTx at the time of reperfusion, with

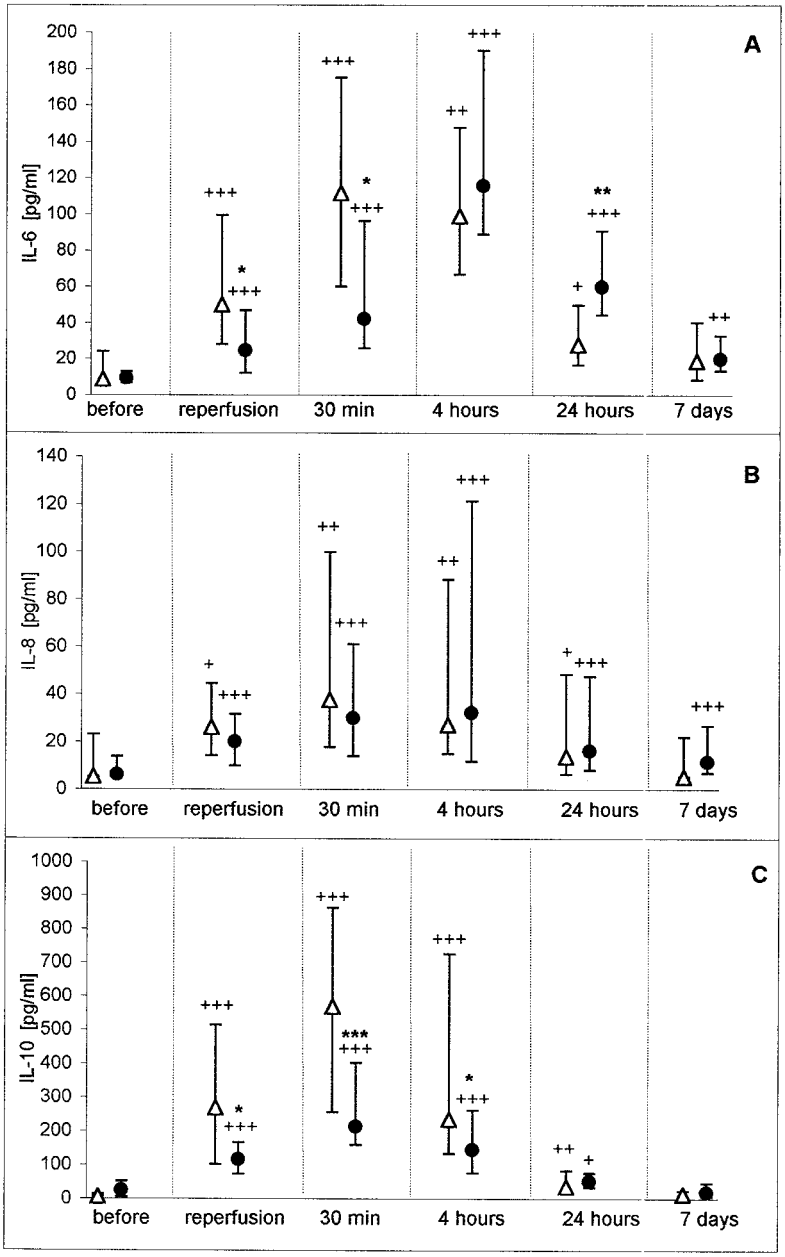

Figure 1. Time profiles of IL-6 (A), IL-8 (B), and IL-10 (C) plasma levels. The ordinate represents actual values in both the HTx *(open triangles)* and non-HTx *(filled circles)* groups at different sampling times: before the operation; at the time of reperfusion; 30 minutes, 4 hours, and 24 hours after reperfusion; and 7 days after operation. *Asterisks* mark the sampling times when the values of a specified parameter differed significantly between the HTx (n = 24) and non-HTx (n = 30) groups (Mann-Whitney *U* test; ***$P < .001$; **$.001 < P < .01$; *$.01 < P < .05$). *Crosses* mark the sampling times when the values of a specified parameter differed significantly from the preoperative values (Wilcoxon matched paired test; +++$P < .001$; ++$.001 < P < .01$; +$.01 < P < .05$).

further remarkable increases 30 minutes after reperfusion (Figure 1). Whenever a stimulation of IL release occurred, it decreased sharply 24 hours after reperfusion and returned to preoperative levels on the seventh day after the operation. All the observed cytokines were increased in a similar pattern in the non-HTx group. However, IL-6 and IL-8 plasma levels reached their peak values later (4 hour after reperfusion) and did not return to the preoperative levels on the seventh day after the operation in the non-HTx group. The plasma levels of IL-6 were higher in the HTx group

than in the non-HTx group in the early phase of reperfusion (up to 30 minutes after reperfusion), whereas IL-6 levels were higher in the non-HTx group 24 hours after reperfusion (Figure 1, *A*). The plasma levels of IL-8 did not remarkably differ between the HTx and non-HTx groups (Figure 1, *B*). IL-10 plasma levels were higher in the HTx group than in the non-HTx group from reperfusion to 4 hours after reperfusion (Figure 1, *C*).

Time Course of Changes in Total and Differential Counts of Leukocytes

Total leukocyte counts were increased in both groups of patients from reperfusion to the seventh day after the operation when compared with the preoperative levels (Figure 2, *A*). Leukocyte numbers were higher in the HTx group at all sampling intervals.

The sharp increase in the total number of leukocytes was associated with a relative increase in neutrophils (mainly of not fully maturated neutrophils; Figure 2, *B*). They increased remarkably at the time of reperfusion and remained higher up to 24 hours after reperfusion in both groups of patients. The percentage of segmented-maturated neutrophils was increased from the time of reperfusion to 24 hours after reperfusion only in the non-HTx group (Figure 2, *C*). In the HTx group the segmented neutrophils were increased only 24 hours after reperfusion and on the seventh day after the operation.

Time Profiles of Spontaneous and Activated Chemiluminescence

Spontaneous chemiluminescence was decreased during reperfusion and 30 minutes after reperfusion in the HTx group when compared with the preoperative level (Figure 3, *A*). After that, the chemiluminescence activity oscillated around the preoperative value. On the other hand, spontaneous chemiluminescence was increased when compared with its preoperative levels in the non-HTx group at all sampling intervals. OZP-activated chemiluminescence was not increased to greater than the preoperative level in the HTx group at any of the sampling intervals (Figure 3, *B*). However, in the non-HTx group the activated chemiluminescence was increased at all sampling intervals compared with the preoperative level. Both spontaneous and OZP-activated chemiluminescence were higher in the non-HTx group than in the HTx group at all perioperative and postoperative sampling times.

Time Course of Malondialdehyde Concentration

Plasma levels of thiobarbituric acid reactive substances were increased 30 minutes and 4 hours after reperfusion in both groups of patients (Table 2).

Correlation Among Monitored Parameters

Correlation analysis detected positive correlation between IL-6 and IL-8 in samples from both the HTx and non-HTx groups at the time of reperfusion and 30 minutes, 4 hours, and 24 hours after reperfusion (Table 3). No other consistent correlations were found among the evaluated parameters.

Discussion

Cardiac operations involving CPB is associated with a systemic inflammatory response, which is an extremely complex phenomenon related to the release of various cytokines.[1] Their release can be triggered by many factors, such as surgical procedure, duration of the ischemia and reperfusion in the heart, exposure of blood to the extracorporeal circuit, or release of endotoxin.[6] The release of cytokines is important because such a release is implicated in the development of postoperative complications.[2,3,7,8,11,14] A better understanding of cytokine responses might lead to new therapeutic implications.

The increase in the plasma levels of IL-6, IL-8, and IL-10 within the early phase of reperfusion in patients undergoing cardiac operations involving CPB has already been described in many studies.[3,5,6,9-12,15-17] However, only several studies have evaluated these parameters in patients undergoing HTx[18,19] or compared both groups of patients.[6] The cytokine levels mostly normalized within the first or second postoperative days in both HTx and cardiac operations. These results were confirmed in our study, in which IL-6, IL-8, and IL-10 kinetics were measured during and after HTx and non-HTx operations involving CPB. Heart storage and longer duration of acute ischemia starting from aortic crossclamping are important factors differentiating the early phase of HTx from non-HTx.[6] Therefore a stronger inflammatory response might be expected during HTx. On the other hand, the HTx group received the immunosuppressive drugs azathioprine and methylprednisolone during the operation. It is well documented that early steroid administration before CPB can reduce the inflammatory response and enhance the release of IL-10.[8,10,18,20]

We found IL-6 and IL-8 plasma concentrations to be approximately on the same level in both groups of patients. On the other hand, the plasma concentration of IL-10 was remarkably higher in the HTx group. Therefore we suggest that immunosuppressive therapy prevented the expected more intensive release of IL-6 and IL-8 in these patients. To the contrary, the extended IL-10 release in the HTx group could also be caused by both immunosuppressive therapy and a more profound ischemic period. The absence of immunosuppressive therapy in the non-HTx group might be a reason for the higher IL-6 and IL-8 concentrations found in these patients on day 7 after the operation. Contrary to our results, Wan and colleagues[6] found higher IL-8 and IL-6 release in patients having undergone HTx than in patients after non-HTx. IL-10 was at the same level in both groups. In our study the cytokine levels and blood phagocyte ROS production had relatively low variability before surgical treatment and did not differ significantly between the 2

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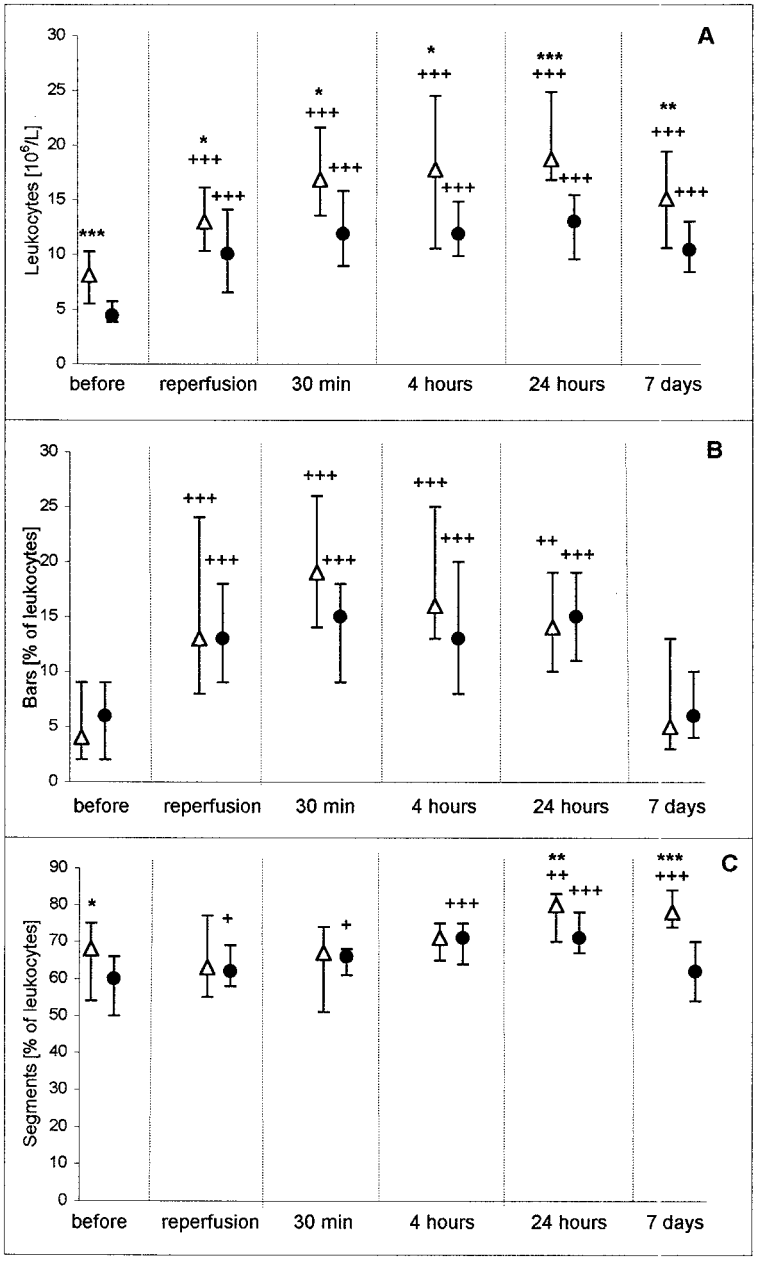

Figure 2. Time profiles of leukocyte counts. Panel A shows the total leukocyte counts corrected for hemodilution. Panel B shows the relative counts of not fully maturated neutrophils *(bars)*, and panel C shows the relative counts of fully maturated neutrophils *(segments)*. The ordinate represents actual values in both the HTx *(open triangles)* and non-HTx *(filled circles)* groups at different sampling times: before the operation; at the time of reperfusion; 30 minutes, 4 hours, and 24 hours after reperfusion; and 7 days after operation. *Asterisks* mark the sampling times when the values of a specified parameter differed significantly between the HTx (n = 24) and non-HTx (n = 30) groups (Mann-Whitney *U* test; ***$P < .001$; **$.001 < P < .01$; *$.01 < P < .05$). *Crosses* mark the sampling times when the values of a specified parameter differed significantly from the preoperative values (Wilcoxon matched paired test; +++$P < .001$; ++$.001 < P < .01$; +$.01 < P < .05$).

groups of patients. However, a relatively high variability of the parameters studied was observed in each group of patients during the perioperative and postoperative periods. Patients in the HTx group were younger than those in the non-HTx group, but there is no evidence that the cytokine release is influenced by age.[6,15] The duration of CPB and the

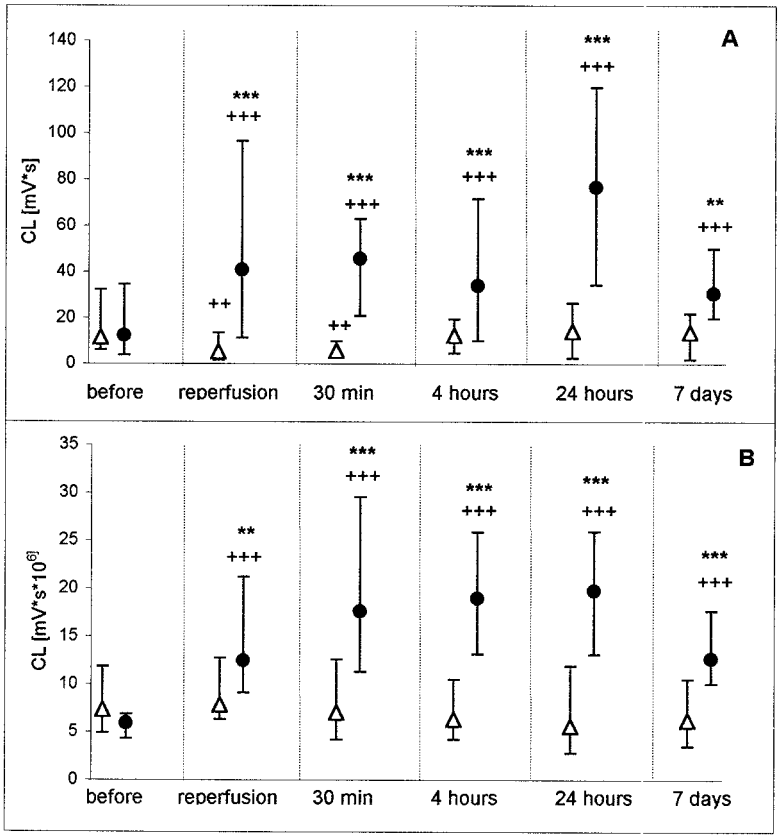

Figure 3. Time profiles of spontaneous (A) and OZP-activated (B) whole-blood chemiluminescence activities of phagocytes. The ordinate represents actual values in both the HTx *(open triangles)* and non-HTx *(filled circles)* groups at different sampling times: before the operation; at the time of reperfusion; 30 minutes, 4 hours, and 24 hours after reperfusion; and 7 days after operation. *Asterisks* mark the sampling times when the values of a specified parameter differed significantly between the HTx (n = 24) and non-HTx (n = 30) groups (Mann-Whitney *U* test; ***$P < .001$; **$.001 < P < .01$. *Crosses* mark the sampling times when the values of a specified parameter differed significantly from the preoperative values (Wilcoxon matched paired test; +++$P < .001$; ++$.001 < P < .01$).

TABLE 2. Time profiles of lipid peroxidation

		Sampling times					
	Patients	Before	Reperfusion	30 min	4 h	24 h	7 d
TBARS (μmol/L)	HTx	6.1 (3.7; 8.2)	6.2 (4.4; 8.3)	8.0 (6.6; 11.3)*	8.8 (5.0; 11.1)*	6.8 (4.4; 8.6)	6.5 (5.3; 8.1)
	Non-HTx	5.6 (3.4; 8.2)	6.2 (4.2; 8.2)	8.1 (4.9; 11.4)*	8.4 (5.1; 12.0)*	5.8 (3.6; 8.1)	6.0 (3.9; 6.2)

Time profiles of the Thiobarbituric acid reactive substances plasma concentrations in both the HTx (n = 24) and non-HTx (n = 30) groups of patients at different sampling times: before the operation; at the time of reperfusion; 30 minutes, 4 hours, and 24 hours after reperfusion; and 7 days after the operation. Data are expressed as medians and lower and upper quartiles. *Asterisks* mark the sampling times when the values of a specified parameter differed significantly from the preoperative values (Wilcoxon matched paired test; *$.001 \leq P < .01$).
TBARS, Thiobarbituric acid reactive substances.

ischemic period (starting from aortic crossclamping on the donor heart in the HTx group) was longer in the HTx group than in the non-HTx group. Several authors have found a correlation between the ischemic period or the duration of CPB and IL-6 or IL-8 concentrations.[6,10,11,14] It suggests an important role of ischemia duration, as well as a role of the inflammatory consequences of longer periods in which blood is exposed to artificial materials or abnormal shear

TABLE 3. Interrelationships between IL-6 and IL-8 at the time of reperfusion, 30 minutes after reperfusion, 4 hours after reperfusion, and 24 hours after reperfusion in the HTx and non-HTx groups

		Sampling times			
	Patients	Reperfusion	30 min	4 h	24 h
IL-6 × IL-8 correlation	HTx	0.505 $P = .02$	0.703 $P < .001$	0.645 $P = .002$	0.747 $P < .001$
	Non-HTx	0.510 $P = .004$	0.618 $P < .001$	0.642 $P < .001$	0.548 $P = .001$

The values of the Spearman correlation coefficient with level of significance are shown for each sampling time.

forces.[6,10,11] On the other hand, no correlation was found with IL-10.[6,12,17] Some authors failed to find any correlation between the peak levels of IL-6 or IL-8 and CPB or aortic crossclamping duration.[3,9]

The site of cytokine release during and after HTx and non-HTx is unclear. Fujiwara and associates[3] suggested that cytokines in serum did not originate from a specific location and that they could be released from leukocytes, macrophages, endothelial cells of systemic vasculature, or the parenchyma of tissue, such as muscles. On the other hand, the myocardium was suggested as an important source of IL-6 and IL-8 in CPB after reperfusion of the heart,[8] and hypoperfused liver has been shown to be the main source of IL-10 during CPB.[12] We found a positive correlation between the plasma levels of IL-6 and IL-8 in both groups of patients, which suggests an activation of their common source (monocytes-macrophages). Although the kinetics of IL-6, IL-8, and IL-10 releases measured in individual groups of patients were similar, no correlation with IL-10 was found. Frering and coworkers[9] failed to observe any correlation between IL-6 and IL-8 levels in patients after CPB. On the other hand, Wan and associates[12] found a positive correlation between IL-10 plasma levels and IL-8 and IL-6 in the non-HTx group.

In the present study a remarkable increase in lipid peroxidation measured on the basis of thiobarbituric acid reactive substances has been found in both groups of patients 4 and 24 hours after the operation. At the cellular level, the excessive oxidative stress causes damage of cell membrane lipids (lipid peroxidation), denaturation of proteins, breakdown of carbohydrates, and, consequently, lysis of endothelium and tissue injury.[21] Our results are consistent with several previous studies in which oxidative stress and subsequent lipid peroxidation were demonstrated in patients undergoing cardiac operations involving CPB.[16,21-24]

In our study the significant increase in leukocyte numbers was observed in both groups of patients starting at the beginning of the operation. The higher leukocyte numbers in the HTx group were found at all sampling intervals, and this difference could result from the high doses of steroids in the HTx group.[25] An inhibitory effect of methylprednisolone on neutrophil sequestration in the pulmonary vascular system has been shown by other authors.[26] Activated polymorphonuclear leukocytes, which are sequestered into the pulmonary circulation and produce increased amounts of ROS, have been shown to be a causal factor of CPB's deleterious effects.[5,7,14] We confirmed, in this study, the results from our previous study that the blood phagocyte ROS production is remarkably increased during reperfusion up to the seventh postoperative day in patients undergoing cardiac operations.[4] The concurrent increase in neutrophil numbers, together with their boosted metabolic activity, contributed to the increased ROS production. Neutrophil priming with an increased production of superoxide during CPB has also been described by other authors.[27,28] On the contrary, we have not found any increase in activated ROS production (spontaneous ROS production was even reduced) at an early phase of reperfusion in patients in the HTx group. It means that although neutrophil numbers were increased, their metabolic activity was suppressed. One of the possible explanations is the relative increase of not fully maturated neutrophils with lower potential of ROS production, whereas fully maturated neutrophils remained without a change at early times of reperfusion in patients in the HTx group. On the other hand, the relative counts of both forms of neutrophils were increased at these time intervals in the non-HTx group. Another possible explanation for the depressed activity of blood phagocytes in the HTx group could be the immunosuppressive therapy. There is a lack of studies describing the influence of immunosuppressive agents on the spontaneous and activated oxidative burst of phagocytes. However, we did not confirm a direct effect of immunosuppressive agents on the production of ROS by neutrophils in in vitro experiments in our previous study.[29] Another possible explanation could be an influence of IL-10 on ROS production by blood phagocytes. There have been some reports showing inhibitory effects of IL-10 on the activity and ROS production by phagocytes,[30] but contrary reports also exist.[31] We did not confirm a direct effect of IL-10 on the whole-blood production of ROS in in vitro experiments in concentrations up to 10 times higher than the average levels occurring during operations (unpublished data). The low chemiluminescence reactivity of phagocytes during HTx remains to be elucidated.

In conclusion, we proved that the processes associated with HTx and non-HTx operations involving CPB are connected with increased oxidative stress and with increased production of both proinflammatory and anti-inflammatory cytokines. Concentrations of proinflammatory cytokines were on the same level in both groups, whereas the anti-inflammatory IL-10 level was higher in the non-HTx group. We confirmed the contribution of circulating phagocytes to the increased oxidative stress in the non-HTx group. On the contrary, the activity of circulating phagocytes was depressed in patients in the HTx group. The observed differences between HTx and non-HTx operations could be caused mainly by the immunosuppressive therapy in HTx operations. However, other factors, such as a longer duration of ischemia in HTx, could be involved.

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ORIGINAL ARTICLE

Phagocyte-derived oxidants and plasma antioxidants in haemodialysed patients

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Abstract

Objective. Oxidative stress is one of the important complications occurring in haemodialysis. The aim of the study was to determine haemodialysis-induced changes in oxidative burst of phagocytes and the antioxidative properties of plasma. ***Methods.*** Twenty-seven patients and 50 healthy controls were examined. Oxidative burst of phagocytes and plasma antioxidative potential were measured luminometrically. Concentrations of major plasma antioxidants (vitamin E, bilirubin and uric acid) were also determined. ***Results.*** Phagocyte chemiluminescence was higher in patients before haemodialysis compared with that in controls and decreased after haemodialysis compared with predialysis status. A significant increase in plasma antioxidative potential and uric acid was found in patients before haemodialysis. These parameters decreased after haemodialysis compared with both predialysis and control values. ***Conclusions.*** The higher generation of phagocyte-derived oxidants and the decline in plasma antioxidative properties after haemodialysis confirm insufficient antioxidant defence in patients with chronic renal failure.

Key Words: *Chemiluminescence, hematology, immunity, kidney diseases, markers of oxidative stress*

Introduction

Haemodialysis (HD) is a life-sustaining procedure for many people worldwide. Growing evidence has accumulated suggesting that oxidative stress may be one of the important complications occurring in HD [1,2]. Oxidative stress has been postulated to be an important risk factor for cardiovascular disorders, and cardiovascular diseases are the major cause of mortality in patients receiving HD [3]. Owing to the non-self nature of the dialysis membrane materials, which come into contact with various effector systems in blood, various humoral and cellular inflammatory reaction cascades can be triggered [4].

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Equilibrium that is established between reactive oxygen species (ROS) and antioxidant defence factors under physiological conditions can be impaired by extracorporeal renal replacement mechanisms which can provoke the formation of ROS or weaken the antioxidant defence, e.g. by eliminating substances with antioxidant properties [5]. In this way, oxidative stress may contribute to the increased risk of atherosclerosis in HD patients [6].

The aim of the present study was to measure and compare HD-induced changes in the plasma antioxidative potential, in the concentrations of major plasma antioxidants, and in both spontaneous and activated whole blood phagocyte-derived production of reactive oxygen species in patients with chronic renal failure, and to compare with healthy subjects.

Methods

Patients and blood collection

Twenty-seven patients (9 M, 18 F) undergoing regular maintenance HD were included in the study. Their mean age was 63.5 years (range 30–78 years) and they had been on chronic HD for 3.5 ± 2.9 years (range 1–13 years). Their chronic dialysis protocol was of 3–4 h thrice weekly depending on residual renal function. Renal failure was caused by glomerulonephritis ($n=9$), tubulointerstitial nephritis ($n=7$), diabetic nephropathy ($n=5$), polycystic liver and kidney disease ($n=4$) and pyelonephritis ($n=2$). Patients included in this study underwent bicarbonate HD with hemophan membrane (GFS+16, GAMBRO, Lund, Sweden). Standard unfractionated heparin as an anticoagulant in the dose 3000–5000 IU was used. All patients received phosphate binders, recombinant human erythropoietin (4000–8000 IU/week) and a low i.v. dose of iron once a week (iron supplementation was not carried out before blood sampling on the day of the study). The patients were treated with antihypertensive drugs (ACE inhibitors, beta-blockers, calcium channel inhibitors, diuretics). None of HD subjects presented with evidence of infectious disease during at least 1 week prior to blood sampling for the laboratory analysis. Baseline laboratory results of the patients were as follows: 39.6 ± 0.59 (g/L) albumin, 3.02 ± 0.09 (10^{12}/L) erythrocytes and 9.75 ± 0.28 (g/L) hemoglobin. Each patient gave informed consent to participation in the study. Heparinized blood samples were drawn at the beginning and at the end of the HD procedure from the arteriovenous shunt. Fifty healthy controls (laboratory staff; 34 F and 16 M), mean age 53.2 (range 23–67) years were also examined. Based on laboratory tests and clinical findings, they were determined to be free of disorders of the kidney, liver, heart, endocrine system and none of them showed signs of systemic infection (leukocytosis, fever).

Reagents

Luminol was obtained from Molecular Probes (USA) and 2,2-azo-bis-amidinopropane hydrochloride from Polyscience (USA). All other highest-grade chemicals were purchased from local distributors or Sigma-Aldrich (USA).

Phagocyte-derived ROS measurement

The kinetics of ROS production by blood phagocytes was analysed by luminol-enhanced chemiluminescence (CL) for a period of 60 min using an automated, multiple sample luminometer BioOrbit 1251 (BioOrbit, Finland). Spontaneous CL was measured in

samples containing 1 μL of whole blood and 1 mM luminol (Molecular Probes, USA); the total reaction volume of 500 μL was adjusted with Hanks balanced salt solution (pH 7.4). To activate ROS generation, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 5 μmol/L) or zymosan from *Saccharomyces cerevisiae* opsonized with human serum (OZP, 0.1 g/L) was used (for details, see [7]). The recorded values included the intensity of CL (expressed as millivolts – mV) emitted during the time interval studied. The integral of the CL kinetic curves is expressed as mV per 60 min (mV/60 min). Because the whole blood CL activity is caused almost exclusively by neutrophils, the integral of CL was also corrected to 1000 neutrophils.

Antioxidative potential of plasma

Total peroxyl radical-trapping antioxidative potential (TRAP) of plasma was also measured using CL assay. This assay is based on the measuring of peroxyl radical production at a constant rate by a thermal decomposition of 2,2-azo-bis-amidinopropane hydrochloride [8]. While the peroxyl radical production was monitored by luminol-enhanced CL, the TRAP value was determined from the duration of the time period during which the CL signal was diminished by plasma antioxidants. Trolox, a water-soluble analogue of α-tocoferol, was used as a reference inhibitor.

The blood count

The red blood count, hemoglobin, blood leukocyte and the differentiation counts were determined using Coulter STKS (Coulter, UK).

Individual antioxidant determinations

Plasma bilirubin, uric acid and albumin were determined using Hitachi 911 analyser (spectrophotometric method, commercial kits Roche). Vitamin E concentration was measured using a fluorescence spectrophotometer (Perkin-Elmer).

Statistical analysis

All values were expressed as means $\pm$ SEM. All variables were tested for Gaussian distribution using the Kolgomorov-Smirnov and Shapiro-Wilk's tests. Paired *t*-test for normally distributed values or Wilcoxon's matched pairs test (for values which were not distributed normally) for within-group testing of differences (pre-dialysis versus post-dialysis values) were used. A p-value < 0.05 was considered statistically significant.

Results

While the total leukocyte count in the blood was not changed in patients before HD in comparison with healthy controls, both absolute and relative neutrophil counts were significantly increased. HD treatment itself did not influence the leukocyte and neutrophil counts significantly (Table I).

Spontaneous ROS production by blood phagocytes, measured as CL activity, was not significantly changed before HD compared with that of healthy controls (Figure 1, upper panel). However, HD treatment caused a significant decrease in total CL activity of blood compared to pre-HD values. After a correction of total CL for the number of neutrophils,

Table I. Leukocytes (total count) and neutrophils (total count and percentage of total leukocyte count) in blood before and after haemodialysis. Asterisks mark values that differed significantly from the relevant healthy control ($p < 0.05$).

	Healthy controls	Patients before HD	Patients after HD
Leukocytes [10^9/L]	6.60 ± 0.29	6.93 ± 0.41	6.44 ± 0.34
Neutrophils [10^9/L]	3.67 ± 0.21	$4.89 \pm 0.33^{\star}$	$4.75 \pm 0.31^{\star}$
Neutrophils [%]	55.50 ± 1.53	$70.30 \pm 2.02^{\star}$	$73.10 \pm 1.99^{\star}$

the CL activity decreased after HD and differed significantly from both the controls and from samples collected before HD. On the other hand, significantly higher total CL of blood activated by OZP was observed in samples collected before HD and after HD compared to healthy controls. OZP-activated CL of blood phagocytes before HD corrected to the number of neutrophils still differed significantly from the controls in contrast to samples after HD, where no significant differences were detected (Figure 1, middle panel). The most obvious differences were observed in FMLP-activated CL (Figure 1, lower panel), where samples before as well as after HD were significantly higher than the values of healthy controls even when the HD procedure significantly decreased FMLP-activated CL activity. These changes remained significant even after the correction of CL to the number of neutrophils.

As can be seen in Figure 2, the patients' TRAP values before HD treatment were significantly higher compared with those of healthy controls. However, after HD treatment, the TRAP values went down and became significantly lower than TRAP values of healthy controls. The difference between pre-dialysis and post-dialysis values was also statistically highly significant (1208 ± 33 versus 578 ± 21 μmol/L). We found a significant increase in uric acid and a decrease in albumin, bilirubin and vitamin E in HD subjects. While uric acid decreased significantly during the HD procedure, even under the level of healthy controls, the concentration of bilirubin increased during HD while still significantly lower compared with that of healthy controls (Table II). There were no significant differences in laboratory results between males and females.

Discussion

It has been widely accepted that there is a physiological steady-state between the production of oxidants and their neutralization by antioxidants established under normal conditions. Overproduction of free radicals causes tissue damage after overwhelming the endogenous antioxidant defence system when the oxidant-antioxidant balance is disturbed [9]. Neutrophils, the effector cells of the first-line of defence against foreign invaders, produce ROS via a multi-component enzyme complex – NADPH oxidase [10]. That is why neutrophils have an irreplaceable role in the proper performance of the immune system. However, excessive ROS production can damage the body's own cells and tissues and contribute to the development of a number of diseases. All these factors make an analysis of oxidative burst of neutrophils important in clinical practice. Among the several existing methods, recording the CL activity is the most convenient for measuring the oxidative burst of neutrophils.

As published previously, neutrophils obtained from HD patients immediately before HD treatment showed a higher spontaneous rate of ROS production than neutrophils from healthy subjects [2]. On the other hand, the high frequency of bacterial infections in end-stage renal patients suggests that neutrophil dysfunction may be involved in the immune

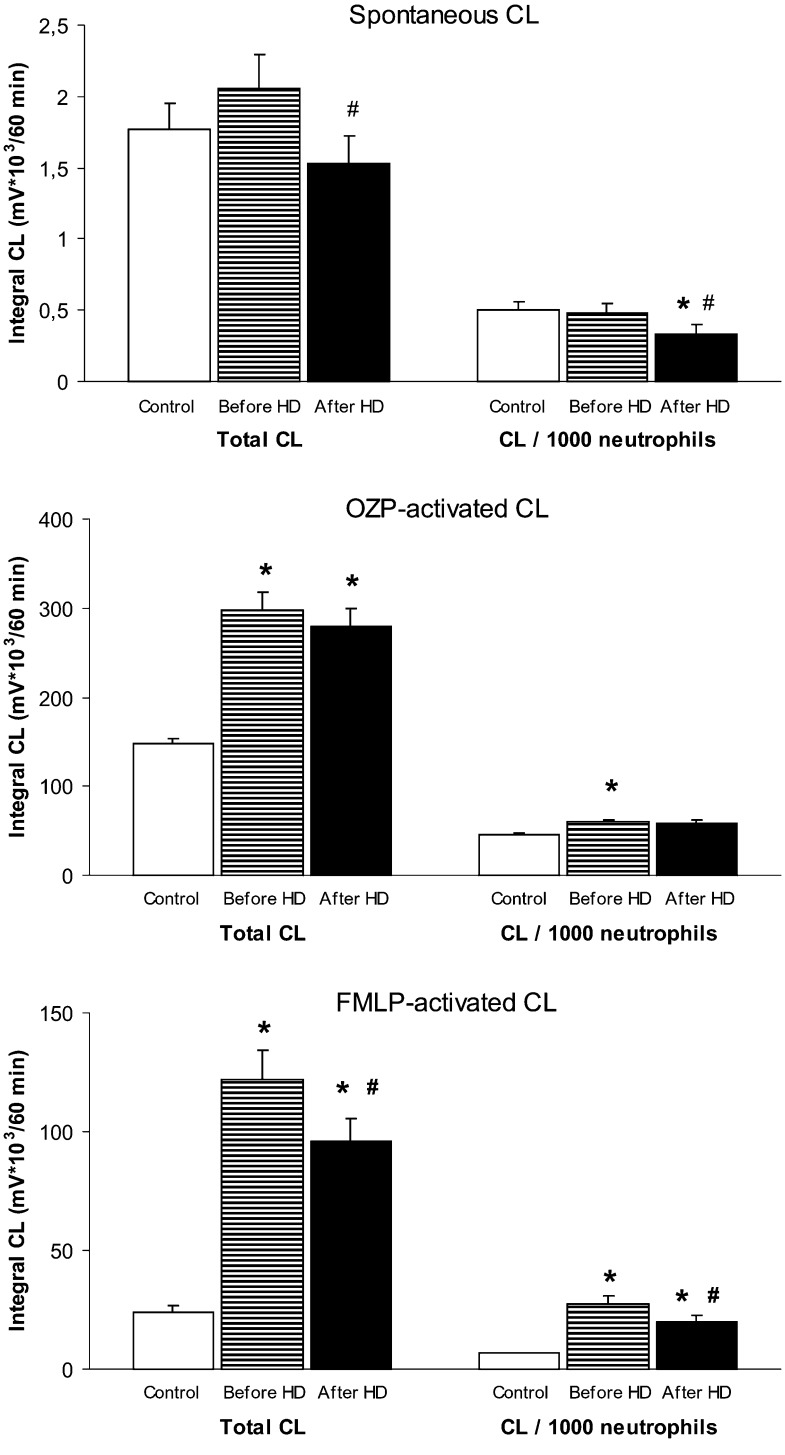

Figure 1. Integral of blood phagocyte chemiluminescence emitted during a 60-min period. Data are expressed as the mean ± SEM. Asterisks mark values that differed significantly from the relevant healthy control ($p < 0.05$), hash symbols mark values after HD that differed significantly from the values before HD ($p < 0.05$).

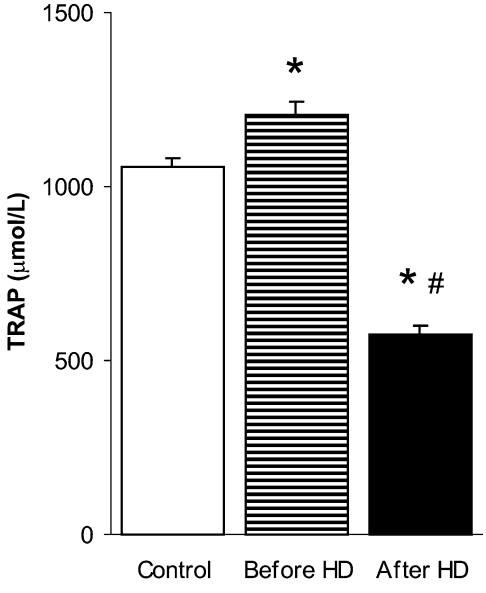

Figure 2. Total peroxyl radical-trapping antioxidative potential of plasma samples collected from patients before and after haemodialysis treatment in comparison with healthy subjects. Data are expressed as the mean ± SEM. Asterisks mark values that differed significantly from the relevant healthy control ($p < 0.05$), hash symbol marks value after HD that differed significantly from the value before HD ($p < 0.05$).

deficiency observed in patients with uraemia [1]. Actually, Perianayagam et al. [11] reported increased basal ROS production, lower mitochondrial membrane potential and impaired IL-10 synthesis in monocytic cells exposed to uraemic plasma. We found increased ROS production by activated phagocytes in HD patients before HD. According to published articles, this increase could be caused by guanidino compounds retained during uraemia. As discussed by Vanholder et al. [12], guanidino compounds such as guanidine and methylguanidine are highly increased in uraemic biological fluids. Both these compounds stimulate the proliferation of undifferentiated HL-60 cells. Moreover, guanidine potentiated PMA-activated CL of differentiated HL-60 cells and methylguanidine enhanced the lipopolysaccharide-stimulated production of inflammatory mediator TNF-α by normal human monocytes [13]. The decrease in ROS production at the end of HD, which was found in our study, is consistent with the hypothesis that the metabolic response of blood phagocytes is adversely affected by HD. Decreased ROS production during and at the end of the HD procedure measured by different methods has been described previously [14,15]. On the other hand, Descamps-Latscha et al. [16] found that both spontaneous and activated CL were unchanged during the HD procedure. Compared

Table II. Antioxidants measured in blood before and after haemodialysis. Data are expressed as the mean ± SEM. Asterisks mark values that differed significantly from the relevant healthy control ($p < 0.05$), hash symbols mark values after HD that differed significantly from the values before HD ($p < 0.05$).

	Healthy controls	Patients before HD	Patients after HD
Bilirubin (μmol/L)	10.4 ± 0.69	6.5 ± 0.39*	7.9 ± 0.44*#
Uric acid (μmol/L)	256 ± 11.7	294 ± 16.4*	97 ± 59*#
Vitamin E (μmol/L)	30.37 ± 0.92	23.85 ± 0.53*	25.1 ± 0.18*

with healthy subjects, elevated activated CL before HD corresponds to our previous work, in which we found increased lipoperoxide (malondialdehyde) serum concentration in haemodialysis subjects [17]. The cause of the decreased metabolic activity of phagocytes during regular HD is not clear yet. One of the possible explanations may be the degranulation of polymorphonuclear neutrophils during HD [18,19]. Degranulation of neutrophils is followed by a decreased amount of myeloperoxidase, which may be important in luminol-induced CL. Our data support the hypothesis that the decrease in blood phagocyte metabolic activity after HD is myeloperoxidase dependent. On the other hand, Cohen et al. [20] found no differences between MPO concentration in PMNs before and after HD. ROS generation during HD can also be alleviated by heparin [21]. The second possibility is that a pre-existing stimulation or exhaustion of blood phagocytes exposed to activated complement could subsequently lower their metabolic activity. This hypothesis is corroborated by the finding of decreased spontaneous ROS production without any stimulation, and by the finding of no differences in ROS generation irrespective of the receptors and mechanism through which the activators stimulate the cells: OZPs are recognized by complement and immunoglobulin receptors, FMLP binds to a specific formyl peptide receptor. Regardless of the up-regulation of the complement activity [22], we found a decreased OZP-stimulated metabolic activity of blood phagocytes after HD.

We found an elevated plasma TRAP in HD patients before regular HD. But although the serum TRAP is increased in patients with chronic renal failure, a depletion of some key chain-breaking antioxidants may result in redox imbalance with oxidative stress and consequently in accelerated atherogenesis due to increased lipid peroxidation [23]. Prakash et al. found increased plasma protein oxidation (protein thiol oxidation) and lipid peroxidation in both chronic renal failure and HD patients [24]. Also, the deficient antioxidant pool of the erythrocyte glutathione-defence system, which may be significantly affected by HD, can contribute to the redox imbalance in HD patients [25,26]. Elevated TRAP in patients before HD is likely to be connected with the high predialysis uric acid level [27]. The pre- and post-dialysis decrease in other physiological antioxidants (bilirubin, vitamin E) in comparison with healthy controls may contribute to ineffective neutralization of ROS in subjects with renal failure. Bilirubin is considered to be a potent physiological antioxidant [28]. Its slight elevation after HD is probably only due to concentration effect. Dramatically decreased TRAP after HD (mainly due to a lower level of uric acid) may indicate insufficient antioxidant defence in patients with chronic renal failure and the need for therapeutic support. Schettler et al. [29] reported that chronic stress caused by uraemia and/or HD may influence the gene induction of enzymatic defence systems. It can be assumed that disturbances in intracellular and extracellular redox systems that occur in chronic renal failure and HD may provide an explanation for the high risk of cardiovascular complications in these patients rather than the high reactive oxygen species production. Although it is not strictly defined how big the changes in redox steady state should be before tissue damage occurs, any prolonged oxidative stress could contribute to the long-term complications in uraemic patients [30].

It can be concluded that the generation of phagocyte-derived ROS probably does not pose a major threat to patients with chronic renal failure. The decrease in TRAP and physiological antioxidants after HD treatment suggests insufficient antioxidant defence in the patients. Thus, the increment in endogenous and exogenous antioxidant capacities in plasma might be thought to prevent possible radical-induced damage in patients undergoing regular HD treatment. A CL method for the detection of total ROS production

seems to be a very useful tool for obtaining a complex view of the HD-induced changes in the phagocyte ROS production (activation and metabolic status of blood phagocytes). Information on phagocyte metabolic activity and the TRAP potential is also of great importance in other pathophysiological conditions because it provides the basic data needed for the indication of proper therapy improving immune defence mechanisms.

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Influence of Polysulfone and Hemophan Hemodialysis Membranes on Phagocytes

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Abstract. The aim of the study was to compare the effect of hemophane and polysulfone membranes on the phagocyte-derived production of reactive oxygen species (ROS) as well as on neutrophil CD11b and CD62L expression in patients undergoing regular hemodialysis. The effects of hemodialysis membranes were also studied in *in vitro* conditions after coincubating them with differentiated HL-60 cells. ROS production was measured using chemiluminometric and flow cytometric methods. Expression of CD11b, CD62L and mitochondrial membrane potential were detected by monoclonal antibodies and by the JC-1 fluorescent probe, respectively. Depressed ROS production was observed in patients already before dialysis. Further decrease in ROS production and an increase in CD11b expression were observed especially in patients after hemophan hemodialysis. Decreased ROS production and increased CD11b expression were observed also after incubation of HL-60 cells with hemophan membranes. Mitochondrial membrane potential dropped only after incubating cells with hemophan membranes proving its more serious adverse effects in comparison with the polysulfone membrane. In conclusion, deleterious effects of hemodialysis on the metabolic activity of phagocytes were proved. Combining chemiluminescent and flow cytometric methods for the detection of ROS production and determining mitochondrial membrane potential can be useful tools for the analysis of material biocompatibility.

Key words: Hemodialysis — Hemophan — Polysulfone – Reactive oxygen species — CD11b

Abbreviations: ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorodihydrofluorescein diacetate; HE, hydroethidine; PMA, phorbol myristate acetate; FMLP,

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N-formyl-L-methionyl-L-leucyl-L-phenylalanine; OZP, zymosan opsonized in human serum; MRF, median of relative fluorescence.

Introduction

Hemodialysis is a life sustaining procedure for hundreds of thousand patients world wide. Due to the non-self nature of dialysis membrane materials which come to contact with various effector systems in blood, various humoral and cellular inflammatory reaction cascades can be triggered (Kormoczi et al. 1999). Although cellulose based membranes are frequently used in clinical practice, their use is associated with transient marked neutropenia during the initial phase of dialysis (Nguyen et al. 1985; Himmelfarb et al. 1992; Rosenkranz et al. 1994) followed by rebound neutrophilia (Descamps-Latscha et al. 1991; Iijima et al. 1999). Cellulose membranes also induce marked complement activation, which is proposed to be a dominant mechanism for granulocyte activation during hemodialysis (Descamps-Latscha et al. 1991; Himmelfarb et al. 1995).

Since neutrophils are effector cells of the immune system, their proper numbers and function are essential for a defense against infective agents. Selectins, which are expressed on their surface mediate initial tethering and rolling of leukocytes along the endothelial lining of the postcapillary venules in inflamed tissue. CD62L (L-selectin) is constitutively expressed on leukocytes and rapidly shed from plasma membranes upon cell activation. β_2-integrins CD11a/CD18 and CD11b/CD18 (also known as MAC-1) are responsible for polymorphonuclear cell anchoring to the endothelium. Moreover, CD11b/CD18 and CD11c/CD18 also function as complement receptor 3 (CR3). The transformation of CD11b/CD18 from an inactivated to a transiently activated state with concomitant cell surface upregulation can be observed during leukocyte activation (Kormoczi et al. 1999). Complement activation caused by cellulose membranes is associated with a rapid and dramatic upregulation of CD11b/CD18 receptors (Himmelfarb et al. 1992). Increased cell surface expression of CD11b (Himmelfarb et al. 1992) and shedding of CD62L (Iijima et al. 1999) may be involved in increased granulocyte adhesiveness to the endothelium resulting in granulocytopenia (Kormoczi et al. 1999).

A major function of activated phagocytic cells in host defense is the production of ROS which play a major role in killing pathological microorganisms, but can also cause damage to the surrounding tissue (Bauer and Bauer 1999). It was proved that increased amounts of neutrophil-derived ROS are produced in patients dialyzed with cellulose membranes (Nguyen et al. 1985; Haag-Weber et al. 1989; Descamps-Latscha et al. 1991; Himmelfarb et al. 1993; Rosenkranz et al. 1999). A direct correlation between the nadir of leukocyte counts and ROS formation was shown by Rosenkranz et al. (1999). On the other hand, it was shown that cellulose membrane-induced neutrophil-derived ROS formation can occur *in vitro* (Rosenkranz et al. 1999) as well as during hemodialysis procedure (Kormoczi et al. 1999) independently of complement activation.

A growing body of evidence has accumulated suggesting that oxidative stress may be one of the important complications occurring in hemodialysis (Galli and Ronco 2000). A significantly increased oxidative stress associated with an insufficient activity of extracellular antioxidant mechanisms was observed in hemodialysed patients in comparison with healthy subjects in our previous studies (Lojek et al. 1998; Soška et al. 1995). However, these studies did not differentiate the effects of the hemodialysis procedure due to the type of membrane used. The aim of the present study was to examine and compare the effects of the hemodialysis procedure performed using hemophan (a frequently used cellulose membrane) and polysulfone (a synthetic membrane) on the phagocyte-derived production of ROS as well as on the expression of blood phagocyte surface antigens (CD11b and CD62L). To clarify the effects of the membranes, they were also evaluated *in vitro* while co-incubating the membranes with promyelocytic HL-60 cells differentiated to cells with neutrophil-like morphology.

Materials and Methods

Reagents

Phorbol myristate acetate (PMA), N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), zymosan from Saccharomyces cerevisiae, dimethyl sulfoxide, RPMI-1640 medium, gentamycin sulfate, heat inactivated fetal calf serum and luminol were obtained from Sigma-Aldrich (USA). Lysing solution Cal-Lyse, fluorescein isothiocyanate-labeled anti-human CD11b murine monoclonal antibody, phycoerythrin-labeled anti-human CD62L murine monoclonal antibody and appropriate control isotype murine antibodies were purchased from Caltag Laboratories (USA). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) and hydroethidine (HE) were purchased from Molecular Probes (USA). All other chemicals were purchased in the highest grade p.a. from local distributors.

Patients and related analyses

Patients: Twenty one patients undergoing chronic hemodialysis (8 men and 13 women), who had given informed consent, were included into the study. The mean age was 64.7 years (range of 45 to 85 years). The chronic dialysis protocol consisted of 4 h three times a week. The membrane materials used for chronic dialysis was polysulfone with surface area of 1.6 m^2 (F7HPS, Fresenius, Germany) and hemophan with surface area of 1.7 m^2 (GFS Plus 16, Gambro Dialysatoren, Germany). Dialyzers and lines were steam sterilized, and no patients had a dialyzer reuse. All patients underwent bicarbonate dialysis and anticoagulation with heparin (the mean dose being 4200 $\pm$ 1800 IU). Twenty five healthy volunteers (10 men and 15 women) were used as age matched control subjects.
Blood collection: Heparinized blood samples (50 $\times$ 10^3 IU/l) were drawn at the beginning and at the end of the hemodialysis procedure from the arterial line. Pre- and post-hemodialysis samples from each patient were examined 3 times for

the hemophan membrane (the beginning of the study, and 3 and 6 months after that) and 3 times for the polysulfone membrane (9, 12 and 15 months after the beginning of the study). The number of leukocytes in the blood and their relative differentiation counts were determined using Coulter counter STKS (Coulter, England).

Oxidative burst measurement of blood phagocytes: Luminol-enhanced chemiluminescence of whole blood phagocytes was measured using BioOrbit 1251 Luminometer (BioOrbit, Finland). The principle of the method is based on luminol interaction with the phagocyte-derived free radicals, which results in large measurable amounts of light. Briefly: The reaction mixture consisted of whole blood (1 μl for zymosan opsonized in human serum (OZP) and 10 μl for FMLP), 1×10^{-3} mol/l luminol (stock solution of 0.01 mol/l luminol in 0.2 mol/l borate buffer) and one of the activators (FMLP – 2×10^{-6} mol/l or OZP – 0.1 g/l). The total reaction volume of 500 μl was adjusted with Hanks balanced salt solution, pH 7.4. The assays were run in duplicates. Spontaneous chemiluminescence measurements in samples containing 10 μl of whole blood and other substances except any activator were included in each assay. The chemiluminescence emission of each vial was followed up for 60 minutes at 37°C. The integral value of the chemiluminescence reaction, which represents the total ROS production by blood phagocytes, was corrected to the number of granulocytes.

Determination of the cell surface expression of adhesion molecules: The measurements were performed according to the manufacturer's protocol (Caltag Laboratories, USA) using unfixed whole blood. Briefly: 100 μl of blood were incubated in plastic tubes (Falcon, USA) with anti-CD11b and anti-CD62L monoclonal antibodies (7.5 μl of each) at room temperature for 15 minutes. 100 μl of blood incubated with fluorescein isothiocyanate- or phycoerythrin-conjugated murine immunoglobulins of the same isotype were used as the negative controls. Then the samples were fixed by Cal-lyse and the red blood cells were lysed by distilled water. The remaining cells were resuspended in phosphate buffered saline, placed on ice and analyzed within 2 h. Ten thousand blood granulocytes selected on the basis of their typical scattering characteristics were analyzed by flow cytometer FACSCalibur (Becton Dickinson, USA). The median of relative fluorescence (MRF) was determined and corrected to the background fluorescence of the isotype control. The results were expressed as the means of MRF and standard deviations.

In vitro determinations

Cell line: Promyelocytic HL-60 cell line was obtained from the American Type Culture Collection (USA). Cells were grown in RPMI-1640 medium supplemented with heat inactivated fetal calf serum (10%) and gentamicine (0.045 g/l) at 37°C in a CO_2 incubator (Heraeus, Germany). Cells were seeded for 96 hours with dimethyl sulfoxide (1%) to differentiate them to cells with neutrophil like morphology. The correct stage of cell differentiation was verified by a flow cytometric analysis of CD11b expression and by the chemiluminescence measurement of ROS production.

Only cells with a viability higher a 95 % as assessed by the trypan blue exclusion test were used for experiments.

Co-incubation of cells with membranes: Differentiated HL-60 cell suspension (1×10^8 cells/l of RPMI-1640 with 10 % heat inactivated fetal calf serum) was prepared for tests. Hollow fibers of the membranes were cut in small pieces (1–3 mm) and 1 g of these was placed in a 50 ml plastic test tube. Then 40 ml of the cell suspension was added to each tube. The tubes were shaken gently in horizontal position for 3 hours and the HL-60 cells were then harvested for further analyses. Cells incubated without membrane were used as a negative control.

ROS production measurement by chemiluminescence: The reaction mixture consisted of 2×10^8 HL-60 cells, 1×10^{-3} mol/l luminol and one of the following activators PMA (5×10^{-6} mol/l) or OZP (0.1 g/l). The total reaction volume was adjusted to 250 μl with Hanks balanced salt solution. Spontaneous chemiluminescence was measured without the activators. The chemiluminescence emission expressed as relative light units was recorded continuously for 90 min at 37 ℃ by microplate luminometer LM-01T (Immunotech, Czech Republic).

Flow cytometric analyses: Determination of the cell surface expression of adhesion molecules: 40 μl of HL-60 cells (1×10^9 cells/l) were incubated in plastic tubes with anti-CD11b and anti-CD62L monoclonal antibodies (4 μl of each) at 4 ℃ for 30 minutes. Then the cells were washed twice in phosphate buffered saline and resuspended in 0.4 ml of phosphate buffered saline, placed on ice and analyzed by flow cytometer within 30 min. Negative isotype controls were also analyzed.

Oxidative burst measurement *in vitro*: 300 μl of HL-60 cells (1×10^8 cells/l) were incubated in sterile plastic tubes with DCFH-DA (2×10^{-5} mol/l) or HE (1×10^{-5} mol/l). DCFH-DA and HE were dissolved in dimethyl sulfoxide (less than 1 % in the final cell suspension). The tubes were shaken gently and incubated in dark at 37 ℃ for 20 min. Then 100 μl of either PMA (2×10^{-5}mol/l) or OZP (0.4 g/l), or just Hanks balanced salt solution were added and the cell suspensions were incubated in dark at 37 ℃ for additional 20 min. At the end of the incubation period, the tubes were placed on ice and analyzed.

Detection of mitochondrial membrane potential: fluorescent probe JC-1 (dissolved in dimethyl sulfoxide) was used for detection of mitochondrial membrane potential as described by Salvioli and co-workers (Salvioli et al. 1997). Briefly, 300 μl of HL-60 cells (1×10^8 cells/l) were incubated in plastic tubes with JC-1 (a final concentration of 2.5 mg/l) in dark at room temperature for 20 min. At the end of the incubation period, the cells were washed twice with Hanks balanced salt solution and analyzed by flow cytometer immediately.

Procedure of analysis of HL-60 cells by flow cytometer: ten thousand live cells selected on the basis of scattering characteristics were analyzed by flow cytometer FACSCalibur (Becton Dickinson, USA) and MRF was determined. The fluorescence of monoclonal antibodies was corrected for background fluorescence of isotype control. The results were expressed as the means of the MRF and the standard error of the mean, or as a mean ratio of the MRF of samples and the control cells plus standard error of the mean.

Statistical analysis:

Results obtained from *in vivo* experiments were analyzed by the Student's t-test for independent or dependent samples, and significances were verified by the non-parametric Mann-Whitney U-test or Wilcoxon test. These non-parametric tests were applied for the analysis of results obtained from *in vitro* experiments.

Results

Clinical study

No significant differences in the expression of CD11b and CD62L on blood granulocytes were found between healthy controls and patients before hemodialysis either with polysulfone or with hemophan membranes (data not shown). The expression of CD11b in patients after hemodialysis was significantly upregulated only in the case of hemophan hemodialysis when compared with the levels determined before the hemodialysis procedure (949 ± 113 MRF *vs.* 1457 ± 128 MRF, $p = 0.01$) whereas polysulfone membrane caused only a mild non-significant increase (890 ± 116 MRF *vs.* 1014 ± 168 MRF). No significant changes in CD62L expression were observed either in polysulfone or hemophan dialyzed patients (data not shown).

When compared with healthy controls a significant decrease in the spontaneous and in the OZP-activated production of ROS measured by chemiluminescence was observed in the patients before hemodialysis with polysulfone or with hemophan membranes (Table 1). No differences in spontaneous and in activated chemiluminescence were found in the patients before hemodialysis either with polysulfone or hemophan membranes. The spontaneous whole blood neutrophil-derived ROS production measured by chemiluminescence was not changed at the end of polysulfone hemodialysis compared with the pre-hemodialysis level, while it was decreased after the hemophan hemodialysis procedure (Table 2). However, hemodialysis reduced the activated chemiluminescence independently of the type of membrane used. This decrease was much more profound after the hemophan hemodialysis procedure, where both FMLP and OZP-activated chemiluminescence were significantly lower in comparison with the pre-hemodialysis levels and even in comparison with the chemiluminescence determined after polysulfone hemodialysis.

There were no significant differences in the total numbers of neutrophils and monocytes between healthy controls and patients before either polysulfone or hemophan hemodialysis. The total numbers of either neutrophils or monocytes did not change significantly after both the hemophan and the polysulfone hemodialysis procedures in comparison with the pre-hemodialysis levels (data not shown).

In vitro experiments

The spontaneous chemiluminescence of HL-60 cells at the end of their 3 hour incubation with polysulfone or hemophan hollow fibers did not changed significantly when compared with controls (Table 3). Activation of the cells with OZP or PMA

Table 1. ROS production in whole blood detected by chemiluminescence in healthy controls and patients dialyzed with hemophan or polysulfone membranes before hemodialysis procedure. Data are expressed as $V \cdot s \cdot 10^{-3}$ (mean ± S.E.M.). Asterisks indicate statistically significant differences ($p < 0.05$) compared with controls

	Spontaneous chemiluminescence (V·s)	Activated chemiluminescence (V·s)	
		FMLP	OZP
control	0.573 ± 0.035	8.649 ± 0.540	45.420 ± 1.826
polysulfone	0.450 ± 0.045*	8.614 ± 0.985	38.188 ± 1.722*
hemophan	0.467 ± 0.038*	8.248 ± 1.137	37.318 ± 2.144*

Table 2. ROS production in whole blood detected by chemiluminescence in patients after hemodialysis procedure with hemophan or polysulfone membranes. Data are expressed as percentages of predialysis levels (mean ± S.E.M.). Crosses indicate statistically significant differences ($p < 0.05$) comparing the effects of the two different membranes, and asterisks indicate statistically significant differences ($p < 0.05$) compared with predialysis levels

	Spontaneous chemiluminescence (%)	Activated chemiluminescence (%)	
		FMLP	OZP
polysulfone	98.4 ± 3.5	90.2 ± 4.8*	93.7 ± 5.2
hemophan	91.9 ± 4.6*	77.3 ± 10.5*$^{+}$	67.7 ± 13.6*$^{+}$

Table 3. Effect of 3 h incubation of HL-60 cells with hemophan or polysulfone membranes on ROS production measured by chemiluminescence. Data are expressed as a percentage of control cells (mean ± S.D. of four independent experiments). Cross indicates statistically significant difference ($p < 0.05$) comparing the effects of the two different membranes, and asterisks indicate statistically significant differences ($p < 0.05$) compared to control cells

	Spontaneous chemiluminescence (%)	Activated chemiluminescence (%)	
		FMLP	OZP
polysulfone	128 ± 24	67 ± 4*	66 ± 8*
hemophan	117 ± 12	58 ± 7*	45 ± 14*$^{+}$

measured by chemiluminescence was significantly reduced in comparison with untreated control cells. The reduction of chemiluminescence was most obvious in the hemophan-treated, PMA-activated cells, where the chemiluminescence was significantly lower not only in comparison with the controls but also with cells treated with polysulfone.

ROS production was also determined by flow-cytometry (Fig. 1). Incubation of HL-60 cells with polysulfone did not cause any significant changes in spontaneous or

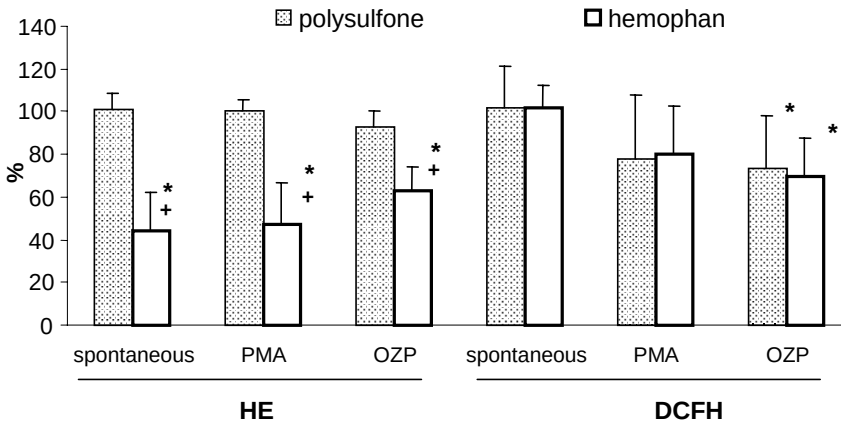

Figure 1. Effect of 3 h incubation of HL-60 cells with hemophan or polysulfone membranes on ROS production measured by HE and DCFH-DA. Data are expressed as percentages of controls (mean ± S.D. of four independent experiments). Crosses indicate statistically significant differences ($p < 0.05$) comparing the effect of the two different membranes, and asterics indicate statistically significant differences ($p < 0.05$) compared with control cells.

activated superoxide production measured with HE. On the other hand, pretreatment with hemophan caused a significant decrease in the spontaneous and in both OZP and PMA-activated production of superoxide. Hydrogen peroxide production measured with DCFH-DA was not significantly changed in the non-activated and the PMA activated HL-60 cells in any cases. In contrast, DCFH-DA fluorescence in the OZP activated cells dropped down after pretreatment with both types of membranes.

As regards the surface molecules, the expression of CD11b was significantly upregulated after 3 hours of incubation with either polysulfone (173 ± 21 MRF *vs.* 219 ± 17 MRF, $p = 0.02$) or hemophan (173 ± 21 MRF *vs.* 227 ± 24 MRF, $p = 0.03$) membranes. Expression of CD62L was not influenced by the membranes (data not shown).

Mitochondrial membrane potential detected by fluorescent probe JC-1 did not change significantly ($113.8 \pm 11.3\,\%$) after a 3 h incubation of the HL-60 cells with polysulfone membrane fibers whereas hemophan caused a significant decrease ($85.2 \pm 4.5\,\%$, $p = 0.01$) compared with control cells.

Discussion

This study was designed to assess and to compare changes in the functional properties and surface molecule expression of neutrophils after their contact with cellulose (hemophan) or synthetic (polysulfone) membranes either in the extracorporeal circulation during hemodialysis or *in vitro*.

The membrane contact with blood elements during the hemodialysis procedure is known to induce neutrophil activation as measured by the release of proinflammatory mediators (Horl et al. 1985), a decrease in viscoelasticity (Iijima et al. 1999), and changes in the surface expression of the integrin protein CD11b/CD18 and the selectin CD62L (Himmelfarb et al. 1992, 1993, 1994, 1995; Hernandez et al. 1998; Rosenkranz et al. 1999). Impaired leukocyte function in response to subsequent stimuli has also been described (Nguyen et al. 1985; Haag-Weber et al. 1989; Descamps-Latscha et al. 1991; Vanholder et al. 1991; Hernandez et al. 1998). The rate of membrane-induced effects depends on the membrane biocompatibility. Numerous investigators have demonstrated that cellulose membranes cause marked complement activation. They proposed that it could be a dominant mechanism for granulocyte activation during hemodialysis (Nguyen et al. 1985; Descamps-Latscha et al. 1991; Himmelfarb et al. 1995; Iijima et al. 1999).

The increased expression of CD11b (CR3) found in our study at the end of the hemodialysis procedure with hemophan is in good agreement with the literature (Himmelfarb et al. 1994, 1995; Hernandez et al. 1998; Iijima et al. 1999; Rosenkranz et al. 1999). Results of Himmelfarb et al. (1995) strongly support the hypothesis that upregulation of CD11b is connected with complement activation. No major changes in the expression of CD11b were detected in patients dialyzed with polysulfone complement non-activating membrane in our study as well as in the study of Rosenkranz et al. (1999).

The main parameter observed in our study was the production of ROS by blood phagocytes. Their metabolic response is altered generally in patients with chronic renal failure who do not receive hemodialysis (Ritchey et al. 1981; Haag-Weber et al. 1989; Vanholder et al. 1991; Cendoroglo et al. 1999). The hemodialysis procedure could further worsen this pathological state.

We extend this knowledge by findings that spontaneous and OZP-activated chemiluminescence were lower before both the polysulfone and the hemophan hemodialysis procedure when compared with the chemiluminescence of healthy controls. Moreover, the hemodialysis procedure further reduced the neutrophil chemiluminescence independently of the type of membrane used, however, this decrease was much more profound after hemophan hemodialysis. The decreased spontaneous generation of ROS after hemophan membrane hemodialysis measured by chemiluminescence was confirmed by the detection of intracellular superoxide production measured by flow cytometry with HE and by the detection of intracellular hydrogen peroxide production measured with DCFH-DA in selected patients (unpublished data). Our results are in good agreement with findings that the metabolic response of blood phagocytes measured by chemiluminescence is adversely affected by hemodialysis (Ritchey et al. 1981; Cohen et al. 1982; Nguyen et al. 1985). The significant decrease in leukocyte response to phagocytic challenge and metabolic activity was caused mainly by cellulose membranes and was not seriously affected by complement non-activating membranes (Nguyen et al. 1985; Vanholder et al. 1991; Descamps-Latscha et al. 1991; Himmelfarb et al. 1993). These findings support a theory that the defect of phagocytes is dependent on complement activation.

On the other hand, respiratory burst induction, CD11b/CD18 upregulation or the shedding of CD62L can also occur partially independently of complement activation being induced by other mechanisms (Kormoczi et al. 1999; Rosenkranz et al. 1999). Experiments of Himmelfarb et al. with aprotinin, complement activation inhibitor and sCR1 suggest that not all the effects of cellulose membranes on granulocyte activation are due strictly to complement activation (Himmelfarb et al. 1994, 1995). The alteration of the metabolic activity of phagocytes caused by the polysulfone membrane, although weaker than that caused by the hemophan membrane, are in good agreement with this theory.

The reason for the decreased phagocyte ROS production measured by chemiluminescence is unclear. One of the possible explanations can be a degranulation of polymorphonuclear cells and consequent decreased amount of myeloperoxidase, which can play key role in luminol-induced chemiluminescence. Degranulation of polymorphonuclear cells during the hemodialysis procedure is relatively well described (Horl et al. 1985; Haag-Weber et al. 1989). In contrary, Cohen et al. (1982) failed to detect any differences between pre- and intra-dialytic myeloperoxidase concentrations in polymorphonuclear cells. These data together with our results where the superoxide and hydrogen peroxide production as well as luminol-induced chemiluminescence were depressed similarly support an idea that the decrease in blood phagocyte metabolic activity after hemodialysis is myeloperoxidase-independent and is caused rather by a true defect in oxidative metabolism.

We used two activators of the oxidative burst of phagocytes which stimulate cells through different receptors and mechanisms. OZP are recognized by complement and immunoglobuline receptors, and FMLP binds to a specific formyl peptide receptor. Regardless the upregulation of complement receptor, a decreased metabolic activity of blood phagocytes by OZP was found in our study after hemodialysis. That is why another explanation for the depressed metabolic activity of phagocytes after hemodialysis can be a pre-existing stimulation or "exhaustion" of blood phagocytes. The decreased spontaneous ROS production as well as the absence of significant differences in ROS production induced by the used activators also support this theory.

The decreased production of ROS at the end of hemodialysis procedure is in contrast to observed increased oxidative stress during hemodialysis (Galli and Ronco 2000) which was even suggested to be caused by increased production of ROS by blood phagocytes (Lojek et al. 1998). Several authors described activation of neutrophils and increased ROS production by neutrophils from the beginning of hemodialysis procedure up to 60 min (Nguyen et al. 1985; Rosenkranz et al. 1994; Iijima et al. 1999; Kormoczi et al. 1999) However, it returned to predialysis levels at later phases of hemodialysis procedure. At the end of hemodialysis the capability of neutrophils to produce ROS was even reduced as was described above. It could be speculated that ROS production by blood phagocytes is an important source of oxidants contributed to increased oxidative stress during hemodialysis.

Beside *in vivo* and *ex vivo* studies, few articles evaluating *in vitro* effects of hemodialysis membranes on isolated neutrophil metabolic activity and sur-

face molecule expression have appeared during last years (Kuwahara et al. 1988; Rosenkranz et al. 1994, 1999). Though, results of these studies could be influenced by the fact that the expression of immunoglobuline and complement receptors on cell membranes, and also the functional properties of neutrophils, are changed during the separation processes (Lojek et al. 1997). It was the reason why we decided to use the HL-60 cell line differentiated by dimethyl sulfoxide to obtain cells exhibiting a phenotype very similar to normal human blood neutrophil granulocytes (Yen 1990; Narayanan and Robinson 1998; Sedlák et al. 1999). This allowed us to obtain sufficient amount of a homogenous population of resting cells without any activation for *in vitro* experiments (Horáková 1999).

A three hour incubation of HL-60 cells with both types of membranes induced an increased expression of CD11b, the expression of CD62L was without significant changes. This effect was similar as for *in vivo* hemodialysis. The activated chemiluminescence was reduced by both membranes, which was most obvious in the hemophan treated cells. Incubation of cells with hemophan also caused a significant decrease in ROS production measured with HE. In contrast, DCFH-DA fluorescence in the OZP-activated cells dropped after pretreatment with both types of membranes. It was already demonstrated in *in vitro* experiments that incubation of polymorphonuclear cells with cellulose membranes had more profound effects on spontaneous ROS production while polysulfone membranes did not elicit radical formation (Rosenkranz et al. 1999). The partial differences in the production of ROS measured by chemiluminescence and flow cytometry in our study can be explained by different specificites of these methods. Luminol-enhanced chemiluminescence detects all types of produced reactive oxygen and nitrogen species and oxidants included in the myeloperoxidase system (Lojek et al. 1997). HE and DCFH-DA-based flow cytometric analyses are considered as specific probes for the intracellular production of superoxide and hydrogen peroxide, respectively (Bass et al. 1983; Rothe and Valet 1990).

It was reported earlier, that the expression of CD11b increased and CD62L decreased only in the presence of normal serum with complement compounds (Rosenkranz et al. 1999). No significant effect was found in the presence of complement component deficient serum and in the absence of serum. In our experiments we used heat inactivated fetal calf serum, therefore an influence of complement components was excluded. These *in vitro* data for CD11b expression and ROS production again suggest the involvement of a complement-independent pathway. Our results are in agreement with the results of Kuwahara et al. (1988). These authors suggested that neutrophil activation occurred by direct membrane-neutrophil interaction and the produced ROS might play an initial and/or additional role in the events occurring at the initiation of hemodialysis.

The mechanism of direct membrane activation of neutrophils in absence of complement is not fully clarified. One of the possible explanations is that neutrophils are activated due to their adhesion to cellulose-based hemodialysis membranes *via* L-fucose-inhibitable lectine-like binding (Kormoczi et al. 1999). We suggest that also other non covalent low force interactions may play important role in

neutrophil activation by hemodialysis membranes in absence of complement. This is supported by such significant increase of neutrophil adhesiveness during hemodialysis which is not fully correlated with increase of main adhesion molecules (Iijima et al. 1999).

The influence of polysulfone and hemophan membranes on neutrophil metabolic activity was detected in our study also on the basis of changes in mitochondrial membrane potential. Mitochondrial membrane potential, which is generated by mitochondrial electron transport chain, is in turn responsible for the formation of ATP molecules by ATP synthase. For this reason, mitochondrial membrane potential is an important parameter for mitochondrial functionality and an indirect evidence of the energy status of the cell. We failed to observe any sharp collapse of mitochondrial membrane potential which is known to be typical for apoptotic process (Cossarizza et al. 1993). On the other hand, the decreased mitochondrial membrane potential of cells after incubation with hemophan membrane could indicate impaired metabolic functions of these cells. These results are in good consent mainly with the decreased ROS production detected with HE.

It was shown that neutrophils from uremic patients either without or with regular hemodialysis undergo accelerated *in vitro* apoptosis (Jaber et al. 1998; Cendoroglo et al. 1999) This phenomenon was caused mainly by uremic toxins and the apoptosis-inducing activity of uremic plasma could be modulated by the material of dialysis membrane. Only a contact of neutrophils with hemodialysis membranes in the absence of uremic toxins does not induce apoptotic process as was shown in our *in vitro* experiments.

In conclusion, a better biocompatibility of polysulfone membranes in comparison with hemophan membranes was proved in our study. Adverse effects of hemophan could be associated with activation of complement cascade but we also proved complement independent effects in *in vitro* experiments with polysulfone membrane. Differentiated neutrophil-like HL-60 cells had been shown to be sensitive to co-incubation with membrane fragments. A combination of chemiluminescence methods for the detection of total ROS production, flow cytometric methods for the intracellular production of ROS using different fluorescent probes and the detection of changes in mitochondrial membrane potential can be a useful tool to obtain a complex view of the activation and metabolic status of blood phagocytes. Therefore, it can be employed for the analysis of membrane biocompatibility.

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Evidence that C-Reactive Protein or IL-6 Are Not Surrogates for All Inflammatory Cardiovascular Risk Factors in Hemodialysis Patients

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Abstract
Background/Aims: In otherwise healthy adults, high C-reactive protein (CRP) levels are associated with cardiovascular disease and have been linked to an inflammatory state. The presence of vascular disease is also associated with increased expression of adhesion molecules, including soluble intercellular adhesion molecule (sICAM), vascular endothelial growth factor (VEGF) and leukocyte-derived myeloperoxidase (MPO). These associations suggest potential mechanisms whereby inflammation may injure the vascular endothelium, but the recognition of how these mediators act in concert remain poorly characterized. That the prevalence of atherosclerosis and markers of inflammation are increased in renal failure patients suggests that inflammation causes accelerated vascular disease. ***Methods:*** In hemodialysis patients, we examined the relationships between plasma CRP and sICAM, VEGF and MPO longitudinally. We determined whether episodes of a high CRP value were paralleled by simultaneous increases in mediators of inflammatory injury or molecules associated with endothelial cell adhesion or growth and whether CRP levels correlated with those of VEGF and MPO. ***Results:*** Episodic increases in CRP were accompanied by higher levels of VEGF, sICAM and MPO. However, there was no correlation between serum CRP levels or other acute phase proteins and either MPO or VEGF, nor was there a constant temporal relationship between MPO and CRP. By contrast, MPO and VEGF levels were closely correlated with one another during episodes of inflammation ($p = 0.0001$), and CRP and interleukin-6 levels were also correlated. Increases in MPO tended to be restricted to patients with grafts or catheters, and not those with AV fistulas. ***Conclusions:*** These results suggest that high plasma levels of CRP or other acute phase proteins in cross-sectional studies should be interpreted cautiously when defining mechanisms underlying cardiovascular disease in the hemodialysis patient population. One, or more than one inflammatory repertoire may be activated, one involving hepatic acute phase proteins and the other neutrophil activation and each may contribute separately to outcomes. Better prognostic information may be obtained by measurement of more markers than CRP alone, such as MPO and VEGF.

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Introduction

In otherwise healthy subjects [1] and patients with kidney failure [2], inflammation is associated with cardiovascular disease and stroke. While the levels of acute phase proteins remain stable in non-dialysis populations [3], inflammation occurs episodically in hemodialysis patients [4]. The specific roles played by various proteins that increase during the acute phase response in initiating or indicating the presence of vascular injury remain controversial. Assessment of inflammation as a variable affecting outcome among dialysis patients has generally relied on measurements of C-reactive protein (CRP) [2, 4, 5] or interleukin (IL)-6 [6] making the assumption that increased levels of either T-helper-1 cytokines (IL-2 and IFN-γ), inflammatory cytokines (IL-6, TNF-α) report a biologically consistent event, specifically the acute phase response, and that elements of that response combine to promote vascular injury, or report existing vascular lesions. It is commonly proposed that CRP [7] is an important mediator linking inflammation and subsequent vascular injury.

There are other mediators that are closely linked to the presence or cause of atherosclerosis [8]. For example, the leukocyte-derived enzyme myeloperoxidase (MPO) is abundantly expressed in neutrophils, monocytes, and tissue-associated macrophages, and has been implicated as a potential factor in the pathogenesis of atherosclerosis. Indeed, MPO is observed in human atherosclerotic lesions [9], and circulating leukocyte levels of MPO are positively associated with increased risk of developing coronary artery disease [10]. Moreover, a single nucleotide polymorphism in the promoter region of the MPO gene confers decreased expression of MPO and is associated with a lower prevalence of cardiovascular disease in ESRD patients [11]. Consistent with this notion, a protective effect of MPO deficiency against cardiovascular disease was observed when a group of 100 totally or subtotally MPO-deficient individuals was compared to a normal reference population selected at random [12]. Additionally, during acute coronary syndromes a single serum measure of MPO serves as a strong predictor of future risk of death from myocardial infarction [13].

Atherosclerosis is also associated with increased expression of adhesion molecules, including soluble intracellular adhesion molecule (sICAM) and vascular endothelial growth factor (VEGF). VEGF plays an important role in the maintenance of endothelial integrity, vascular permeability to serum proteins and is associated with atherosclerosis in patients with cardiac allografts [14]. VEGF levels are increased in conditions associated with increased risk of vascular disease, and decrease when risk factors are removed. For example, plasma VEGF changes in conditions that are common in patients with kidney disease; it is elevated in poorly controlled diabetic patients [15] and levels decrease following control of diabetes [16]. Among diabetic patients, microalbuminuria is significantly associated with VEGF [17] and plasma levels of VEGF are higher in non-diabetic hypertensive patients and decrease following successful antihypertensive treatment [18].

The biological roles of these proteins provide potential mechanisms whereby inflammation may cause vascular injury, lipoprotein oxidation, or increased adhesion of macrophages and other leukocytes to the vascular endothelium. The hypothesis that MPO is causally linked to atherosclerosis is based largely upon correlations of levels with outcomes. MPO can function as a catalytic 'nitric oxide (NO) oxidase' which blunts the vasodilatory and presumably the vasoprotective functions of NO [19]. In addition, MPO is a source of reactive oxygen, nitrogen and chlorine species, which have the propensity to damage biological macromolecules such as lipids and proteins within the arterial wall.

Like MPO, VEGF is also found in neutrophils and is released upon neutrophil activation and degranulation [20, 21]. By contrast, CRP is primarily liver-derived and is regulated by a group of upstream cytokines, the most proximal of which is IL-6 [22, 23]. Thus at least two reservoirs of 'inflammatory proteins' may contribute to changed plasma protein concentration following an inflammatory event. This model would suggest that more than one repertoire of inflammatory events may occur and contribute to downstream vascular injury. Epidemiologic studies have utilized one protein or several proteins from one group (fibrinogen, CRP, IL-6) while not including proteins that may be expressed by a different tissue. If indeed more than one type of inflammatory response is triggered, measurements of at least one protein from each repertoire may be of use.

Since high CRP levels have been commonly used as an indicator of an inflammatory reaction that causes atherogenesis and explains the prevalence of cardiovascular disease in hemodialysis patients, we decided to test whether CRP is indeed a surrogate for other acute inflammatory mediators such as MPO and VEGF. The intermittent nature of the inflammatory response in dialysis patients permitted us the opportunity of testing whether intermittent episodes of inflammation are ac-

companied by simultaneous increases in blood levels of MPO, VEGF and sICAM, and whether these components of inflammatory responses that are highly associated with cardiovascular disease [8] are necessarily always interrelated. Should changes in the levels of all of these markers occur simultaneously then one could serve as a surrogate for all facilitating the use of epidemiologic information gleaned from various studies in which only one of the markers was measured.

Methods

Patient Selection

Our protocol was approved by our individual Institutional Review Boards and informed consent was obtained from each patient. All patients were enrolled in the HEMO study sponsored by the National Institutes of Health [24] and were studied longitudinally at three of the participating centers: the University of California, Davis (UC Davis Medical Center, Sacramento, Calif., USA), Beth Israel Medical Center (New York, N.Y., USA) or Emory University (Atlanta, Ga., USA). Blood samples from patients in the HEMO study were taken every 6 months according to the approved experimental protocol. In this ancillary study, blood was sampled monthly. The samples were obtained from patients without regard to current clinical status as they were scheduled to avoid sampling bias.

Patients: Patients who had been randomized into one of the four HEMO treatment groups were eligible for enrollment in this study. Inclusion criteria for the HEMO Study included patients were between 18 and 80 years of age, receiving in-center hemodialysis 5 times per week, and had a dialysis history of at least 3 months. Exclusion criteria included a urine urea clearance of >1.5 ml/min/35 l urea volume, the presence of serious comorbid medical conditions such as active malignancies requiring chemotherapy or radiation therapy, treated class IV congestive heart failure, unstable angina pectoris, symptomatic AIDS, active systemic infections such as tuberculosis or systemic fungal infection, chronic pulmonary disease requiring supplemental oxygen, and cirrhosis with encephalopathy or abnormal prothrombin time and there were no plans for a living donor transplant within the period of the study. Samples from 40 patients of the 79 patients enrolled in the ancillary study were analyzed. The samples were selected from those patients who had elevations of CRP that were at least 5 times higher than the minimal value recorded for that patient and that the level also returned to the minimum value. We defined this as a period of inflammation; the absence of inflammation (as defined by CRP levels without such changes in the plasma levels were also identified. Twenty of the 40 patients were men. Nineteen were African-American. Mean age was 57.7 ± 16.5 years. Sixteen patients were diabetic. Vascular access was transcutaneous catheter in 6 patients, AV fistula in 15 patients and a polytetrafluoroethylene graft in 19 patients. Seventeen patients were dialyzed with an F8 dialyzer, 18 with an F80 (FMC Walnut Creek, Calif., USA), 2 patients with a CA 210, and 3 patients with a CT 190 G (Baxter). None of the patients who were studied in this cohort had any residual renal function.

Plasma levels of acute phase proteins, CRP, and α_1-acid glycoprotein (α_1-AG), were each measured initially and then weekly using a Beckman Array automated nephelometer. Plasma levels of IL-6, sICAM and VEGF were measured by ELISA (Hemagen). All nephelometric and ELISA measurements were made in duplicate and the average of the values was used for calculations. The intraassay CV for α_1-AG was 0.14%, with an interassay CV ranging from 3.07 to 5.50% over the usable range of the assay. The intraassay CV for CRP was 0.35% and interassay CV 5.5%. The intraassay CV for IL-6 was 0.32% and interassay CV 8.7%. The intraassay CV for VEGF was 5.4% and interassay CV 7.3%. The intraassay CV for sICAM was 1.7% and interassay CV 15.9%. The limit of detection for CRP, sICAM and VEGF, IL-6 and α_1-AG are 0.4 mg/dl, 0.35 ng/ml and 5.0 pg/ml, 0 pg/ml and 35 mg/dl respectively.

To establish if episodes during which CRP values were high (i.e., an inflammation period) were accompanied by increased values of MPO, sICAM or VEGF, plasma specimens that had not been previously thawed were chosen for analysis of MPO and VEGF in order to avoid any artifact of repeated freeze-thaw cycles; a second frozen sample was chosen from a period when CRP was at least 5-fold below its peak value. The differences between peak and trough CRP values were either not of similar magnitude or patients did not have periods in which CRP was not elevated in the other 39 subjects. Samples representing control (i.e., no inflammation) were chosen for analysis at time intervals either before or after the periods with a high CRP level in order to avoid a time bias. The range was from 707 days prior to an episode of a high CRP to 590 days following an increase in CRP, with a median value of 56 days following the increase in the CRP level. The shortest duration of time between the two measurements was 18 days.

Quantitation of Plasma MPO Activity

Peroxidase activity was determined by measuring the rate of H_2O_2-dependent oxidation of tetramethylbenzidine (TMB) as described [25]. Aliquots of each plasma sample, containing 1 mg of total protein, were mixed with sodium acetate buffer (300 mM, pH 5.4, containing 1.2 mM TMB prepared in dimethylformamide and 0.3 mM H_2O_2 in a total of 200 μl in microtiter plate wells. The rate of TMB oxidation was monitored at 630 nm over 2 min on a PowerWave$_x$ UV-Vis plate reader (Bio-Tek Instruments, Inc.). Rates are expressed as the ΔOD_{630}/min/mg protein. Values were calculated based upon linear TMB oxidation rates. If linearity was not achieved over 2 min, samples were diluted accordingly. The intraassay COV of the MPO assay was 2.7% and the interassay COV was 6.0%

Statistical Analyses

The levels of serum proteins during a period of high vs. low plasma CRP values were compared by paired Student's t test. Cross-sectional analysis was performed by stepwise multivariable regression using CRP, IL-6, serum albumin, α_1-AG, patient age, ethnicity, presence of diabetes, access type, and body mass index (height/m^2) as independent variables. CRP, IL-6, VEGF and MPO levels were not normally distributed and were log-transformed prior to analysis.

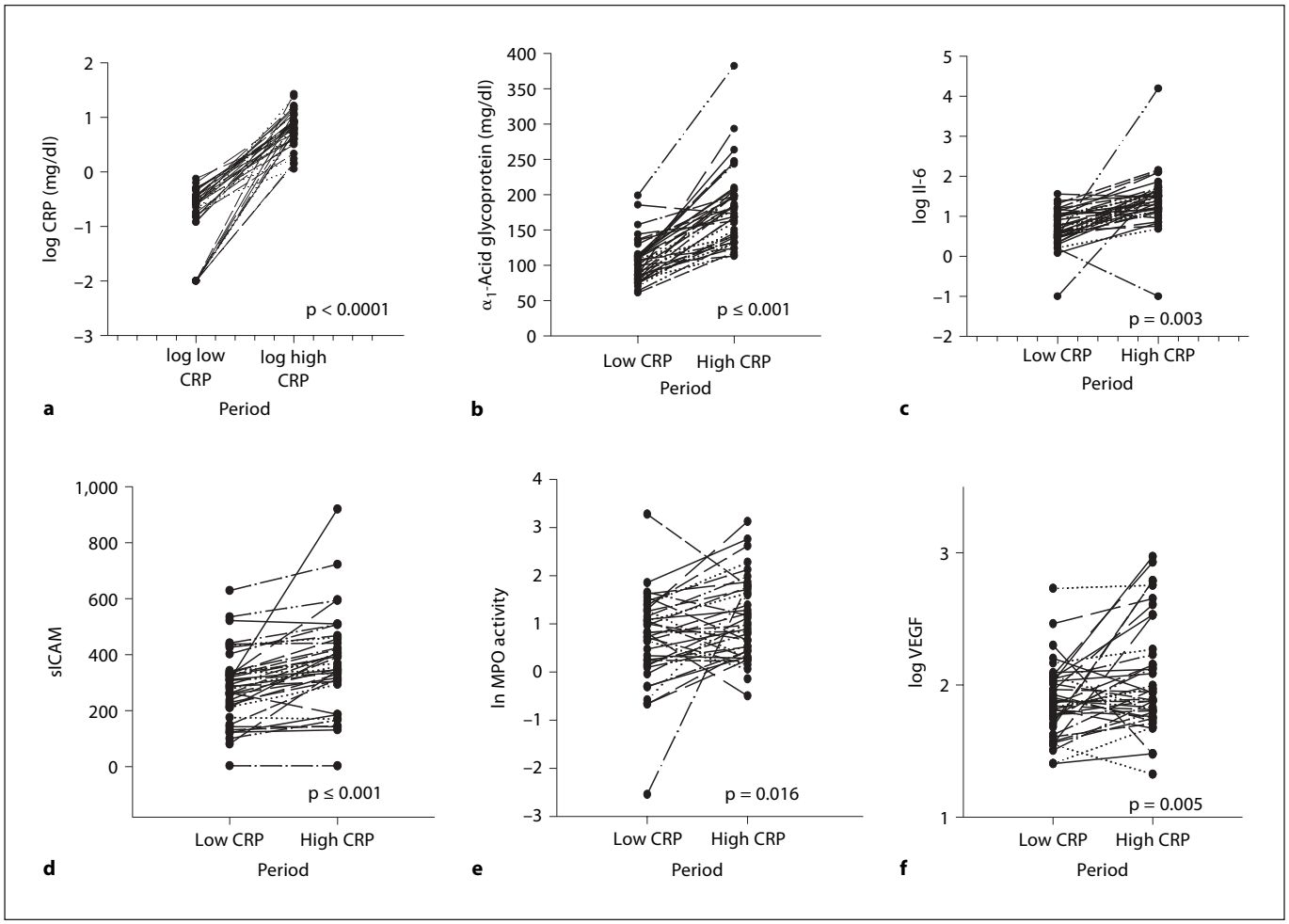

Fig. 1. a CRP levels at low and high values chosen. **b** α_1-AG levels during periods when CRP was at minimal or elevated values. **c** IL-6 levels during periods when CRP was at minimal or elevated values. **d** MPO activity during periods when CRP was at minimal or elevated values. **e** sICAM levels during periods when CRP was at minimal or elevated values. **f** VEGF levels during periods when CRP was at minimal or elevated values.

Results

Hemodialysis patients followed longitudinally have revealed that CRP levels increase and decrease in an episodic manner [2, 4]. By design, we have chosen sample intervals which reflected both low and high CRP levels for each individual patient (fig. 1a). Sample analyses in this patient population revealed that CRP levels were collectively significantly greater during the period when maximal CRP was selected. The levels of α_1-AG, IL-6, VEGF and MPO were all elevated during the period when CRP was maximal (fig. 1b–f). During these periods of increased inflammation, the levels of CRP correlated positively with the acute phase protein α_1-AG ($p = 0.0213$) (data not shown) and the cytokine IL-6 ($p = 0.0182$, $r^2 = 0.4044$) (fig. 2a), but not with either VEGF ($p = 0.87$, $r^2 = 0.00$ data not shown) or MPO (fig. 2b).

However, we did observe a strong and positive correlation between VEGF levels and MPO ($r^2 = 0.384$, $p < 0.001$) (fig. 3) in samples where CRP was high, but MPO was not statistically associated with CRP level nor with that of any other acute phase protein measured. Once it became apparent that MPO levels did not correlate with the levels of acute phase proteins, we analyzed serial serum samples for MPO to establish whether there were temporal associations between increase in MPO and those of CRP, as we have described previously for other acute phase proteins [4]. While some episodes during which CRP levels

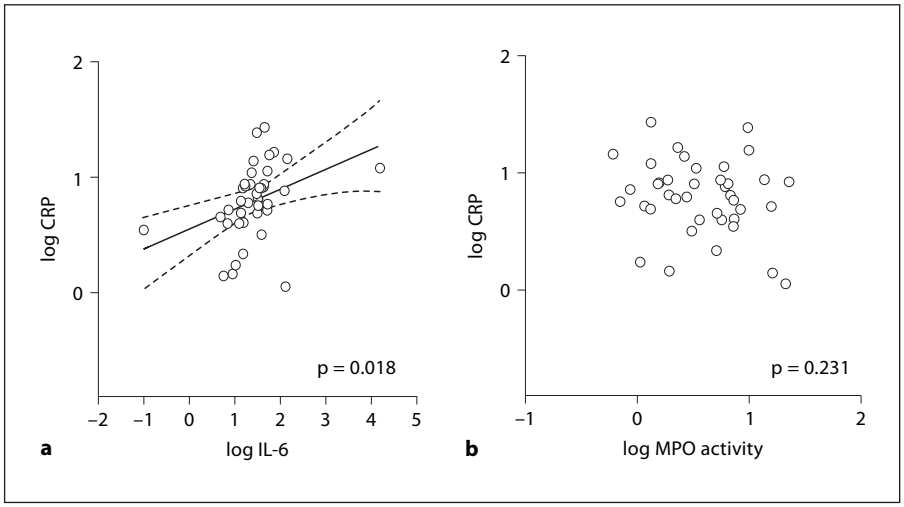

Fig. 2. Relationship between CRP and IL-6 levels (following log transformation) during periods of elevated CRP (**a**) and between CRP and MPO (following log transformation) during periods of elevated CRP (**b**).

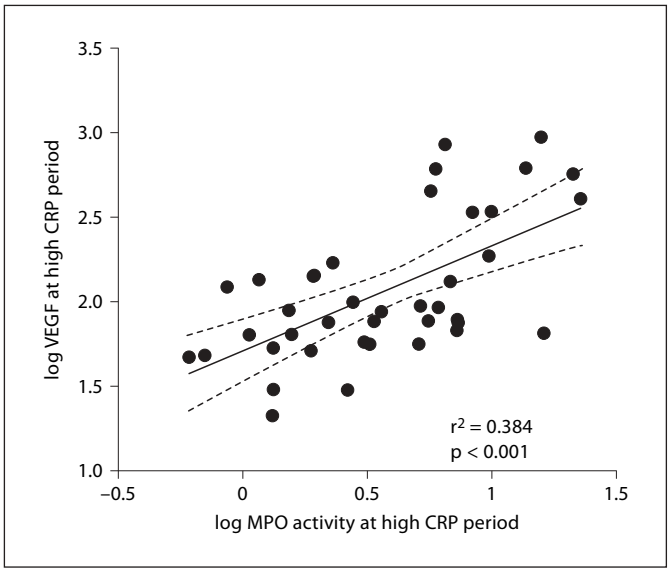

Fig. 3. Relationship between VEGF levels and MPO activity (following log transformation) during periods of elevated CRP.

were increased were associated temporally with an increase of MPO, others were not (fig. 4), and similarly episodes characterized by increased MPO level were as likely as not to be accompanied by increases in CRP or in other acute phase proteins (data not shown).

When the associations were controlled for age, ethnicity, gender and diabetic status, sICAM levels were negatively associated with VEGF ($p = 0.0373$) and positively with IL-6 ($p = 0.0276$, r^2 for the model 0.3462).

Thus, while periods of inflammation may be characterized by increased levels of VEGF, MPO, sICAM, acute phase proteins and cytokines, the magnitude of change in the acute phase proteins and cytokines are unrelated to changes found in MPO and VEGF. When the patient population was assessed in terms of the type of vascular access utilized (i.e. fistula vs. graft/catheter), no differences were observed with regard to the fold change in CRP levels for the two groups (fig. 5). However, and while not statistically significant, MPO levels (as assessed by fold change) revealed that patients having grafts or catheters had an ~3-fold increase in MPO, whereas those with fistulas did not show an increase in MPO at times of high CRP. Therefore, increases in MPO tended to be restricted to patients with grafts or catheters, and suggests that the type of inflammatory responses or the inciting factors are inherently different.

Discussion

In dialysis patients, cardiovascular disease is the leading cause of mortality. In the general population, the relative risks for all-cause as well as cardiovascular mortality is closely associated with high plasma levels of inflammatory markers in the general population [1], and similar associations are present in dialysis patients [2, 5, 6]. These findings are consistent with a pathophysiologic state in which inflammation plays a key role in atherogenesis [8] and inflammation alters endothelial structure and plasma protein composition in ways that independently promote vascular disease. It is difficult to establish a cause-

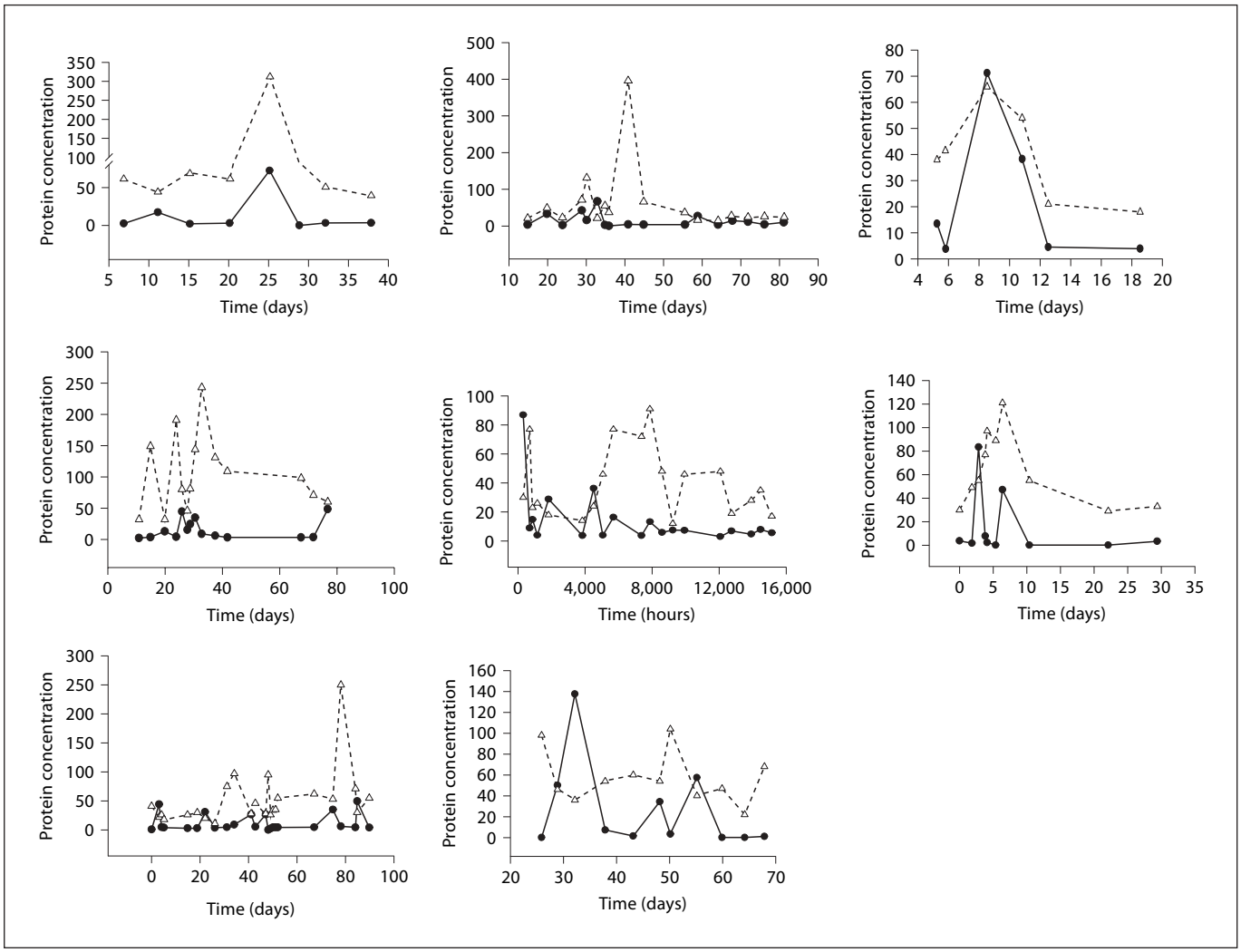

Fig. 4. Temporal relationship between serum CRP levels (●) and MPO activity (△) in plasma of 8 hemodialysis patients.

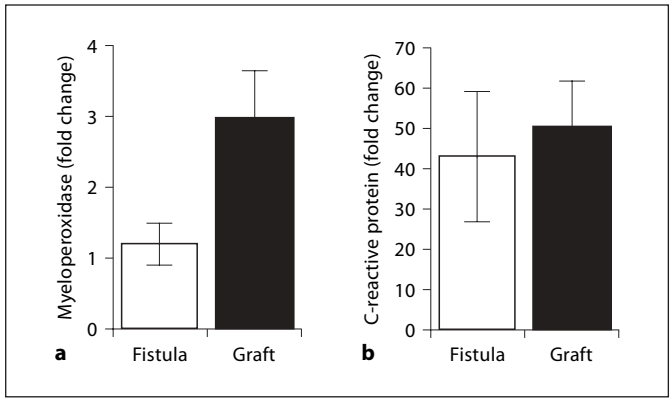

Fig. 5. Relationship between access type and fold change in MPO (**a**) and CRP (**b**). MPO and CRP were quantified in plasma at baseline and during periods of elevated inflammation. Data are expressed as the average ± SEM. Fistula = AV fistula; Graft = catheter or graft.

effect relationship between inflammation and atherosclerosis because there are no selective methods of blocking inflammation in humans [26]. Several proteins, including CRP serum amyloid A and fibrinogen, have been suggested to play a direct role in atherogenesis based on extensive knowledge of the biology of these proteins. All of these are acute phase proteins regulated by the cytokines IL-1, IL-2 and IL-6 [23] and are for the most part derived from de-novo synthesis by the liver [22].

The acute phase response is a component of the innate immune system. It is not a single response by a single organ but instead is a result of a change in expression of a group of genes expressed in several tissues, including the liver [27], the vascular endothelium [28] and circulating monocytes, macrophages and neutrophils. Some of these proteins, specifically CRP, have been identified in atherosclerotic lesions as well, suggesting that some changes in plasma levels of some of the proteins are reflective of changes in gene expression in blood vessels rather than in the liver, however the contribution of vascular production of acute phase proteins to changing plasma levels is not well defined. The bulk of synthesis of all of these proteins takes place in the liver.

By contrast, MPO and VEGF are primarily derived from neutrophils, and finding increased levels of these proteins is suggestive of an event that causes neutrophil degranulation. Thus an increased level of these proteins in plasma does not necessarily require de-novo hepatic protein synthesis and a liver-derived acute response need not be accompanied by neutrophil degranulation. VEGF is released from neutrophils following activation during bacterial infection or following stimulation with soluble proinflammatory activators [29]. Inflammatory events may activate both hepatic acute phase protein synthesis and neutrophil degranulation, or either pathway to varying degrees and at different times during the course of inflammation.

MPO can modify low density lipoprotein to increase its binding to macrophages and hence its atherogenicity. MPO also catalytically consumes endothelial-derived NO to impair vascular relaxation responses [19]. VEGF is also expressed in and around smooth muscle cells/myofibroblasts within the venous neointima of dialysis grafts, in macrophages lining the graft, and within the neointima and adventitia [30]. Moreover, VEGF alters vascular permeability [31] and is a downstream effector for angiotensin II-dependent alterations in vascular permselectivity [32]. The strong association between VEGF and MPO levels during periods of inflammation in hemodialysis patients suggests that these factors however reflect events characterized by neutrophil activation and degranulation [20].

The finding that CRP levels during episodes of inflammation are not correlated with the activity of leukocyte-derived MPO suggests that not all aspects of the acute phase response are symmetrically activated during each episode of inflammation, or that the temporal production and/or release of these mediators may reflect differences in the type of inflammatory response experienced by the individual at a given point in time. Increases in CRP, IL-6 and α_1-acid glycoprotein are released as part of a repertoire that does not necessary involve leukocyte degranulation, while MPO and VEGF are released by episodes that do. CRP has been found to inhibit leukocyte degranulation [33], so it is possible that maximal degranulation of leukocytes may not correspond with maximal CRP levels. Indeed, CRP and MPO levels tended to be negatively associated with one another when CRP was elevated suggesting a complex relationship between MPO and the acute phase response.

By contrast to the lack of relationship between CRP and either MPO or VEGF, CRP was highly correlated to IL-6, a cytokine that plays a role in initiating hepatic release of CRP [23]. Thus some episodes of inflammation appear to recruit a cytokine acute phase protein repertoire of response with little evidence of neutrophil degranulation, while other episodes of inflammation are associated with increased levels of MPO and VEGF, products released by neutrophils [20, 21]. Each class of inflammation has been associated with cardiovascular disease [2, 5, 8, 13], however, these responses appear to represent different biological phenomena that are not interchangeable. The recent observations of Smeeth et al. [34] demonstrating a significant increase in cardiovascular events immediately following infectious hospitalizations are consistent with an association of acute inflammation with subsequent cardiac events, rather than a link between chronic inflammation and outcome.

Our observations that elevations in MPO during times of inflammation (high CRP) tend to be restricted to patients with grafts/catheters (but not AV fistulas), suggest that the cause of the inflammatory response may be quite different. It is likely that the increases in MPO (and thus VEGF) in patients with grafts/catheters may reflect infection, a well-established complication of grafts/catheters, as the cause of inflammation in this patient group.

Considerable epidemiologic information has been compiled using CRP values and causal inferences have been made based upon observations that specific ele-

ments associated with inflammation are increased in patients, while other studies suggest that MPO may be more predictive of outcome during acute events such as myocardial infarction and chest pain [13]. Our finding that MPO activity does not correlate either with CRP level or that of other acute phase proteins suggests that different episodes of the acute phase response differ in their effects both on leukocytes and non-leukocytic cells. Therefore, our data suggest that all episodes of inflammation are not the same with regard to alteration in plasma protein composition, neutrophil degranulation or release of specific growth factors, each of which may play a specific role in inducing and/or perpetuating vascular injury. Better prognostic information, as well as mechanistic insight, may be obtained by measurement of multiple markers of inflammation other than CRP alone.

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Original Contribution

Modulation of arachidonic and linoleic acid metabolites in myeloperoxidase-deficient mice during acute inflammation

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ABSTRACT

Acute inflammation is a common feature of many life-threatening pathologies, including septic shock. One hallmark of acute inflammation is the peroxidation of polyunsaturated fatty acids forming bioactive products that regulate inflammation. Myeloperoxidase (MPO) is an abundant phagocyte-derived hemoprotein released during phagocyte activation. Here, we investigated the role of MPO in modulating biologically active arachidonic acid (AA) and linoleic acid (LA) metabolites during acute inflammation. Wild-type and MPO-knockout (KO) mice were exposed to intraperitoneally injected endotoxin for 24 h, and plasma LA and AA oxidation products were comprehensively analyzed using a liquid chromatography–mass spectrometry method. Compared to wild-type mice, MPO-KO mice had significantly lower plasma levels of LA epoxides and corresponding LA- and AA-derived fatty acid diols. AA and LA hydroxy intermediates (hydroxyeicosatetraenoic and hydroxyoctadecadienoic acids) were also significantly lower in MPO-KO mice. Conversely, MPO-deficient mice had significantly higher plasma levels of cysteinyl-leukotrienes with well-known proinflammatory properties. In vitro experiments revealed significantly lower amounts of AA and LA epoxides, LA- and AA-derived fatty acid diols, and AA and LA hydroxy intermediates in stimulated polymorphonuclear neutrophils isolated from MPO-KO mice. Our results demonstrate that MPO modulates the balance of pro- and anti-inflammatory lipid mediators during acute inflammation and, in this way, may control acute inflammatory diseases.

Acute inflammation is a common sign of many acute life-threatening pathologies, including septic shock and multiple organ failure [1]. Polymorphonuclear neutrophils (PMNs) play an important role in the pathogenesis of sepsis, producing a wide range of inflammatory mediators. However, the exact mechanisms by which these cells participate in sepsis remain incompletely characterized. Activated PMNs release myeloperoxidase (MPO), an abundant hemoprotein that represents up to 5% of total PMN proteins. MPO is thought to mediate primarily host defense reactions [2–4]. Recent evidence suggests that, aside from its role in host defense, reactive intermediates formed by MPO-catalyzed reactions may modify signaling mediators, leading to alterations in cellular signaling [3,5,6]. During acute inflammation, MPO contributes to vascular dysfunction by nitric oxide catalytic consumption and suppression of nitric oxide bioavailability, an effect that disrupts nitric oxide-dependent signaling pathways [7]. Further, MPO catalyzes posttranslational modification of proteins (e.g., chlorination, nitration, and dityrosine bridge formation), in a way that may affect their structure and function [2,3,8–10]. The ability of MPO to modulate redox-sensitive signaling pathways may point to a role for this protein in regulating the inflammatory process [5,7].

Lipid peroxidation is a characteristic feature of acute inflammation, including inflammation associated with septic shock [11]. Oxidative metabolites of arachidonic acid (AA) and linoleic acid (LA) are potent inflammatory mediators, and an increase in their synthesis generally correlates with the severity of sepsis or trauma [11]. Epoxides of AA and LA are epoxyeicosatrienoic acids (EETs) and epoxyoctadecenoic

Abbreviations: AA, arachidonic acid; DHETE, dihydroxyeicosatrienoic acid; DHOME, dihydroxyoctadecenoic acid; EET, epoxyeicosatrienoic acid; EpOME, epoxyoctadecenoic acid; H(P)ETE, hydroxyeicosatrienoic acid and hydroperoxyeicosatrienoic acid; H(P)ODE, hydroxyoctadecadienoic acid and hydroperoxyoctadecadienoic acid; LA, linoleic acid; LOX, lipoxygenase; LPS, lipopolysaccharide; MPO, myeloperoxidase; MPO-KO, MPO-deficient mice; oxo-EET, oxoepoxyeicosatrienoic acids; oxo-ODE, oxo-octadecadienoic acid; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear neutrophil; SEM, standard error of the mean.

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acids (EpOMEs). EETs have the ability to decrease inflammatory responses, including fever [12,13]. EETs and EpOMEs are further metabolized by soluble epoxide hydrolase to their corresponding diols, dihydroxyeicosatrienoic acids (DHETEs) and dihydroxyoctadecenoic acids (DHOMEs) [12–16]. In contrast to epoxides, DHETEs possess fewer anti-inflammatory properties and DHOMEs are mostly proinflammatory [13,17]. Other initial products of AA and LA metabolism are hydroperoxyeicosatrienoic acids (HPETEs) and hydroperoxyoctadecadienoic acids (HPODEs), respectively, and their corresponding reduced forms, hydroxyeicosatetraenoic acids (HETEs) and hydroxyoctadecadienoic acids (HODEs) [13,18]. H(P)ETEs and H(P)ODEs stimulate both pro- and anti-inflammatory pathways depending on the positional isomer of the metabolite, cell type, and bioactivity levels [18]. Hydroperoxy fatty acids can be converted to specific epoxy leukotrienes (LTs). Primarily, 5-hydroperoxy arachidonate (5-HPETE) is metabolized to the highly unstable epoxide intermediate LTA4. Further, LTB4 is formed from LTA4 or LTA4 conjugates with glutathione to yield LTC4, which is additionally metabolized to LTD4 and LTE4 [18,19]. LTs have long been recognized as potent inducers of the inflammatory response [19].

Cytochrome P450 mono-oxygenases, cyclo-oxygenases, and lipoxygenases (LOXs) are widely recognized as the primary enzymes participating in the formation of biologically active lipid mediators [11,13,18]. However, various heme peroxidases that are structurally similar to the family of cytochrome P450 enzymes may also participate in the metabolism of biologically active lipids [20]. Among these is MPO. The MPO/hydrogen peroxide system or hypochlorous acid (a MPO-derived oxidant) promotes the formation of epoxides, peroxides, and chlorohydrins from polyunsaturated fatty acids and cholesterol in isolated PMNs, high-density lipoprotein particles, or chemical systems [21–33]. Recent studies of MPO-KO mice also show that MPO catalyzes the initiation of lipid peroxidation and lipoprotein oxidation in vivo [8,9,34,35]. However, whether MPO participates in the formation of AA- and LA-derived lipid mediators that are involved in the regulation of the inflammatory response is unclear.

Here, we investigated MPO-dependent modulation of selected biologically active AA and LA metabolites in mice with acute inflammation induced by intraperitoneal (ip) application of lipopolysaccharide (LPS), a noninfectious model of systemic sepsis in mice. We focused on AA and LA metabolites that are generally considered products of cytochrome P450 mono-oxygenase and lipoxygenase pathways. Alterations in a wide spectrum of AA- and LA-derived lipid oxidation products were simultaneously monitored in the plasma of MPO-KO and wild-type mice and isolated PMNs using a unique liquid chromatography–mass spectrometry method [14,15]. Our findings show that MPO modulates the formation of pro- and anti-inflammatory lipid mediators and suggest that this enzyme is a direct systemic regulator of the acute inflammatory response.

Material and methods

Materials

The following AA and LA metabolites were purchased from Cayman Chemicals (Ann Arbor, MI, USA): (±)9(10)-epoxy-12*Z*-octadecenoic acid [9(10)-EpOME], (±)12(13)-epoxy-9*Z*-octadecenoic acid [12(13)-EpOME], (±)5(6)-epoxy-8*Z*,11*Z*,14*Z*-eicosatrienoic acid [5(6)-EET)], (±)8(9)-epoxy-5*Z*,11*Z*,14*Z*-eicosatrienoic acid [8(9)-EET], (±)14(15)-epoxy-5*Z*,8*Z*,11*Z*-eicosatrienoic acid [14(15)-EET], (±)11(12)-epoxy-5*Z*,8*Z*,14*Z*-eicosatrienoic acid [11(12)-EET], (±)13-hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE), (±)9-hydroxy-10*E*,12*Z*-octadecadienoic acid (9-HODE), 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, 15-HETE, 19-HETE, 20-HETE, 5,6-DHETE, 8,9-DHETE, 11,12-DHETE, 14,15-DHETE, 9-oxo-10*E*,12*Z*-octadecadienoic acid (9-oxo-ODE), 13-keto-9*Z*,11*E*-octadecadienoic acid (13-oxo-ODE), 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-oxo-ETE), 15-oxo-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid (15-oxo-ETE), leukotriene B4 (LTB4), LTC4, LTD4, LTE4, *N*-acetylleukotriene E4 (Na-LTE4), and 6-oxo-9α,11α,15*S*-trihydroxyprost-13*E*-en-1-oic-3,3,4,4-d4 acid (6-keto prostaglandin F1α–d4). Lordan Fine Lipids provided (±) 9,10-dihydroxy-12(*Z*)-octadecenoic acid (9,10-DHOME) and (±) 12,13-dihydroxy-9(*Z*)-octadecenoic acid (12,13-DHOME). The remainder of the metabolites were synthesized in-house [12-(3-cyclohexylureido)dodecanoic acid, 10(11)-epoxyheptadecanoic acid, and 10(11)-dihydroxynonadecanoic acid] as described previously [36]. Omni-Solv acetonitrile and methanol were from EM Science (Gibbstown, NJ, USA). All other chemical reagents of a high-performance liquid chromatography grade or better were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Fisher Scientific (Pittsburgh, PA, USA).

Animal experiment

All animal protocols were approved by the Animal Research Committee of the University of California at Davis. MPO-KO mice backcrossed on a C57BL/6J background have been comprehensively characterized elsewhere [37–39]. Age- and sex-matched wild-type C57BL/J6 mice and 4-month-old male MPO-KO mice weighing 22–28 g were given a single ip injection of *Escherichia coli* LPS (10 mg/kg), which was freshly prepared in sterile phosphate-buffered saline (pH 7.4). Control mice received an equivalent amount of sterile phosphate-buffered saline. The LPS murine model of acute systemic inflammation was selected because we have previously observed extensive activation of the P450-epoxygenase/soluble epoxide hydrolase and LOX lipid metabolism pathways in this model [14,15]. Twenty-four hours after LPS administration, the mice received an overdose of pentobarbital (ip), and blood was collected via cardiac puncture with an ethylenediaminetetraacetic acid-rinsed syringe. The plasma was immediately separated, and a combination of triphenylphosphine, ethylenediaminetetraacetic acid (1 mM), and butylated hydroxytoluene (0.2% w/w) was added to stabilize the samples. All samples were stored at less than −70°C until analysis.

In vitro experiments with isolated PMNs

Isolation of mouse neutrophils was performed as described previously, with modifications [40,41]. A sterile solution of casein (2% w/v in phosphate-buffered saline) was injected into the peritoneal cavity of the mouse (1 ml per mouse) to enrich the exudate of PMNs. The mice were euthanized by carbon dioxide 24 h later, and peritoneal lavage was performed using repeated applications of phosphate-buffered saline. The resulting cells were washed twice with ice-cold phosphate-buffered saline (200 *g* for 10 min at 4°C), and contaminating erythrocytes were lysed in 0.45% NaCl. PMNs were purified using Histopaque-1077 and -1119 (800 *g* for 30 min at room temperature), washed three times with ice-cold phosphate-buffered saline (200 *g* for 10 min at 4°C), and resuspended in Hanks' balanced salt solution containing 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid. All samples were >93% pure as determined by Diff-Quik staining and had >96% viable PMNs as determined by trypan blue dye exclusion. For determination of AA and LA metabolites, PMN samples were pooled from four or five wild-type or MPO-KO mice to obtain a sufficient amount of PMNs. PMNs (2×10^6) in 1 ml of Hanks' balanced salt solution were preincubated with or without AA (50 μM) and LA (50 μM) at 37°C for 5 min. Stock solutions of AA and LA were in ethanol and the final concentration of ethanol in cell suspension was <0.05%. A subset of samples was incubated for an additional 30 min (control PMNs). Another subset (activated PMNs) was stimulated first with PMA (phorbol 12-myristate 13-acetate; 100 nM) for 5 min and then with the calcium ionophore A23187 (2 μM) for 25 min. Stimulation of samples was

terminated with 0.5 ml ice-cold methanol. A combination of triphenylphosphine, ethylenediaminetetraacetic acid (1 mM), and butylated hydroxytoluene (0.2% w/w) was then immediately added to stabilize the samples. All samples were stored at less than −70°C until analysis.

Oxylipin profile analysis

The analysis was performed as described previously [42–44]. Immediately before solid-phase extraction, 250 μl serum or 1.5 ml PMN suspension was diluted 1:1 (v/v) with 2.5 mM phosphoric acid. Each sample was then spiked with the surrogates 26.7 nM 6-keto prostaglandin F1α–d4, 26.7 nM 10(11)-epoxyheptadecanoic acid, and 26.7 nM 10(11)-dihydroxynonadecanoic acid. Oasis HLB 60-mg cartridges (Waters, Milford, MA, USA) were preconditioned with 2 ml of methanol and 2 ml of solution containing 2.5 mM phosphoric acid and 10% methanol (pH 3.8). After the samples were applied, the cartridges were washed with 2 ml of the 2.5 mM phosphoric acid–10% methanol mixture. The analytes were eluted with 2 ml of ethyl acetate. The ethyl acetate residue was evaporated under nitrogen gas, and the samples were resuspended in 100 μl of methanol containing 26.7 nM internal standard 12-(3-cyclohexylureido)dodecanoic acid. The samples were then vortexed for 5 min, transferred to autosample vials, and stored at −80 °C until analysis. A Waters 2790 separation module equipped with a Luna C18 column (2.0×150 mm, 5 μm; Phenomenex, Torrance, CA, USA) and a Quattro Ultima tandem-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization source were used for high-performance liquid chromatography separation and electrospray tandem mass spectrometry, respectively. Mobile phase A was water with 0.1% glacial acetic acid. Mobile phase B consisted of acetonitrile:methanol (84:16) with 0.1% glacial acetic acid. Gradient elution was performed at a flow rate of 400 μl/min. Chromatography was optimized to separate all analytes in 21 min. The Quattro Ultima tandem-quadrupole mass spectrometer was operated in multiple reaction monitoring modes, and both negative and positive ion electrospray was used as the means of ionization.

Data collection and processing were performed using MassLynx software version 4.0, as described previously [14,15,43,44]. Surrogates were added to samples before extraction to mimic the extraction of AA and LA metabolites. Internal standard was used to verify surrogate recovery and to monitor instrument response. The analytes were linked to their corresponding surrogates for the purpose of quantification. In addition, if the surrogate recoveries, as monitored by internal standards, were not between 80 and 120%, the sample was considered invalid and not included in the dataset. Calibration curves were prepared to generate a series of six calibration points spanning a concentration range from 0.1 to 100 nM for all analytes, with the surrogates and internal standards at a constant concentration of 26.7 nM.

Statistical analysis

Data are expressed as the means ± standard error of the mean (SEM). Groups were compared using an unpaired Student *t* test. Values of *p* less than 0.05 were considered significant.

Results

MPO deficiency suppresses endotoxemia-induced formation of LA epoxides and vicinal dihydroxy metabolites of AA and LA

To model acute inflammation associated with systemic endotoxemia/sepsis, we injected mice ip with LPS and measured AA and LA metabolites 24 h later. In wild-type animals, LPS-induced endotoxemia greatly increased the plasma levels of LA epoxides (EpOMEs) as well as vicinal dihydroxy metabolites of AA and LA (DHETEs and DHOMEs, respectively), confirming that the P450-epoxygenase/soluble epoxide hydrolase lipid metabolism pathways are activated in our noninfectious model of systemic sepsis (Table 1). Interestingly, after induction of sepsis MPO-KO mice had significantly lower plasma levels of 12(13)-EpOME, 9,10-DHOME, and 12,13-DHOME. Septic MPO-KO mice also had significantly lower levels of 5,6-DHETE, 8,9-DHETE, 11,12-DHETE, and 14,15-DHETE. The plasma concentration of another LA epoxide, 9(10)-EpOME, was not significantly different in WT and MPO-KO mice. The legend to Table 1 lists other metabolites belonging to these metabolic pathways that fall below the detection limit in both wild-type and MPO-KO mice.

MPO deficiency suppresses endotoxemia-induced increases in HETEs and HODE

In wild-type mice, endotoxin challenge also significantly increased the formation of lipid metabolites from LOX-catalyzed pathways (Table 2). However, induction of these AA (5-HETE, 11-HETE, 12-HETE, and 15-HETE) and LA (9-HODE and 13-HODE) metabolites was significantly suppressed in MPO-KO mice. The 12-HETE plasma concentrations were approximately 2 orders higher compared to other metabolites and associated with high variability. This was most likely due to contamination from platelets in the isolated plasma samples. The legend to Table 2 lists other metabolites belonging to these metabolic pathways that fall below the detection limit in both wild-type and MPO-KO mice.

Table 1
MPO deficiency suppresses endotoxemia-induced increases in EpOMEs, DHOMEs, and DHETEs

Metabolite	Control		LPS	
	WT	MPO-KO	WT	MPO-KO
(±)9(10)-Epoxy-12*Z*-octadecenoic acid [9(10)-EpOME]	3.5 ± 1.0	4.3 ± 0.7	5.5 ± 1.1	3.0 ± 0.5
(±)12(13)-Epoxy-9*Z*-octadecenoic acid [12(13)-EpOME]	6.0 ± 0.9	6.9 ± 1.1	13.0 ± 2.13†	7.4 ± 0.8*
(±)9,10-Dihydroxy-12(*Z*)-octadecenoic acid (9,10-DHOME)	2.7 ± 0.4	4.0 ± 1.0	19.4 ± 4.4†	11.0 ± 1.4*,‡
(±)12,13-Dihydroxy-9(*Z*)-octadecenoic acid (12,13-DHOME)	9.1 ± 2.1	12.1 ± 3.8	50.6 ± 9.4†	30.4 ± 3.1*,‡
(±)5,6-Dihydroxy-8*Z*,11*Z*,14*Z*-eicosatrienoic acid (5,6-DHETE)	2.3 ± 0.5	1.6 ± 0.5	6.4 ± 1.7†	3.0 ± 0.6*
(±)8,9-Dihydroxy-5*Z*,11*Z*,14*Z*-eicosatrienoic acid (8,9-DHETE)	5.0 ± 1.3	4.9 ± 0.3	12.2 ± 1.2†	7.7 ± 1.1*,‡
(±)11,12-Dihydroxy-5*Z*,8*Z*,14*Z*-eicosatrienoic acid (11,12-DHETE)	3.2 ± 0.5	3.5 ± 0.3	10.6 ± 1.8†	6.0 ± 0.6*,‡
(±)14,15-Dihydroxy-5*Z*,8*Z*,11*Z*-eicosatrienoic acid (14,15-DHETE)	5.4 ± 0.7	4.5 ± 0.3	15.8 ± 2.1†	7.9 ± 0.8*,‡

Plasma levels (nM) of EpOMEs (epoxides of LA), DHOMEs (dihydroxy metabolites of LA), and DHETEs (dihydroxy metabolites of AA) at 24 h after induction of endotoxemia are shown. Results represent the means ± SEM derived from at least four animals per group. Plasma levels of other determined metabolites including epoxides of AA (EETs) and the degradation products 9-oxo-ODE, 13-oxo-ODE, 5-oxo-ETE, and 15-oxo-ETE were under the detection limits in both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.

* $p<0.05$ MPO-KO vs wild type.

† $p<0.05$ control wild type vs LPS-treated wild type.

‡ $p<0.05$ control MPO-KO vs LPS-treated MPO-KO.

Table 2
MPO deficiency suppresses endotoxemia-induced increases in HETEs and HODEs

Metabolite	Control		LPS	
	WT	MPO-KO	WT	MPO-KO
(±)5-Hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HETE)	3.1 ± 0.4	2.2 ± 0.2*	18.4 ± 2.8†	8.0 ± 1.3*,‡
(±)11-Hydroxy-5*Z*,8*Z*,12*E*,14*Z*-eicosatetraenoic acid (11-HETE)	1.38 ± 0.34	1.01 ± 0.27	2.39 ± 0.21†	0.89 ± 0.25*
(±)12-Hydroxy-5*E*,8*Z*,10*Z*,14*Z*-eicosatetraenoic acid (12-HETE)	3485 ± 786	3202 ± 374	4555 ± 665	3869 ± 746*
(±)12-Hydroxy-5*E*,8*Z*,10*Z*,14*Z*-eicosatetraenoic acid (15-HETE)	1.24 ± 0.18	1.19 ± 0.08	2.03 ± 0.31†	0.97 ± 0.08*,‡
(±)9-Hydroxy-10*E*,12*Z*-octadecadienoic acid (9-HODE)	5.5 ± 1.2	5.2 ± 0.9	10.6 ± 0.7†	5.8 ± 1.0*
(±)13-Hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE)	8.4 ± 1.3	7.6 ± 1.3	22.3 ± 2.2†	11.8 ± 1.2*,‡

Plasma levels (nM) of HETEs (AA-derived lipid metabolites) and HODEs (LA-derived lipid metabolites) generated by LOX-catalyzed pathways. HETEs and HODEs were analyzed 24 h after induction of endotoxemia. Results represent the means ± SEM derived from at least four animals per group. Plasma levels of other determined metabolites, including 8-HETE, 9-HETE, 19-HETE, and 20-HETE, were under the detection limits in both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.
* $p<0.05$ MPO-KO vs wild-type.
† $p<0.05$ control wild type vs LPS-treated wild-type.
‡ $p<0.05$ control MPO-KO vs LPS-treated MPO-KO.

MPO deficiency increases endotoxemia-induced formation of LTs

The involvement of the enzyme 5-LOX in AA and LA metabolism is complex, because its primary metabolite 5-HPETE feeds multiple biosynthetic pathways, including pathways for the synthesis of LTs and 5-HETE. In an alternate metabolic pathway, 5-HPETE can undergo stereospecific dehydration to LTA4 by a second 5-LOX-catalyzed step. LTA4 can then be converted to LTB4 or by LTC4 synthase into the cysteinyl-LTs LTC4, LTD4, and LTE4. In contrast to other AA and LA mediators, cysteinyl-LTs were revealed to be higher in the plasma of MPO-KO mice than in the plasma of wild-type mice (Table 3). Levels of LTA4 were not detectable in this study and LTB4 did not differ significantly among WT and MPO-KO mice. However, particularly interesting were significantly higher levels of LTC4, LTD4, LTE4, and LTE4-NA in the plasma of septic MPO-KO mice. LTC4 and LTE4 levels were also higher in control MPO-KO mice than in wild-type mice, suggesting that these cysteinyl-LTs accumulate in MPO-KO mice even in the absence of an acute inflammatory response. This could due to a disturbance of the peritoneum by the ip application of sterile saline in control mice.

Activated PMNs from wild-type mice contain significantly higher levels of AA and LA epoxides, vicinal dihydroxy metabolites, HETEs, and HODEs than those from MPO-KO mice

Knowing that the MPO deficiency affects systemic fatty acid metabolism in septic mice, we evaluated AA and LA mediator profiles in PMNs isolated from MPO-KO and wild-type mice. In these experiments, purified AA (50 μM) and LA (50 μM) were added to a suspension of isolated PMNs, which were then activated by successive exposure to PMA and a calcium ionophore. Samples containing nonactivated PMNs and lacking exogenous AA and LA served as controls. The AA and LA concentrations selected, as well as the use of the combination of PMA and calcium ionophore, were intended to maximize the production of AA and LA mediators by isolated PMNs and were based on preliminary data and literature [41,45,46].

Consistent with our in vivo findings, PMNs isolated from MPO-KO mice formed, in the presence of exogenous AA and LA, significantly lower levels of AA epoxides [5(6)-EET, 8(9)-EET, and 11(12)-EET] and LA epoxides [9(10)EpOME and 12(13)-EpOME)] than wild-type PMNs (Fig. 1). Correspondingly, levels of vicinal diols of AA and LA were significantly lower in MPO-KO PMNs in the presence of exogenous AA and LA (Fig. 2). Concentrations of AA metabolites (5-HETE, 8-HETE, 9-HETE) and LA metabolites (9-HODE and 13-HODE) were significantly lower in PMNs isolated from MPO-KO mice (Fig. 3). Oxo-octadecadienoic acids (9-oxo-ODE and 13-oxo-ODE), which are formed by degradation of unstable LA-derived hydroperoxides (HPODE), were significantly decreased in MPO-KO PMNs in the presence of exogenous AA and LA (Figs. 4A and 4B). The same was true of the AA metabolites oxo-epoxyeicosatrienoic acids (5-oxo-EET and 15-oxo-EET; Figs. 4C and 4D). The complex profile of LTs was not analyzed in the homogenates of PMNs. However, a determination of cysteinyl-LTs in the supernatants of PMNs isolated from wild-type and MPO-KO mice incubated in the absence and presence of PMA for 10, 30, and 120 min showed no significant differences (data not shown).

Table 3
MPO deficiency enhances endotoxemia-induced increases in cysteinyl LTs

Metabolite	Control		LPS	
	WT	MPO-KO	WT	MPO-KO
Leukotriene B4 (LTB4)	0.4 ± 0.5	0.5 ± 0.7	16.2 ± 6.8†	20.6 ± 4.3‡
Leukotriene C4 (LTC4)	2.1 ± 1.4	9.9 ± 0.6*	9.2 ± 1.4†	16.3 ± 1.8*,‡
Leukotriene D4 (LTD4)	3.3 ± 2.1	4.1 ± 1.9	6.6 ± 1.6	24.9 ± 4.9*,‡
Leukotriene E4 (LTE4)	0.9 ± 0.3	4.2 ± 0.8*	3.1 ± 0.6†	14.3 ± 1.5*,‡
N-acetylleukotriene E4 (LTE4-NA)	0.1 ± 0.0	0.1 ± 0.0	6.4 ± 0.5†	14.6 ± 4.2*,‡

LT plasma levels (nM) were analyzed 24 h after endotoxemia induction. Results represent the means ± SEM derived from at least four animals per group. Plasma levels of LTA4 were under the detection limits in both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.
* $p<0.05$ MPO-KO vs wild type.
† $p<0.05$ control wild type vs LPS-treated wild type.
‡ $p<0.05$ control MPO-KO vs LPS-treated MPO-KO.

Wild-type and MPO-KO mice have comparable numbers of blood leukocytes and differentiation counts

The numbers of peripheral blood leukocytes and PMNs were determined in wild-type and MPO-KO mice before sepsis and 24 h after induction of sepsis. Both wild-type and MPO-KO mice exhibited a drop in peripheral blood PMNs after endotoxin challenge. However, no significant differences in total leukocyte blood count and differentials were observed (control wild type, $4.31 \pm 0.54 \times 10^9$/L with 22 ± 9% neutrophil granulocytes; control MPO-KO, $4.05 \pm 0.62 \times 10^9$/L with 25 ± 11% neutrophil granulocytes; LPS-treated wild type, $3.22 \pm 0.32 \times 10^9$/L with 35 ± 10% neutrophil granulocytes; and LPS-treated MPO-KO, $3.48 \pm 0.46 \times 10^9$/L with 34 ± 12% neutrophil granulocytes). Eosinophilic polymorphonuclear granulocytes were under 2% and basophilic polymorphonuclear granulocytes were not found. Similarly, other authors have not found any significant differences in the numbers of leukocytes and PMNs between wild-type and MPO-KO mice in various inflammatory models [35,39,47].

Discussion

Our results illustrate the significance of MPO in the formation of biologically active metabolites of AA and LA. Data suggest that during acute inflammation, MPO plays an important role in the formation of

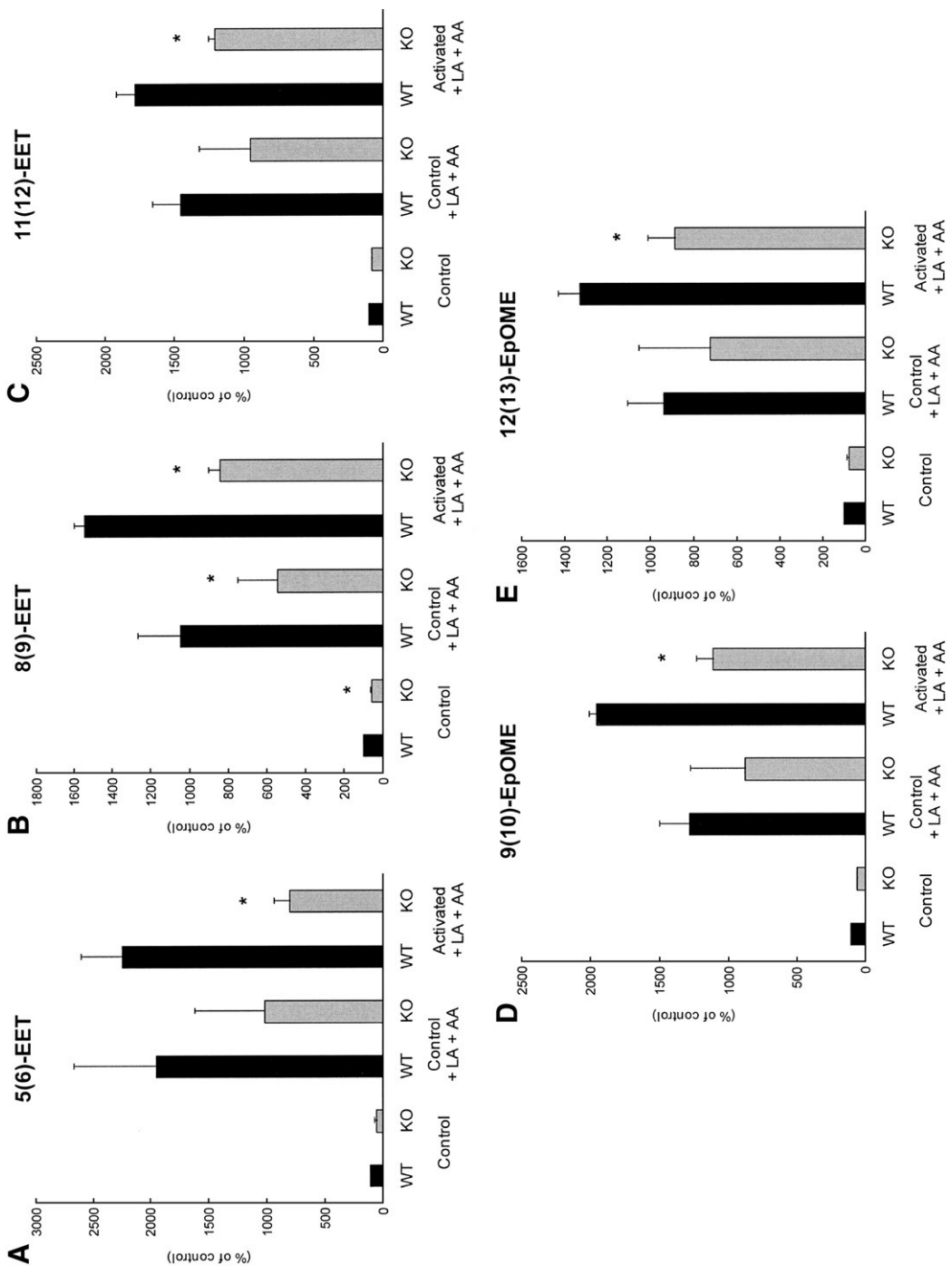

Fig. 1. Increased production of EET and EpOME by PMNs isolated from wild-type compared to PMNs isolated from MPO-KO mice. Relative levels of EETs (AA epoxides) and EpOMEs (LA epoxides) in control and PMA/calcium ionophore-activated PMNs incubated in the absence or the presence of AA and LA are shown. Results are expressed as the percentage (mean ± SEM) of wild-type controls incubated in the absence of AA and LA and are derived from at least three samples (pooled from four or five animals). *$p < 0.05$ MPO-KO vs wild-type, #$p < 0.05$ control MPO-KO vs LPS-treated MPO-KO. Average values in control samples of WT mice were as follows: 5(6)-EET, 4.72 nM; 8(9)-EET, 2.33 nM; 11(12)-EET, 2.02 nM; 9(10)-EpOME, 2.22 nM; and 12(13)-EpOME, 2.97 nM. Concentrations of 14(15)-EET were under the detection limits in samples from both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.

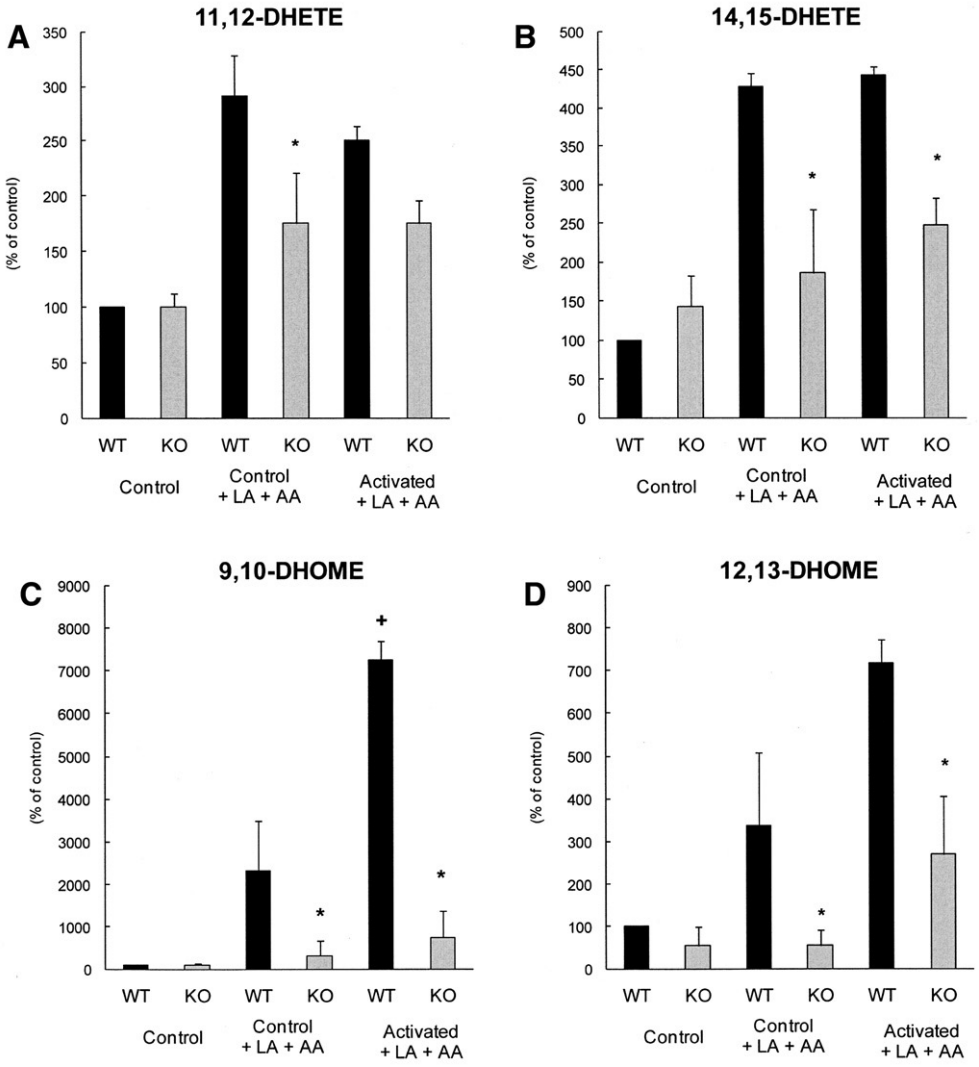

Fig. 2. Increased production of DHETE and DHOME by PMNs isolated from wild-type compared to PMNs isolated from MPO-KO mice. Relative levels of DHETEs (AA dihydroxy metabolites) and DHOMEs (LA dihydroxy metabolites) in control and PMA/calcium ionophore-activated PMNs incubated in the absence or the presence of AA and LA are shown. Results are expressed as the percentage (mean ± SEM) of wild-type controls incubated in the absence of AA and LA. All results are derived from at least three samples (pooled from four or five animals). *$p<0.05$ MPO-KO vs wild-type, +$p<0.05$ control wild type vs LPS-treated wild-type, #$p<0.05$ control MPO-KO vs LPS-treated MPO-KO. Average values in control samples of WT mice were as follows: 11,12-DHETE, 0.10 nM;14,15-DHETE, 0.09 nM; 9,10-DHOME, 0.10 nM; 12,13-DHOME, 0.22 nM. Concentrations of 5,6-DHETE and 8,9-DHETE were under the detection limits in samples from both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.

AA and LA epoxides and hydroxy intermediates together with the catabolism of cysteinyl-LTs. Accordingly, the formation of AA and LA lipid mediators (HETEs, HODEs, and H(P)ODEs) was suppressed in MPO-KO mice and PMNs isolated from these animals. Interestingly, the difference between AA and LA metabolites formed in PMNs from wild-type and MPO-KO mice was also observed in the absence of any direct oxidative burst stimulation. However, PMNs elicited into the peritoneum by casein injection are partially activated although some responsiveness to proinflammatory stimuli remains [40,41]. Thus the activation of PMNs with casein together with the activation of PMNs by the isolation procedure can partly trigger PMNs to produce reactive oxygen species, including hydrogen peroxide. However, this observation suggests that metabolic pathways not directly related to the activation of MPO can also be involved in the observed phenomenon.

Several mechanisms may underlie the initiation of lipid peroxidation by MPO-catalyzed reactions. A wide range of oxidized lipid intermediates were shown to be formed in reactions of cholesterol and unsaturated fatty acids with MPO in the presence of low-molecular-weight intermediates (such as chloride, nitrite, or tyrosine) through MPO-generated diffusible radical species [2,3,8–10]. An alternative diffusible radical species formed by MPO that may participate in lipid peroxidation is nitrogen dioxide [28,29,48,49]. Epoxides can be formed from chlorohydrins that undergo HCl elimination [21,22,50]. Further, MPO as a member of the family of heme peroxidases can also be suggested to directly function as a fatty acid epoxygenase and lipoxygenase [20,51]. However, the epoxidation mechanisms catalyzed by heme proteins are not well understood. The putative mechanism for the epoxidation of alkenes by chloroperoxidases, also known as the ferryl–oxygen transfer mechanism [20,52], is comparable to the oxygen transfer mechanism of cytochrome P450.

The nearly uniform distribution of various isomers of AA and LA lipid peroxides formed during endotoxemia is consistent with a free radical, rather than a regioselective (e.g., involving LOXs and cyclo-oxygenases), mechanism of AA and LA oxidation. However, the employed methodological approach did not allow the analysis of stereoisomer specificity in our study. It was not possible to compare the activity of all enzymes involved in AA and LA in MPO-KO and wild-type mice. Moreover, other studies show that the cellular and enzymatic components capable of affecting lipid peroxidation are comparable between MPO-KO and wild-type mice under acute inflammatory conditions. Zhang et al. showed that cyclo-oxygenase-1, cyclo-oxygenase-2, and 12-LOX levels are similar in isolated

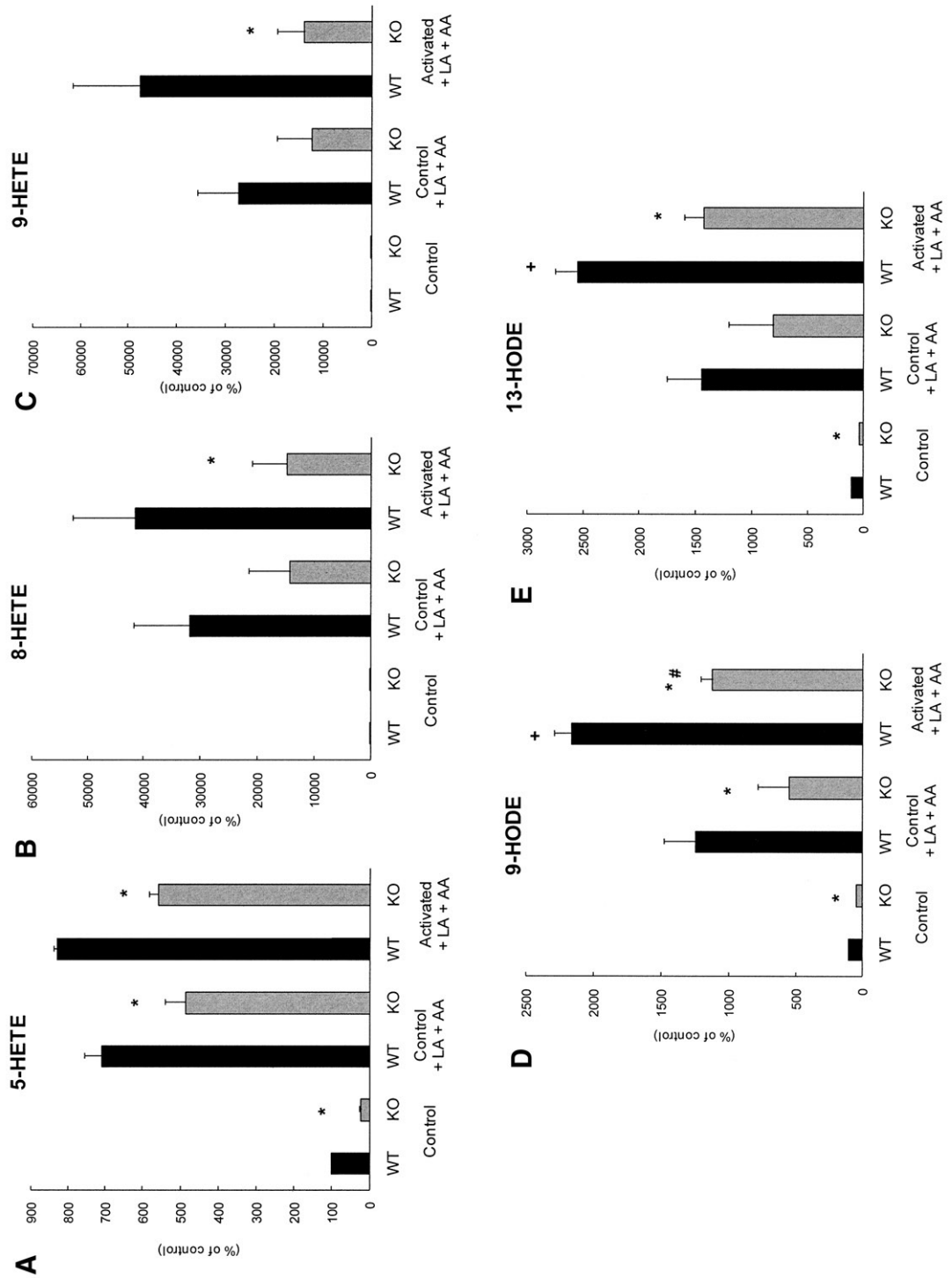

Fig. 3. Increased production of HETE and HODE by PMNs isolated from wild-type compared to PMNs isolated from MPO-KO mice. Relative levels of AA- (HETEs) and LA-derived (HODEs) lipid metabolites in control and PMA/calcium ionophore-activated PMNs incubated in the absence or the presence of AA and LA are shown. Results are expressed as the percentage (mean ± SEM) of wild-type controls incubated in the absence of AA and LA and are derived from at least three samples (pooled from four or five animals). *$p < 0.05$ MPO-KO vs wild-type, +$p < 0.05$ control wild-type vs LPS-treated wild-type, #$p < 0.05$ control MPO-KO vs LPS-treated MPO-KO. Average values in control samples of WT mice were as follows: 5-HETE, 17.72 nM; 8-HETE, 0.1 nM; 9-HETE, 0.1 nM; 9 HODE, 73.07 nM; and 13 HODE, 65.76 nM. Concentrations of 11-HETE, 12-HETE, 15-HETE, 19-HETE, and 20-HETE were under the detection limits in samples from both wild-type and MPO-KO mice. Limits of quantification are in Supplementary Table 1.

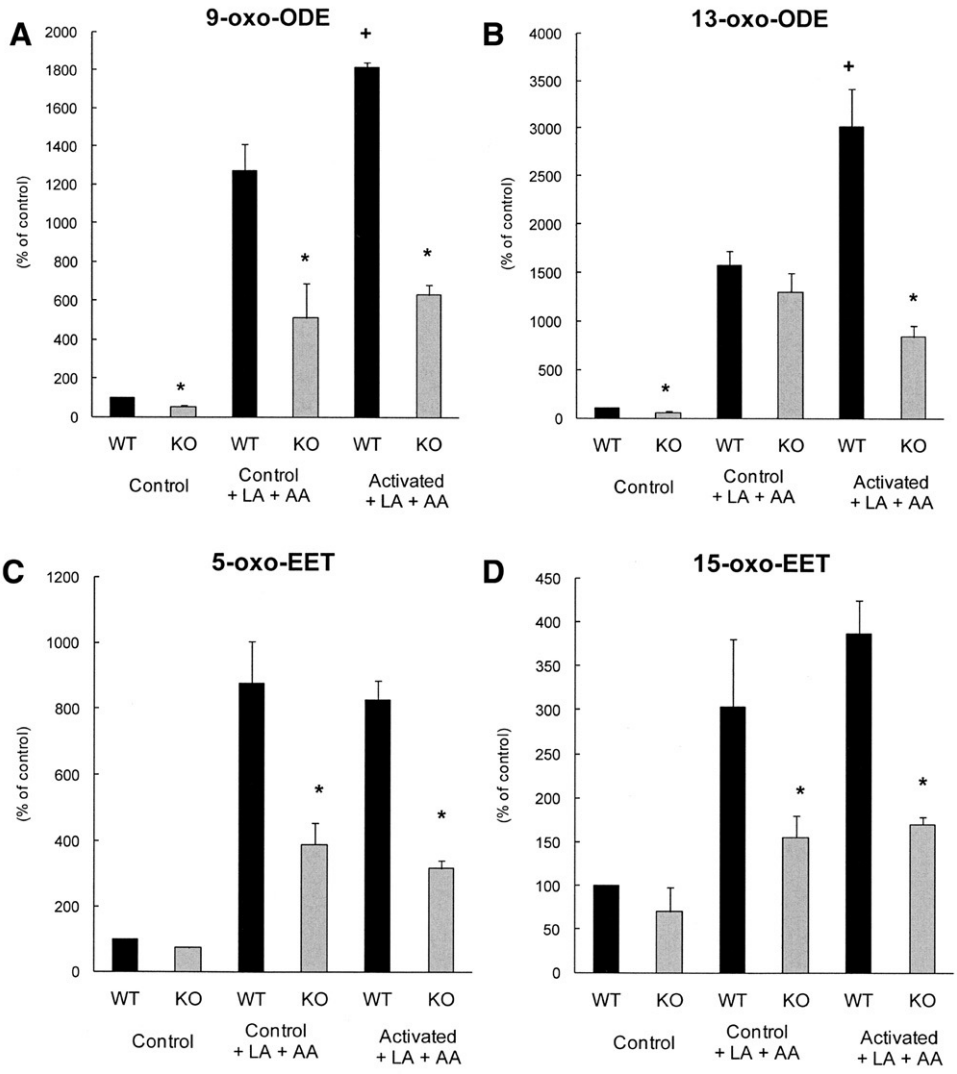

Fig. 4. Increased production of oxo-ODEs and oxo-EETs by PMNs isolated from wild-type compared to PMNs isolated from MPO-KO mice. Relative levels of oxo-ODEs (LA degradation products) and oxo-EETs (AA degradation products) in control and PMA/calcium ionophore-activated PMNs incubated in the absence or the presence of AA and LA are shown. Results are expressed as the percentage (mean ± SEM) of wild-type controls incubated in the absence of AA and LA and are derived from at least three samples (pooled from four or five mice). *$p<0.05$ MPO-KO vs wild-type, +$p<0.05$ control wild-type vs LPS-treated wild-type, #$p<0.05$ control MPO-KO vs LPS-treated MPO-KO. Average values in control samples of WT mice were as follows: 9-oxo-ODE, 10.27 nM; 13-oxo-ODE, 20.62 nM; 5-oxo-EET, 2.11 nM; 15-oxo-EET, 5.75 nM. Limits of quantification are in Supplementary Table 1.

peritoneal leukocytes from MPO-KO and wild-type mice [35]. Likewise, another two in vivo studies of MPO-KO mice have suggested that initiation of lipid peroxidation is dependent upon MPO [34,35]. Interestingly, MPO-KO mice under septic conditions induced by *Candida* revealed significantly reduced plasma levels of isolevuglandins, a family of ketoaldehydes generated by free radical-induced peroxidation of arachidonate-containing lipids [34]. Similarly, activated PMNs isolated from MPO-deficient human subjects form 9-HETE and 9-HODE only after addition of exogenous MPO [30]. Nevertheless, the possibility that MPO-KO alters the activities of various AA and LA metabolic enzymes by changing their tissue-specific expression, posttranslational modification, or cellular localization cannot be excluded.

Our data demonstrate for the first time that MPO deficiency leads to the accumulation of cysteinyl-LTs in systemic circulation during the course of acute inflammation. The finding that these important proinflammatory mediators are deactivated by MPO in an in vivo model of acute inflammation is supported by several in vitro studies [53–55]. MPO is thought to degrade cysteinyl-LTs by their oxidation [53–55]. Previously, the MPO-dependent inactivation of LTs and cysteinyl-LTs in particular was demonstrated in cell-free systems and in activated human phagocytes. In the latter case, human MPO-deficient PMNs and monocytes were unable to degrade LTs unless MPO was added back into the system [53–55]. However, in our experiments we were not able to confirm these findings using PMNs isolated from wild-type and MPO-KO mice.

Interestingly, metabolites that were under the detection limit in plasma of both wild-type and MPO-KO mice and in PMNs isolated from wild-type and MPO-KO mice differ (legends to Tables 1 and 2 and Figs. 1, 2, and 3). For example, epoxides of AA were under the detection limits in the plasma of mice, similar to our other studies [14,15], which is probably related to their low half-life in vivo. Undetectable levels of various degradation products of unstable LA- and AA-derived hydroperoxides in vivo in contrast to in vitro could also be coupled with their short half-life in the blood circulation. On the other hand, the presence of detectable levels of some dihydroxy metabolites and hydroperoxides of AA and LA only in vivo can be connected to a limited range of enzymes involved in AA and LA metabolic pathways present in PMNs in contrast to the complex metabolism of AA and LA by various cell types in vivo. This raises the question of the importance of various cell types and their enzymatic accessories in the formation of biologically active AA and LA

metabolites during the course of acute inflammation. It is not possible to exclude differences in the activity of various enzymes in cells of different origin between wild-type and MPO-KO mice as discussed above; however, our data strongly support the importance of MPO in this process.

The MPO-dependent modulation of AA and LA mediators could significantly affect the physiological functions of the organism. It is interesting to speculate about the role of MPO-dependent formation of polyunsaturated fatty acid epoxides with mostly anti-inflammatory effects during acute inflammation, which could theoretically compensate for the decreased activity of cytochrome P450 enzymes due to inflammation [56]. Simultaneously, MPO can catalyze the degradation of the cysteinyl-LTs LTC4, LTD4, and LTE4, which stimulate various leukocyte functions, including chemotactic movement and tissue infiltration [13,18,19]. Nevertheless, the MPO-dependent formation of lipid mediators with anti-inflammatory properties and the catabolism of proinflammatory LTs may have positive effects on the inflammatory process. In this study, no significant effects of MPO deficiency on mortality or physiological functions such as blood pressure (data not shown) were observed up to the time of blood collection. However, several studies using various types of inflammatory models recently showed an increased level of the inflammatory process in MPO-deficient mice [37,47,57–59]. Thus the specific modulation of AA and LA metabolites by MPO can be included among other previously suggested anti-inflammatory effects, such as clearance of noxious stimuli, oxidative inactivation of proinflammatory mediators, inactivation of proteases, catabolism of NO, and inhibition of inducible nitric oxide synthase expression [60].

This study provides evidence that MPO plays an important role in the formation of both pro- and anti-inflammatory AA- and LA-derived lipid mediators in our a noninfectious model of systemic sepsis in mice. Our findings invite a reappraisal of the enzymatic participants in lipid peroxidation in vivo, with MPO added to the ranks of enzymes like LOXs, cyclo-oxygenases, and cytochrome P450 complexes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2010.02.010.

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Pathogenic Cycle Between the Endogenous Nitric Oxide Synthase Inhibitor Asymmetrical Dimethylarginine and the Leukocyte-Derived Hemoprotein Myeloperoxidase

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Background—The nitric oxide synthase inhibitor asymmetrical dimethylarginine (ADMA) and the leukocyte-derived hemoprotein myeloperoxidase (MPO) are associated with cardiovascular diseases. Activation of monocytes and polymorphonuclear neutrophils (PMNs) with concomitant release of MPO is regulated in a nitric oxide–dependent fashion. The aim of the study was to investigate a potential 2-way interaction between ADMA and MPO.

Methods and Results—Ex vivo, ADMA uptake by isolated human PMNs, the principal source of MPO in humans, significantly impaired nitric oxide synthase activity determined by gas chromatography–mass spectrometry. In humans, short-term ADMA infusion ($0.0125\ mg \cdot kg^{-1} \cdot min^{-1}$) significantly increased MPO plasma concentrations. Functionally, PMN exposure to ADMA enhanced leukocyte adhesion to endothelial cells, augmented NADPH oxidase activity, and stimulated PMN degranulation, resulting in release of MPO. In vivo, a 28-day ADMA infusion ($250\ \mu mol \cdot kg^{-1} \cdot d^{-1}$) in C57Bl/6 mice significantly increased plasma MPO concentrations, whereas this ADMA effect on MPO was attenuated by human dimethylarginine dimethylaminohydrolase1 (hDDAH1) overexpression. Moreover, the MPO-derived reactive molecule hypochlorous acid impaired recombinant hDDAH1 activity in vitro. In $MPO^{-/-}$ mice, the lipopolysaccharide-induced increase in systemic ADMA concentrations was abrogated.

Conclusions—ADMA profoundly impairs nitric oxide synthesis of PMNs, resulting in increased PMN adhesion to endothelial cells, superoxide generation, and release of MPO. In addition, MPO impairs DDAH1 activity. Our data reveal an ADMA-induced cycle of PMN activation, enhanced MPO release, and subsequent impairment of DDAH1 activity. These findings not only highlight so far unrecognized cytokine-like properties of ADMA but also identify MPO as a regulatory switch for ADMA bioavailability under inflammatory conditions. (***Circulation*. 2011;124:2735-2745.**)

Key Words: arteriosclerosis ■ endothelium ■ inflammation ■ leukocytes ■ nitric oxide synthase

Over the past decade, the endogenous nitric oxide (NO) synthase (NOS) inhibitor asymmetrical dimethylarginine (ADMA) has emerged as a novel cardiovascular risk factor.[1] Elevated plasma ADMA concentrations have been demonstrated in a variety of patient cohorts with cardiovascular risk factors and overt cardiovascular disease.[1] Moreover, ADMA appears to be an independent predictor of cardiovascular and all-cause mortality.[2–5] Leukocyte-derived NO has emerged as a critical determinant of the activation state of a cell. Accordingly, elevated ADMA concentrations are associated with increased leukocyte activation.[6,7] The major pathway for elimination of ADMA is its metabolism by

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the enzyme dimethylarginine dimethylaminohydrolase (DDAH), which exists in 2 isoforms (DDAH1 and DDAH2), yielding L-citrulline and dimethylamine.[8,9] A minor portion of circulating ADMA is excreted via the kidneys. Importantly, the activity of the DDAH enzyme has been shown to be regulated in a redox-sensitive fashion.[10] Thus, DDAH inactivation under conditions of increased production of vascular-derived reactive oxygen and nitrogen species may result in increased ADMA concentrations.

Clinical Perspective on p 2745

Endothelium-derived NO plays a crucial role in the regulation of vascular tone, platelet activity, leukocyte adhesion, and development of arteriosclerosis.[11] A decreased NO bioavailability by impaired synthesis resulting from the presence of endogenous NOS inhibitors and/or increased NO inactivation by elevated oxidative stress leads to the development of endothelial dysfunction. Important initial steps in the course of endothelial dysfunction are an impaired release of NO from the endothelium, an increased expression of adhesion molecules, and the consecutive enhanced attachment of polymorphonuclear neutrophils (PMNs) to endothelial cells.

On activation, PMNs release myeloperoxidase (MPO), an NO-oxidizing hemoprotein with various proinflammatory properties. The MPO enzyme is stored within primary granules of neutrophils and, to a lesser extent, in monocytes and macrophages, and is released in the course of degranulation.[12,13] MPO catalyzes the generation of oxygen- and nitrogen-derived reactive species and promotes oxidative damage of host tissue at sites of inflammation, including arteriosclerotic lesions. The enhanced NO consumption by MPO may explain, in part, the importance of MPO in the context of endothelial dysfunction and cardiovascular diseases. Elevated concentrations of circulating MPO are associated with the presence of coronary artery disease and powerfully predict cardiovascular events in patients with acute coronary syndrome.[14,15]

We hypothesized that increased ADMA concentrations lead to enhanced PMN activation, subsequent degranulation, and MPO release. In addition, we hypothesized that MPO—by impairing DDAH activity, which leads to ADMA accumulation and decreased NO production—may contribute to endothelial dysfunction and cardiovascular diseases via an additive pathway. Therefore, the aim of the present study was to investigate a potential 2-way interaction between the ADMA/DDAH pathway and MPO.

Methods

Isolation of Human PMNs

Experiments focusing on the ADMA effect on intracellular NOS enzyme activity and activation of neutrophils were performed in isolated human PMNs. Blood was taken from healthy volunteers, and PMNs were isolated as described previously.[16] Neutrophils were suspended in Hank balanced salt solution buffer containing $MgCl_2$ and $CaCl_2$ (Invitrogen, Karlsruhe, Germany). Isolated PMNs were exposed to saline, ADMA (100 μmol/L), symmetrical dimethylarginine (SDMA; 100 μmol/L), or formyl-methyl-leucyl-phenylalanine (10 μmol/L), an inductor of NADPH oxidase activity.

Determination of the High-Affinity Cationic Amino Acid Transporter-1 in Human PMNs

For detection of the transmembrane cationic amino acid transporter-1 (CAT-1) transport system in isolated human PMNs by Western blot analysis, PMNs were lysed (100 mmol/L sodium dihydrogen phosphate; 0.1% Triton) and sonicated on ice, and polyacrylamide gel electrophoresis and immunoblotting with an anti–CAT-1 antibody (1:500; Abnova, Heidelberg, Germany) were performed according to the manufacturer's protocol.

Determination of Intracellular Dimethylarginine Accumulation in Human PMNs

Isolated human PMNs were preincubated with saline, ADMA (100 μmol/L), or SDMA (100 μmol/L) for 30 minutes at 37°C. Cells were centrifuged, washed in Hank balanced salt solution buffer, and sonicated, and the lysate was centrifuged again. ADMA and SDMA concentrations in the supernatant were measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS) as recently described.[4] Isotope-labeled [$^2H_6$]-ADMA (in-house synthesis) was used as internal standard for quantification of ADMA and SDMA.

Determination of ADMA Uptake by HL-60 Cells

HL-60 cells (ATCC, Wesel, Germany) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum. For differentiation, the cells were incubated with 1% dimethyl sulfoxide for 96 hours and characterized by flow cytometric quantification of CD11b integrin expression and by determination of reactive oxygen species production. After differentiation, 1×10^6 cells/mL were incubated with increasing concentrations of ADMA (0, 0.5, 1, 10, and 100 μmol/L) for 2 hours in Hank balanced salt solution supplemented with L-arginine (400 μmol/L). After repeated washing, HL-60 cells were resuspended in ice-cold phosphatase inhibitor containing PBS and homogenized, and nuclei and membrane fractions were separated. The proteins from cytosolic fraction were removed by centrifugation through 3-kDa filters (Millipore, US, Bedford, MA). ADMA concentrations in the cytosolic fraction were determined by LC-MS/MS and related to total cytosolic protein content determined before filtration of proteins.[4]

Determination of the Effect of ADMA and SDMA on NOS Activity in Human PMNs

To determine the intracellular effect of ADMA and SDMA on NOS in PMNs, the NOS enzyme activity was monitored by gas chromatography–mass spectrometry as described previously.[17] The lysed cells were treated with protease inhibitors, and a sterile solution of L-[guanidine-$^{15}N_2$]-arginine hydrochloride (100 μmol/L; ^{15}N isotope purity >98%; Eurisotop, Saarbrücken, Germany) dissolved in 0.45% sodium chloride was added and incubated for additional 30 minutes. Supernatant samples were collected before and 30 minutes after administration of labeled L-arginine. Reaction products were derivatized with pentafluorobenzyl bromide (Sigma, Hamburg, Germany) and extracted into toluene (Merck, Darmstadt, Germany). The conversion of L-[guanidine-$^{15}N_2$]-arginine to ^{15}N-labeled nitrite was detected as the ^{15}N over ^{14}N isotope ratio with gas chromatography–mass spectrometry. ^{14}N-nitrite concentration was determined subsequently by gas chromatography–mass spectrometry, and ^{15}N-nitrite concentration was calculated from the isotopic ratio. Concentration was normalized to cell number.

Effect of ADMA on Human PMN Degranulation and Release of Oxygen Species

MPO and elastase concentrations in the supernatant of human PMNs were determined by ELISA according to the manufacturer's recommendations (Calbiochem and IBL, Hamburg, Germany, respectively). MPO activity in isolated human PMNs was determined by photometrically assessing the oxidation of 3,3′,5,5′-tetramethylbenzidine as described.[18] The generation of superoxide anion by isolated human PMNs was determined as linear rate of superoxide dismutase–inhibitable reduction of cytochrome c as

described.[19,20] The measurement was initiated by addition of cytochrome c (5 μL, 100 μmol/L) and simultaneous application of superoxide dismutase (50 μL, 300 U/mL) to the samples.

Effect of ADMA on Adhesion of Human PMNs to Human Umbilical Vein Endothelial Cells

Human PMNs were isolated and preincubated with the fluorescent dye acetylmethoxy-calcein.[21] The labeled cells were added to cultured human umbilical vein endothelial cells and coincubated with Hank balanced salt solution buffer, SDMA (100 μmol/L), or ADMA (100 μmol/L) for 30 minutes. The cells were washed and light emission was measured in a fluorometer (Berthold Twinkle LB 970; excitation/emission wavelength, 495/515 nm).

Animals

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (publication No. 85–23, revised 1996). The study protocol was approved by the Administrative Panel on Laboratory Animal Care, Hamburg University (protocol No. 70/07). The in vivo effect of MPO on methylarginine concentrations on lipopolysaccharide (LPS) stimulation was assessed with MPO-deficient ($MPO^{-/-}$) mice. For the experiments, female $MPO^{-/-}$ and control mice (at 12–16 weeks of age; both groups were on a C57Bl/6 background) were generated as previously described.[22] Male human DDAH1 (hDDAH1) transgenic mice were mated with C57Bl/6 female mice (Charles River Laboratories Germany, Sulzfeld, Germany). Offsprings were screened for transgene expression by polymerase chain reaction analysis as described earlier.[23] All experiments investigating the impact of hDDAH1 overexpression on the effects of MPO were conducted in female, 6- to 8-week-old, heterozygous hDDAH1 transgenic mice and age-, sex-, and weight-matched wild-type (WT) littermates housed in a temperature-controlled animal facility with a 12-hour light/dark cycle and free access to tap water and rodent chow.

Assessing the In Vivo Effect of ADMA on MPO Plasma Concentrations in C57Bl/6 Mice

To study the in vivo effect of exogenous ADMA on MPO plasma concentrations, osmotic minipumps (ALZET, model 2002) were implanted in female C57Bl/6 (C57) mice (age, 8–12 weeks; weight, 23–28 g) for 28 days. The minipumps were loaded with either ADMA (250 $\mu mol \cdot kg^{-1} \cdot d^{-1}$) or vehicle (saline and KCl; 136+5 mmol/L). All animals were anesthetized by intraperitoneal injection of 0.12 mg ketamine and 0.016 mg xylazine/10 g body weight. An osmotic minipump was implanted into the abdomen of each animal after disinfection and midline incision. Finally, the wound was closed with nylon sutures. To assess plasma MPO concentrations, blood samples were taken at day 28 and determined by ELISA according to the manufacturer's recommendations (Hycult Biotech, Uden, the Netherlands). ADMA and SDMA plasma concentrations were measured by LC-MS/MS.[4]

Effect of MPO on Recombinant hDDAH1 Activity In Vitro

The effect of MPO and its enzymatic product hypochlorous acid (HOCl) on the activity of recombinant hDDAH1 enzyme was measured by a modified version of the method developed by Knipp and Vasak.[24] Briefly, 1 μg hDDAH1 was incubated for 15 minutes at 37°C with active MPO (0.001, 0.005, 0.01, 0.05, 0.1, and 0.5 μg/100 μL; Planta Natural Products, Vienna, Austria) or heat-inactivated MPO (boiled at 95°C for 30 minutes) and substrate (10 μmol/L hydrogen peroxide [H_2O_2]; Sigma, Germany) or, in a different setting, with HOCl (0.1, 1, 10, and 100 μmol/L; Sigma, Germany). After the addition of 1 mmol/L of the DDAH substrate ADMA in a final reaction volume of 100 μL, the samples were incubated for 1 hour at 37°C. After incubation, 200 μL of fresh prepared color developing reagent solution was added to each sample. After 15 minutes of incubation at 95°C in an oven, the plate was cooled to room temperature, and citrulline formation was measured at 540 nm.

In Vivo Effect of $MPO^{-/-}$ on Methylarginine Concentrations

To study the in vivo effect of MPO on plasma ADMA and SDMA concentrations, $MPO^{-/-}$ and control C57 mice received an intraperitoneal injection of 12.5 mg/kg LPS. Four hours after LPS administration, the mice were anesthetized, and blood samples for determination of methylarginine concentrations were obtained by puncturing the inferior vena cava and drawing the blood to EDTA-rinsed syringes. The blood was immediately centrifuged at 4°C (10 minutes at 4000 rpm), and the supernatant was stored at −80°C. ADMA, SDMA, and N^G-monomethyl-L-arginine (L-NMMA) plasma concentrations were measured by LC-MS/MS with isotope-labeled [$^2H_6$]-ADMA and [$^2H_6$]-L-NMMA used as internal standards for quantification of ADMA and SDMA and of L-NMMA, respectively.[4]

In Vivo Impact of hDDAH1 Overexpression on the Effects of MPO

To assess the potency of DDAH1 on opposing the effects of MPO, we investigated the impact of hDDAH1 overexpression on MPO plasma concentrations after 28 days of ADMA treatment via osmotic minipumps. HDDAH1 transgenic mice and their WT littermates were treated with saline- or ADMA-loaded osmotic minipumps (250 $\mu mol \cdot kg^{-1} \cdot d^{-1}$) over a period of 28 days. MPO plasma concentrations were determined at day 28 as described earlier. In a second approach, we challenged hDDAH1 and WT littermate mice with an intraperitoneal LPS injection (12.5 mg/kg) and determined ADMA and SDMA plasma concentrations by LC-MS/MS 4 hours after injection.

In Vivo Effect of Short-Term ADMA Infusion on MPO Plasma Concentrations in Humans

To assess the effect of short-term ADMA infusion on MPO plasma concentrations in humans, 10 healthy, normotensive men and women were studied before, during, and after a short-term ADMA infusion after written, informed consent. This study was approved by the University of Michigan Institutional Review Board. ADMA was manufactured for human administration by Bachem AG (Bubendorf, Switzerland) and approved by the Federal Drug Administration (investigational new drug 77,330). After a baseline period, ADMA was administered intravenously at 0.0125 $mg \cdot kg^{-1} \cdot min^{-1}$. Plasma MPO, ADMA, SDMA, and L-NMMA concentrations were measured at baseline, at 60 minutes of ADMA infusion, and 20 minutes after the ADMA infusion was discontinued by LC-MS/MS.[4] MPO plasma concentrations were determined by ELISA technique as described earlier.

Statistical Analysis

The data are expressed as mean±SEM. All data were tested with the Kolmogorov-Smirnov test and revealed normal distributions. To test for differences between groups, the Student paired or unpaired *t* test as appropriate or 1-way ANOVA followed by Bonferroni post hoc test was used. A value of $P<0.05$ was considered statistically significant.

Results

ADMA Incubation Leads to Intracellular Accumulation and Impairs NOS Activity in PMNs

In line with previous findings in macrophages,[25] the CAT-1 transport system was detected in human PMNs (Figure 1A). Incubation with ADMA or SDMA (100 μmol/L) resulted in an increase in ADMA and SDMA in the cell lysate, respectively (Figure 1B). Additional experiments in HL-60 cells revealed a significant 6-fold increase in intracellular ADMA

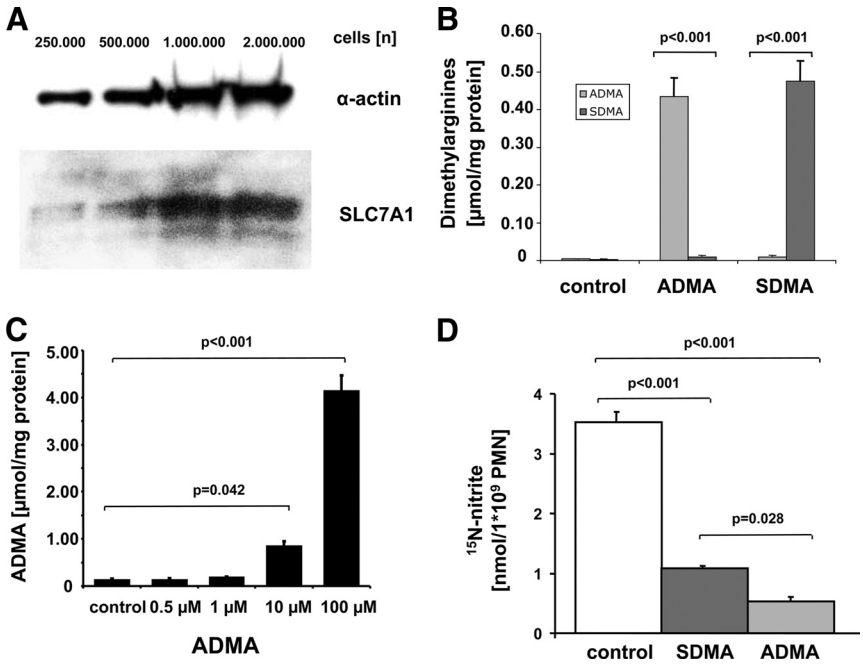

Figure 1. Transport and accumulation of asymmetrical dimethylarginine (ADMA) in human polymorphonuclear neutrophils (PMNs) and ADMA effect on nitric oxide synthase (NOS) activity. Detection of the cationic amino acid transporter-1 (CAT-1) in human PMNs by Western blot analysis (**A**). In conformity with increasing amounts of α-actin, the SLC7A1 protein was detected in PMNs with increasing cell numbers. After incubation with ADMA or symmetrical dimethylarginine (SDMA; 100 μmol/L), the corresponding dimethylarginine accumulated within the PMNs (**B**; n=4 per group). Incubation of PMN-like differentiated HL-60 cells with 10 and 100 μmol/L ADMA for 2 hours revealed a significant increase in cytosolic ADMA concentrations (**C**; n=4 per group). Both ADMA and SDMA incubation (100 μmol/L) significantly impaired ex vivo NOS activity in isolated human PMNs compared with saline controls, whereas the ADMA effect on NOS was significantly stronger compared with SDMA (**D**; n=5–6 per group).

on incubation with 10 μmol/L ADMA (control versus 10 μmol/L ADMA, 0.14±0.02 versus 0.86±0.09 μmol ADMA/mg protein; P=0.042; Figure 1C). In accordance with the enhanced intracellular ADMA or SDMA accumulation, NOS enzyme activity in PMNs was significantly reduced after treatment with either ADMA or SDMA (3.52±0.18, 0.53±0.07, and 1.08±0.04 nmol ^{15}N-nitrite/1×10^9 PMNs for control, ADMA, and SDMA, respectively; each $P<0.001$; ADMA versus SDMA, P=0.028; Figure 1D).

ADMA Leads to Activation of PMN, Resulting in an Increase in MPO and Superoxide Release

MPO and elastase concentrations and MPO activity in the supernatant of PMNs as markers of PMN degranulation and activation were significantly elevated on exposure to ADMA (ADMA versus control; MPO protein: 184.3±26.5 versus 140.0±7.4 ng/mL, P=0.044; elastase: 7.42±0.65 versus 4.89±0.40 ng/mL, P=0.022; MPO activity: 0.06±0.01 versus 0.04±0.01 Δ optical density [OD]/min, P=0.042) or formyl-methyl-leucyl-phenylalanine (MPO protein: 256.4±47.5 ng/mL, P=0.027; elastase: 9.77±0.88 ng/mL, P=0.013; MPO activity: 0.08±0.01 ΔOD/min, P=0.035 versus control) as opposed to SDMA (MPO protein: 137.5±23.9 ng/mL, P=0.812; elastase: 5.73±0.25 ng/mL, P=0.252; MPO activity: 0.04±0.01 ΔOD/min, P=0.927 versus control; Figure 2A–2C).

Besides the effect on PMN degranulation, ADMA enhanced oxidative stress burden in PMNs. In contrast to SDMA (SDMA versus control; superoxide release: 0.47±0.10 versus 0.27±0.08 ΔOD/min; P=0.194), incubation with ADMA (0.72±0.07 ΔOD/min; P=0.034 versus control) significantly increased superoxide production in isolated human PMNs (Figure 2D).

ADMA Enhances Adhesion of Isolated Human PMNs to Human Umbilical Vein Endothelial Cells

As a further marker of PMN activation, adhesion to endothelial cells was tested. Without prior stimulation, only a few PMNs tended to adhere to endothelial cells (Figure 3A). Coincubation with 100 μmol/L SDMA led to a slight increase in PMN adhesion (43.0±2.6 relative fluorescence units, values of control subtracted). In contrast, coincubation with 100 μmol/L ADMA caused a significant, 2-fold increase in PMN adhesion (85.4±17.5 relative fluorescence units, values of control subtracted; P=0.021; Figure 3B) compared with the SDMA effect.

Long-Term In Vivo ADMA Treatment Increases MPO Plasma Concentration in C57 Mice

Long-term treatment with exogenous ADMA via osmotic minipumps resulted in significantly increased MPO plasma concentrations in C57 mice after 28 days of treatment (saline versus ADMA treated, 26.3±1.2 versus 42.6±5.9 ng/mL; P=0.018; Figure 4A and Figure IA in the online-only Data Supplement). The ADMA plasma concentrations significantly increased on ADMA treatment (saline versus ADMA treated, 0.72±0.03 versus 2.00±0.25 μmol/L; $P<0.001$; Figure 4B and Figure IB in the online-only Data Supplement), whereas SDMA plasma concentrations did not change significantly (saline versus ADMA treated, 0.11±0.01 versus 0.13±0.01 μmol/L; P=0.244; Figure 4C and Figure IC in the online-only Data Supplement).

In Vitro Effect of MPO on Recombinant hDDAH1 Activity

H_2O_2 plus MPO treatment resulted in a significant reduction of recombinant hDDAH1 activity by 78% (100% versus 22%; $P<0.001$; Figure 5A). To investigate a direct protein-protein interaction between hDDAH1 and MPO, heat-inacti-

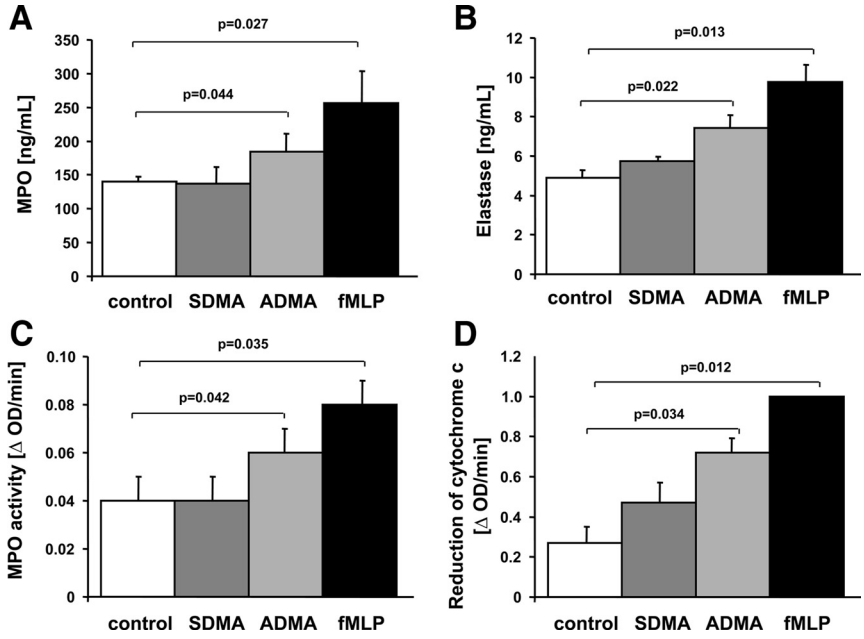

Figure 2. Effect of asymmetrical dimethylarginine (ADMA) on human polymorphonuclear neutrophil (PMN) degranulation and activation. PMNs were stimulated with ADMA (100 μmol/L), symmetrical dimethylarginine (SDMA; 100 μmol/L), and formyl-methyl-leucyl-phenylalanine (fMLP; 10 μmol/L), and indicators of PMN degranulation and superoxide release were determined. **A**, Myeloperoxidase (MPO) release, n=6 per group. **B**, Elastase release, n=5 per group. **C**, MPO activity in PMN supernatant, n=6 per group. **D**, Superoxide release: control, n=6; SDMA, n=7; ADMA, n=13; fMLP, n=13.

vated MPO and H_2O_2 were added, which did not affect hDDAH1 activity (100% versus 99%; $P=1.000$; Figure 5A). Both MPO (plus 10 μmol/L H_2O_2) and the MPO product HOCl itself dose-dependently impaired hDDAH1 activity, starting with an MPO concentration of 0.005 μg/100 μL ($P<0.001$; Figure 5B) and an HOCl concentration of 0.1 μmol/L ($P=0.003$; Figure 5C), respectively.

In Vivo Effect of MPO on ADMA and SDMA Concentrations After Inflammatory Stimulation

Under baseline conditions, no significant differences in ADMA plasma concentrations were observed between $MPO^{-/-}$ and C57 mice ($MPO^{-/-}$ versus C57, 0.82±0.04 versus 0.84±0.04 μmol/L; $P=1.000$; Figure 6A and Figure IIA in the online-only Data Supplement). However, after stimulation with LPS, ADMA plasma concentrations in C57 mice increased significantly (C57+LPS, 1.08±0.08 μmol/L; $P=0.023$ versus baseline), whereas the LPS-induced increase in ADMA in $MPO^{-/-}$ mice was not significant ($MPO^{-/-}$+LPS, 0.95±0.07 μmol/L; $P=0.790$ versus baseline). SDMA plasma concentrations did not show a significant difference between the groups under baseline conditions ($MPO^{-/-}$ versus C57, 0.16±0.01 versus 0.16±0.01 μmol/L; $P=1.000$; Figure 6B and Figure IIB in the online-only Data Supplement). Under LPS treatment, SDMA plasma concentrations increased significantly in both groups without any significant difference between the 2 groups (C57+LPS, 0.25±0.02 μmol/L; $MPO^{-/-}$+LPS, 0.25±0.02 μmol/L; each $P<0.001$ versus baseline). Interestingly, LPS treatment decreased L-NMMA plasma concentrations in both groups of animals ($MPO^{-/-}$ versus $MPO^{-/-}$+LPS: 0.23±0.01 versus 0.10±0.01 μmol/L, $P<0.001$; C57 versus C57+LPS: 0.20±0.03 versus 0.13±0.02 μmol/L, $P=0.080$), whereas the L-NMMA plasma concentrations did not significantly differ between $MPO^{-/-}$ and C57 mice. Creatinine concentrations in the blood samples of all animal groups treated with saline/LPS revealed no significant increase after LPS challenge and no significant difference between the 2 groups.

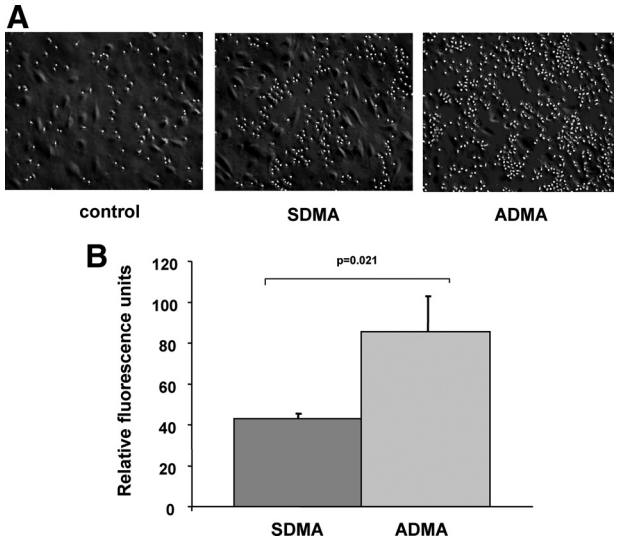

Figure 3. Effect of asymmetrical dimethylarginine (ADMA) on adhesion of human polymorphonuclear neutrophil (PMNs) to human umbilical vein endothelial cells (HUVECs). ADMA incubation increased adhesion of isolated human PMN to cultured HUVECs (**A**, light microscopy 20×; **B**, relative fluorescence units). n=6 per group.

hDDAH1 Overexpression in Mice Attenuates the Effect of Long-Term ADMA Infusion and Short-Term Inflammatory Stimulation In Vivo

ADMA-treated WT mice showed a significant increase in MPO concentrations at day 28 compared with saline-treated WT mice (WT+saline versus WT+ADMA, 95.3±6.3 versus 179.7±17.8 ng/mL; $P=0.005$; Figure 7A and Figure IIIA in the online-only Data Supplement). Interestingly, overexpression of the hDDAH1 transgene protected against the ADMA-induced increase in MPO (hDDAH1+saline versus hDDAH1+ADMA, 125.8±21.6 versus 132.5±16.8 ng/mL;

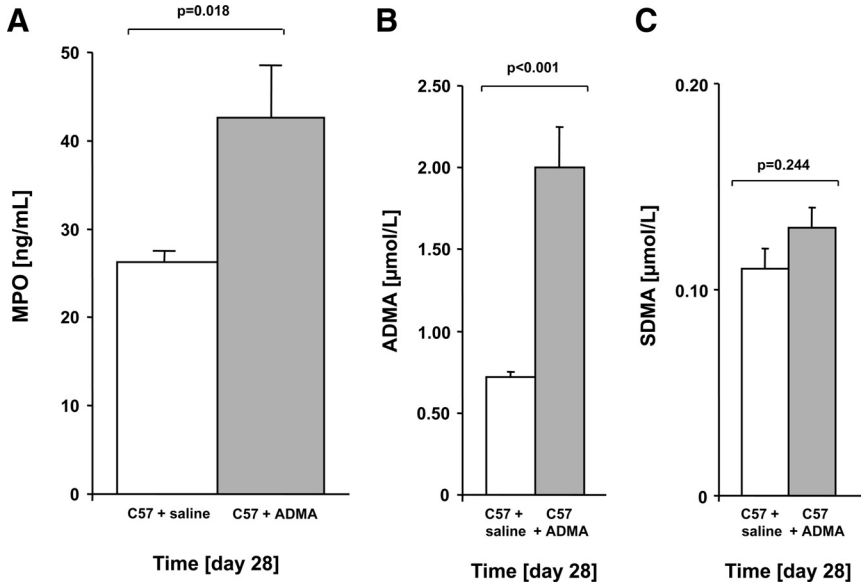

Figure 4. In vivo effect of long-term asymmetrical dimethylarginine (ADMA) infusion on myeloperoxidase (MPO) plasma concentrations. MPO plasma concentrations significantly increased in C57Bl/6 (C57) mice on long-term ADMA treatment (250 μmol · kg^{-1} · d^{-1}; 28 days; **A**). Long-term ADMA infusion significantly increased ADMA (**B**), whereas SDMA plasma concentrations (**C**) were not significantly affected by ADMA treatment. C57+saline, n=14; C57+ADMA, n=13.

P=0.851), underlining the potency of DDAH overexpression in opposing the MPO effect. In terms of the MPO plasma concentrations in WT and hDDAH1 mice treated with a saline-loaded minipump, we did not see significant differences after 28 days of treatment.

Before LPS application, hDDAH1 mice revealed significantly lower ADMA plasma concentrations compared with WT mice (hDDAH1 versus WT at baseline, 0.48±0.02 versus 0.67±0.03 μmol/L; P=0.041; Figure 7B and Figure IIIB in the online-only Data Supplement). Four hours after LPS stimulation, ADMA plasma concentrations in WT mice were significantly elevated (WT+LPS, 0.95±0.08 μmol/L; P=0.019), whereas hDDAH1 mice showed no significant increase in ADMA plasma concentrations (hDDAH1+LPS, 0.52±0.02 μmol/L; P=0.308). In addition, we assessed the SDMA plasma concentrations, showing no significant differences between the hDDAH1 and WT mice either at baseline (hDDAH1 versus WT, 0.17±0.01 versus 0.15±0.01 μmol/L; P=1.000) or after 4 hours of LPS treatment (hDDAH1+LPS versus WT+LPS, 0.25±0.02 versus 0.19±0.02 μmol/L;

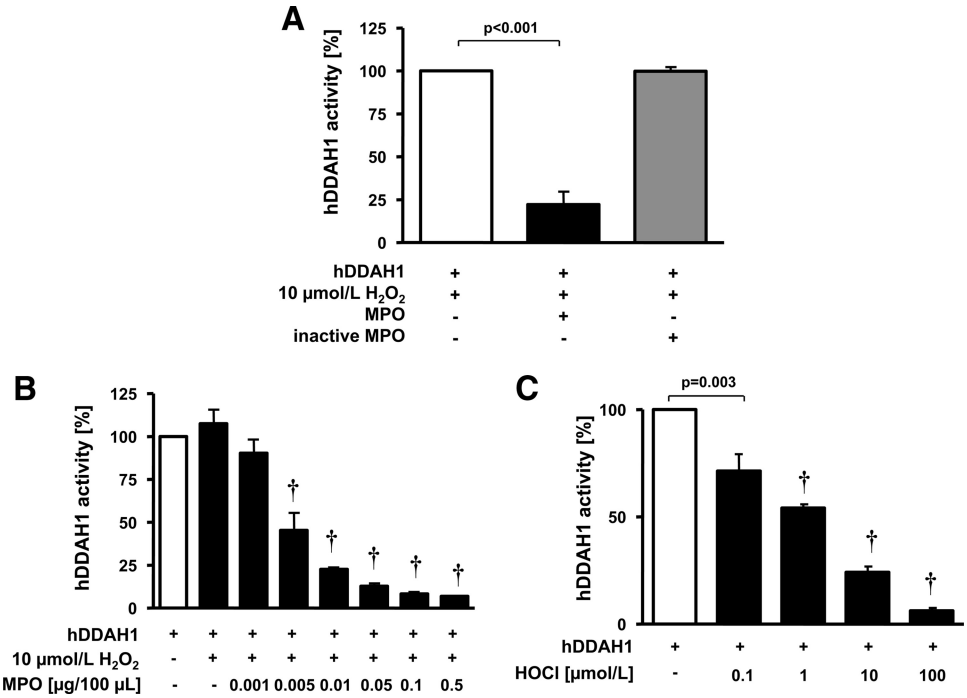

Figure 5. In vitro effect of myeloperoxidase (MPO) on recombinant human dimethylarginine dimethylaminohydrolase1 (DDAH1) enzyme activity. Addition of MPO (10 μg) and its substrate hydrogen peroxide (H_2O_2; 10 μmol/L) resulted in a significant decrease in recombinant human DDAH1 (hDDAH1) activity, whereas heat-inactivated MPO (10 μg) and H_2O_2 did not (**A**). In addition, MPO and H_2O_2 (**B**) and its product, hypochlorous acid (HOCl; **C**), dose dependently impaired hDDAH1 activity. n=3 for controls, heat-inactivated MPO, and each individual concentration; †P<0.001.

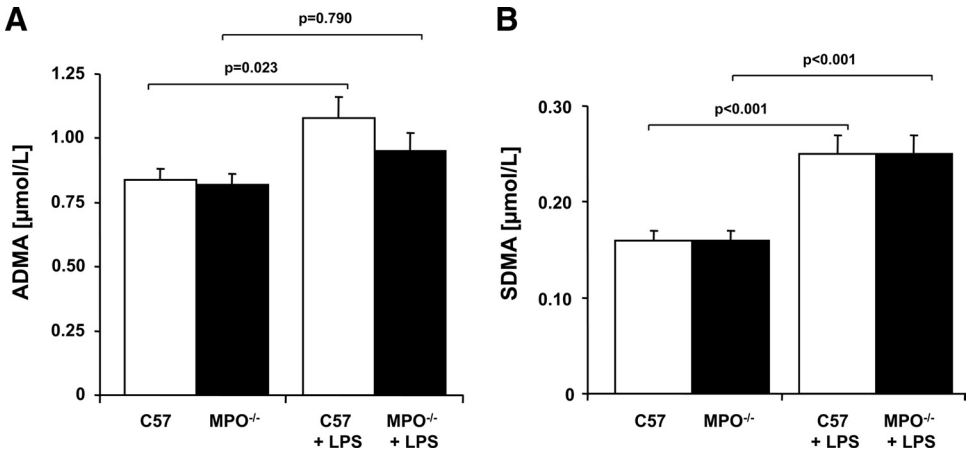

Figure 6. Dimethylarginine concentrations in myeloperoxidase-deficient ($MPO^{-/-}$) mice after lipopolysaccharide (LPS) stimulation. Under baseline conditions, asymmetrical dimethylarginine (ADMA) plasma concentrations showed no significant differences between $MPO^{-/-}$ and C57 mice (**A**). After LPS stimulation, ADMA concentrations in C57 mice increased significantly, whereas the LPS-induced increase in ADMA in $MPO^{-/-}$ mice was attenuated. Symmetrical dimethylarginine (SDMA) plasma concentrations at baseline did not differ significantly between the 2 groups (**B**) and increased significantly in both groups after LPS treatment but without any significant difference between the 2 groups. ADMA and SDMA, respectively: C57, n=20; $MPO^{-/-}$, n=17; C57+LPS, n=19; $MPO^{-/-}$+LPS, n=17.

P=0.095) but with a significant increase after LPS application in both groups. To exclude an artificial finding resulting from the LPS effect in the moribund animal, we determined the creatinine concentrations in all 4 groups, which revealed no significant increase after LPS challenge and no significant difference between the 2 groups.

Short-Term ADMA Infusion Increases MPO Plasma Concentrations in Humans

With ADMA infusions, there was a significant increase in mean plasma MPO concentrations (189.7±10.5 versus 207.7±16.0 pmol/L; P=0.006; the Table) associated with the short-term rise in plasma ADMA concentrations (0.74±0.06

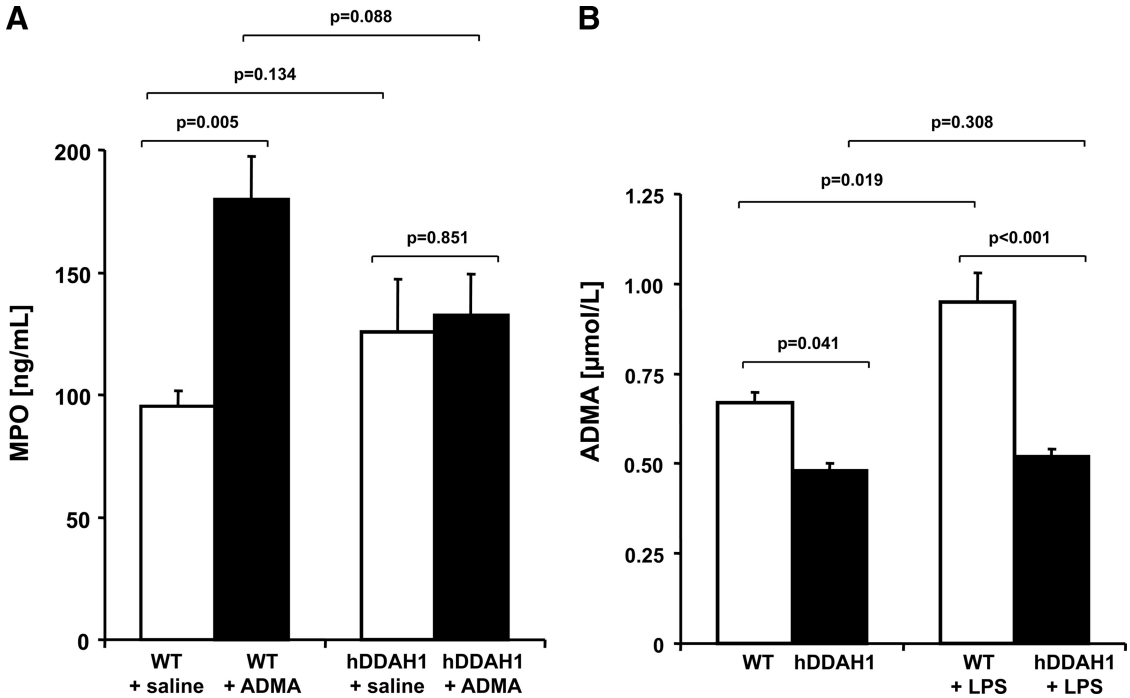

Figure 7. Effect of human dimethylarginine dimethylaminohydrolase1 (hDDAH1) overexpression on long-term asymmetrical dimethylarginine (ADMA) infusion and short-term lipopolysaccharide (LPS) challenge in vivo. ADMA-treated wild-type (WT) mice showed a significant increase in myeloperoxidase (MPO) concentrations at day 28 compared with saline-treated WT mice, whereas hDDAH1 overexpression was able to attenuate the ADMA-induced increase in MPO (**A**). MPO: WT+saline, n=4; WT+ADMA, n=5; hDDAH1+saline, n=2; hDDAH+ADMA, n=7. Four hours after LPS stimulation, ADMA plasma concentrations in WT mice were significantly elevated, whereas hDDAH1 mice showed no significant increase in ADMA concentrations (**B**). ADMA: WT, n=9; hDDAH1, n=9; WT+LPS, n=9; hDDAH1+LPS, n=9.

Table. Concentrations of Myeloperoxidase, Asymmetrical Dimethylarginine, Symmetrical Dimethylarginine, and N^G-Monomethyl-L-Arginine

	Baseline	ADMA Infusion (60 min)	ADMA Discontinued (20 min)
MPO, pmol/L	189.7±10.5	207.7±16.0*	192.6±13.0
ADMA, μmol/L	0.74±0.06	5.82±0.51†	3.28±0.22†
SDMA, μmol/L	0.41±0.03	0.43±0.03	0.46±0.03
L-NMMA, μmol/L	0.07±0.01	0.09±0.01	0.06±0.01

ADMA indicates asymmetrical dimethylarginine; SDMA, symmetrical dimethylarginine; MPO, myeloperoxidase; and L-NMMA, N^G-monomethyl-L-arginine. n=10 healthy, normotensive individuals.

*P=0.006, †P<0.001 for baseline vs ADMA infusion and ADMA infusion vs ADMA discontinued, respectively.

versus 5.82±0.51; P<0.001, baseline versus 60 minutes of ADMA infusion). Conversely, discontinuation of ADMA infusions was associated with normalization of plasma MPO (192.6±13.0 pmol/L; P=0.736 versus baseline), although ADMA concentrations (3.28±0.22 μmol/L; P<0.001 versus baseline) remained significantly above preinfusion levels at 20 minutes. SDMA and L-NMMA plasma concentrations were unaffected by ADMA infusions, as demonstrated in the Table.

Discussion

Leukocyte activation and concomitant release of MPO play a pivotal role in the development of endothelial dysfunction. Because both steps are regulated in an NO-dependent fashion, we investigated the interaction between the endogenous NOS inhibitor ADMA and the leukocyte-derived hemoprotein MPO. The present study provides evidence of the impact of an ADMA-induced pathogenic cycle on increased PMN activation, enhanced MPO release, and subsequent impairment of DDAH activity, which in turn accounts for an increase in ADMA concentrations (Figure 8). Briefly, the salient findings are the following. First, ADMA accumulates in human PMNs and impairs intracellular NOS activity. Second, ADMA accumulation in PMNs leads to PMN degranulation and increases superoxide release, resulting in enhanced PMN adhesion to human umbilical vein endothelial cells ex vivo. Third, the hypothesis of an ADMA/MPO interaction is confirmed in human individuals challenged with a short-term ADMA infusion. Fourth, in vitro, MPO via its product HOCl decreases recombinant hDDAH1 activity. Fifth, in vivo, the LPS-induced increase in plasma ADMA concentrations is attenuated in $MPO^{-/-}$ mice, and overexpression of the hDDAH1 gene is able to oppose the effects of MPO.

NO inhibits PMN activation and thereby exerts antiinflammatory effects.[26] Treatment of mice with the NOS inhibitor N^G-nitro-L-arginine or a soluble guanylyl cyclase inhibitor enhances neutrophil migration, rolling, and adhesion to the endothelium.[27] The NOS substrate L-arginine and methylated arginines, eg, ADMA, are transported into the cells via the high-affinity CAT-1 transporter. In humans, this transporter is encoded by the *SLC7A1* gene and has been demonstrated in neurons, endothelium, and macrophages. In the present study, we were able to detect the expression of the CAT-1 transporter in isolated human PMNs. We hypothesize that increased concentrations of circulating ADMA and subsequent enhanced cellular uptake by CAT-1 cause an intracellular ADMA accumulation in PMNs. This in turn may affect the activation state of PMNs. Indeed, NOS activity of PMNs was significantly impaired by ADMA; subsequently, characteristics of PMN degranulation, ie, MPO and elastase release, increased on ADMA stimulation in our present study. PMN

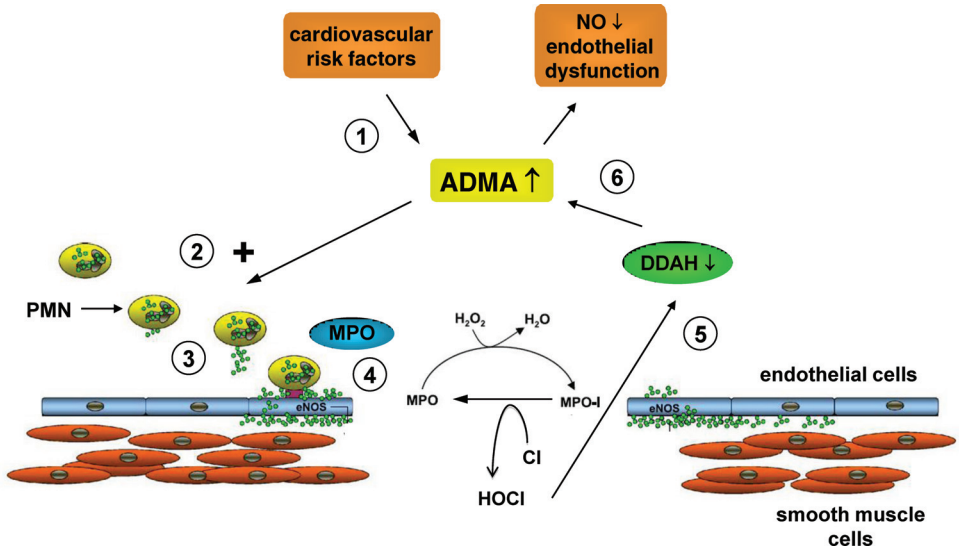

Figure 8. Pathogenic cycle between the nitric oxide (NO) synthase (NOS) inhibitor asymmetrical dimethylarginine (ADMA) and leukocyte-derived myeloperoxidase (MPO). ADMA plasma concentrations are elevated under conditions of increased cardiovascular risk factors (1). Elevated ADMA concentrations accumulate in polymorphonuclear neutrophils (PMNs) and impair intracellular NOS activity (2), resulting in activation, degranulation, and adhesion of PMNs (3). As a consequence, MPO plasma concentrations and local MPO activity are enhanced (4). Subsequently, the MPO product HOCl impairs dimethylarginine dimethylaminohydrolase1 (DDAH) activity (5), resulting in a further increase in ADMA concentrations (6).

degranulation is a crucial process that occurs through tightly controlled receptor-coupled mechanisms, leading to regulated exocytosis. Phosphorylation of p38 mitogen-activated protein kinase (MAPK) is considered a key event affecting the activation state of PMNs.[28] Activation of p38 MAPK induces activation of the NADPH oxidase-dependent respiratory burst.[29] We have previously shown that the addition of MPO to PMNs increased phosphorylation of p38 MAPK.[30] Interestingly, ADMA enhances phosphorylation of p38 MAPK in endothelial cells.[31] In our present study, exposure to ADMA led to degranulation of PMNs; therefore, ADMA acted as a mediator of the activation state of PMNs (ie, enhanced MPO activity, superoxide release). Another possible mechanism by which altered NO bioavailability may influence PMN degranulation has been described by Fortenberry et al.[32] These investigators showed that NO impairs oxidative cell function of PMNs and increases neutrophil cell death in part by enhancing DNA fragmentation, resulting in a markedly impaired release of toxic granula content (ie, MPO). As a result of the ADMA-induced PMN activation in our present study, the isolated human PMNs showed a significantly increased adhesion to human umbilical vein endothelial cells ex vivo. To assess the impact of ADMA on PMN and MPO release in vivo, we investigated the effect of long-term ADMA treatment in C57 mice. In this model, long-term ADMA infusion significantly increased MPO plasma concentrations in mice. Intriguingly, our hypothesis of an ADMA/MPO interaction was confirmed in humans challenged with a short-term ADMA infusion, resulting in significantly increased MPO plasma concentrations.

Studies using hDDAH1 transgenic mice underlined the impact of lowering ADMA plasma concentrations in inflammatory responses within the vessel and/or myocardium.[33,34] Overexpression of the hDDAH1 gene diminished the development of transplant vasculopathy after heterotopic heart transplantation.[33] DDAH1 overexpression in the recipient reduced ADMA plasma concentrations, myocardial oxidative stress, cytokine elaboration, and inflammation in the allograft. These effects were associated with less graft coronary artery disease and improved function of the allograft. In another study, hDDAH1 overexpression markedly reduced myocardial reperfusion injury after occlusion and reopening of the left coronary artery.[34] Decreased NO bioavailability in ischemia reperfusion injury triggers a cascade of pathophysiological events, ie, upregulation of adhesion molecules, leukocyte adhesion to endothelial cells, transmigration of PMNs, and subsequent tissue damage of the reperfused myocardium.[35] Stuhlinger et al[34] demonstrated that ADMA concentrations were elevated and DDAH activity was impaired in the early phase of reperfusion, resulting in reduced NO bioavailability. Interestingly, enhanced leukocyte activity after ischemia reperfusion (determined by enzyme activity of MPO) was markedly reduced in hearts of hDDAH1 transgenic mice. In our present study, hDDAH1-overexpressing mice were protected against an ADMA-induced increase in plasma MPO and an LPS-induced increase in plasma ADMA concentrations. Therefore, the findings of our present study may offer an explanation for the potency of DDAH overexpression in opposing the MPO effect, eg, in myocardial reperfusion injury.

NO serves as a physiological substrate for MPO,[36] and MPO attenuates NO-dependent smooth muscle cell relaxation.[37] Thus, it was discussed that MPO may serve as a catalytic sink for NO, limiting its bioavailability.[13,36] Indeed, our data demonstrate that MPO impairs the production of NO by impairing DDAH activity. Redox-sensitive inactivation of DDAH by superoxide and peroxynitrite has been described by Leiper et al.[38] In our present study, hDDAH1 incubation with MPO and its substrate H_2O_2 or with the MPO product HOCl alone resulted in hDDAH1 inactivation. The possibility that, besides a mechanism-based effect of MPO on DDAH function, MPO may exert its effect on DDAH by direct protein-protein binding was excluded by showing that heat-inactivated MPO and H_2O_2 protein did not affect hDDAH1 activity.

In vivo ADMA plasma concentrations did not differ between $MPO^{-/-}$ and C57 mice at baseline. However, after stimulation with LPS, ADMA concentrations increased significantly in control mice, whereas in $MPO^{-/-}$ mice, this increase was blunted. SDMA plasma concentrations did not differ at baseline and increased significantly to the same extent in both mouse groups after LPS stimulation, suggesting that changes in renal function caused by systemic inflammation cannot explain the demonstrated differences in ADMA concentrations exclusively. More probably, the lack of MPO-induced inactivation of DDAH may have contributed to this effect in $MPO^{-/-}$ mice.

Recently, Wang et al[5] performed an elegant study showing that patients with significant coronary artery disease revealed higher ADMA and SDMA plasma concentrations. Furthermore, dose-dependent increases in systemic ADMA and SDMA concentrations within the individuals were strongly associated with an increased risk for experiencing a major cardiovascular event over a 3-year follow-up period. Intriguingly, these investigators observed an increased arginine methylation index, a ratio indicating an increased posttranslational arginine methylation. These investigators concluded that the relationship between arginine methylation pathways and the development of coronary artery disease extends beyond direct NOS inhibition. Considering our recent results, this might be due to the competition of SDMA and ADMA with L-arginine at the CAT system in leukocytes, leading to a reduction of intracellular L-arginine concentrations in PMNs. In previous studies, impaired systemic NO production was not consistently associated with SDMA concentrations. However, the effect of SDMA on the intracellular L-arginine/NO homeostasis of PMNs may be more fragile, contributing to a decreased NO production, subsequent degranulation, and initiation of cardiovascular disease, additive to the intracellular ADMA accumulation.

Surprisingly, lower L-NMMA plasma concentrations were associated with significant coronary artery disease in the study by Wang et al.[5] The authors argue that this association may reflect a heightened posttranslational modification of proteins through dimethylation reactions and proteolysis, producing ADMA and SDMA, in these patients. Indeed, inflammatory and redox-sensitive pathways (ie, activation

and degranulation of PMNs), which are known to enhance protein arginine residue posttranslational modification by methylation and subsequent proteolysis, may be a key mechanism accounting for the strong association of ADMA and cardiovascular disease. Our experiments in which we challenged C57 mice with LPS further support the hypothesis by Wang et al. In these studies, L-NMMA plasma concentrations decreased whereas ADMA concentrations increased after inflammatory stimulation.

Certainly, apart from the mechanisms described here, alternative mediators may influence the ADMA-MPO pathway. Besides the inactivation of DDAH by HOCl, DDAH is described to be inactivated by the oxidized low-density lipoprotein, which can be generated from low-density lipoprotein in an MPO-dependent fashion.[39,40] Moreover, MPO may affect the accumulation of ADMA by modulating the gene expression and/or activity of protein arginine *N*-methyltransferases. We have observed that the mRNA expression of the protein arginine *N*-methyltransferase isoforms responsible for the synthesis of ADMA (protein arginine *N*-methyltransferase-1, -3, and -6) was significantly downregulated in liver tissue of $MPO^{-/-}$ mice compared with C57 mice (preliminary data not shown). Apart from that, ADMA has been shown to activate the transcription factor nuclear factor-κB in endothelial cells with subsequent production of chemokines.[6] Therefore, it is conceivable that released chemokines and mediators produced by the inflamed endothelium lead to an activation of PMNs with subsequent sequestration of MPO.

Conclusions

Our data reveal an ADMA-induced cycle of PMN activation, MPO release, and subsequent impairment of hDDAH1 activity. In addition, our data not only highlight so far unrecognized cytokine-like properties of ADMA but also identify MPO as a regulatory switch for ADMA bioavailability under inflammatory conditions. We believe that understanding the underlying mechanisms of the ADMA/MPO interaction may open a new avenue for the treatment of cardiovascular disease.

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Disclosures

Drs Maas, Schwedhelm, and Böger are named inventors on patents relating analytical assays for methylarginines and receive royalties from them. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

The nitric oxide synthase inhibitor asymmetrical dimethylarginine (ADMA) and the leukocyte-derived hemoprotein myeloperoxidase (MPO) are associated with cardiovascular diseases. Activation of monocytes and polymorphonuclear neutrophils with concomitant release of MPO is regulated in a nitric oxide–dependent fashion. The present article describes an ADMA-induced cycle of polymorphonuclear neutrophil activation, MPO release, and subsequent impairment of human dimethylarginine dimethylaminohydrolase1 (hDDAH1) activity. In addition, the data not only highlight so far unrecognized cytokine-like properties of ADMA, but also identify MPO as a regulatory switch for ADMA bioavailability under inflammatory conditions. This finding suggests that understanding the underlying mechanisms of the ADMA/MPO interaction may open a new avenue for treatment of cardiovascular disease.

Supplemental Material

Supplemental Figure Legends

Supplemental figure 1:

In vivo effect of chronic ADMA infusion on MPO plasma concentrations

MPO plasma concentrations significantly increased in C57Bl/6 mice upon chronic ADMA treatment (250 µmol/kg/d; 28 days; median at baseline vs. day 28, 26.0 vs. 35.3 ng/ml; 1A). Chronic ADMA infusion significantly increased ADMA (median at baseline vs. day 28, 0.70 vs. 1.64 µmol/l; 1B), whereas SDMA plasma concentrations (median at baseline vs. day 28, 0.13 vs. 0.12 µmol/l; 1C) were not significantly affected by ADMA treatment. C57+saline: N=14, C57+ADMA: N=13.

Supplemental figure 2:

Dimethylarginine concentrations in MPO $^{-/-}$ mice after LPS stimulation

Under baseline conditions, ADMA plasma concentrations showed no significant differences between MPO$^{-/-}$ and C57 mice (MPO$^{-/-}$ vs. C57: median at baseline, 0.79 vs. 0.86 µmol/l; 2A). After lipopolysaccharides (LPS) stimulation, ADMA concentrations in C57 mice increased significantly (C57: median at baseline vs. LPS, 0.86 vs. 0.95 µmol/l), whereas the LPS-induced increase of ADMA in MPO$^{-/-}$ mice was attenuated (MPO$^{-/-}$: median at baseline vs. LPS, 0.79 vs. 0.91 µmol/l). SDMA plasma concentrations at baseline did not differ significantly between the two groups (MPO$^{-/-}$ vs. C57: median at baseline, 0.16 vs. 0.16 µmol/l; 2B), increased significantly in both groups after LPS-treatment (C57: median at baseline vs. LPS; 0.16 vs. 0.25 µmol/l and MPO$^{-/-}$: median at baseline vs. LPS, 0.16 vs. 0.25 µmol/l), however, without any significant difference between the two groups. *ADMA:* C57: N=20, MPO$^{-/-}$: N=17, C57+LPS: N=19, MPO$^{-/-}$+LPS: N=17; *SDMA*: C57: N=20, MPO$^{-/-}$: N=17, C57+LPS: N=19, MPO$^{-/-}$+LPS: N=17.

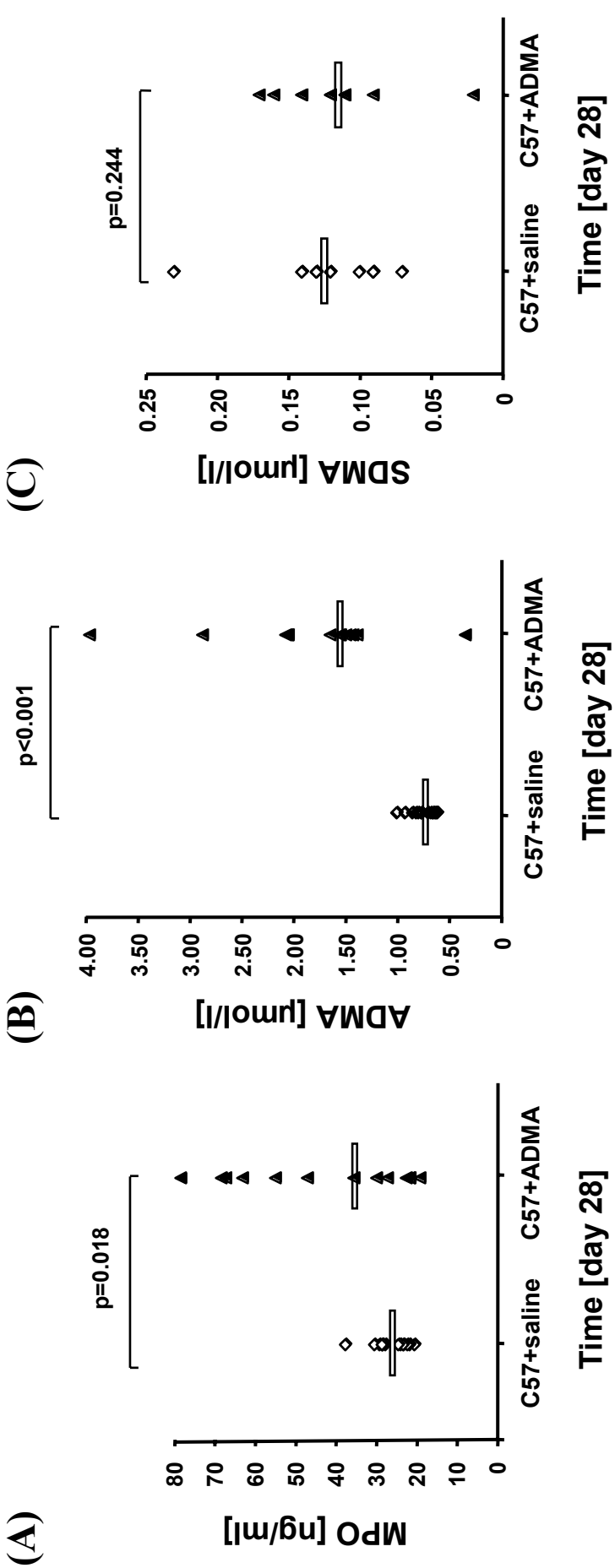
Supplemental figure 3:

Effect of hDDAH1 overexpression on chronic ADMA infusion and acute LPS challenge in vivo

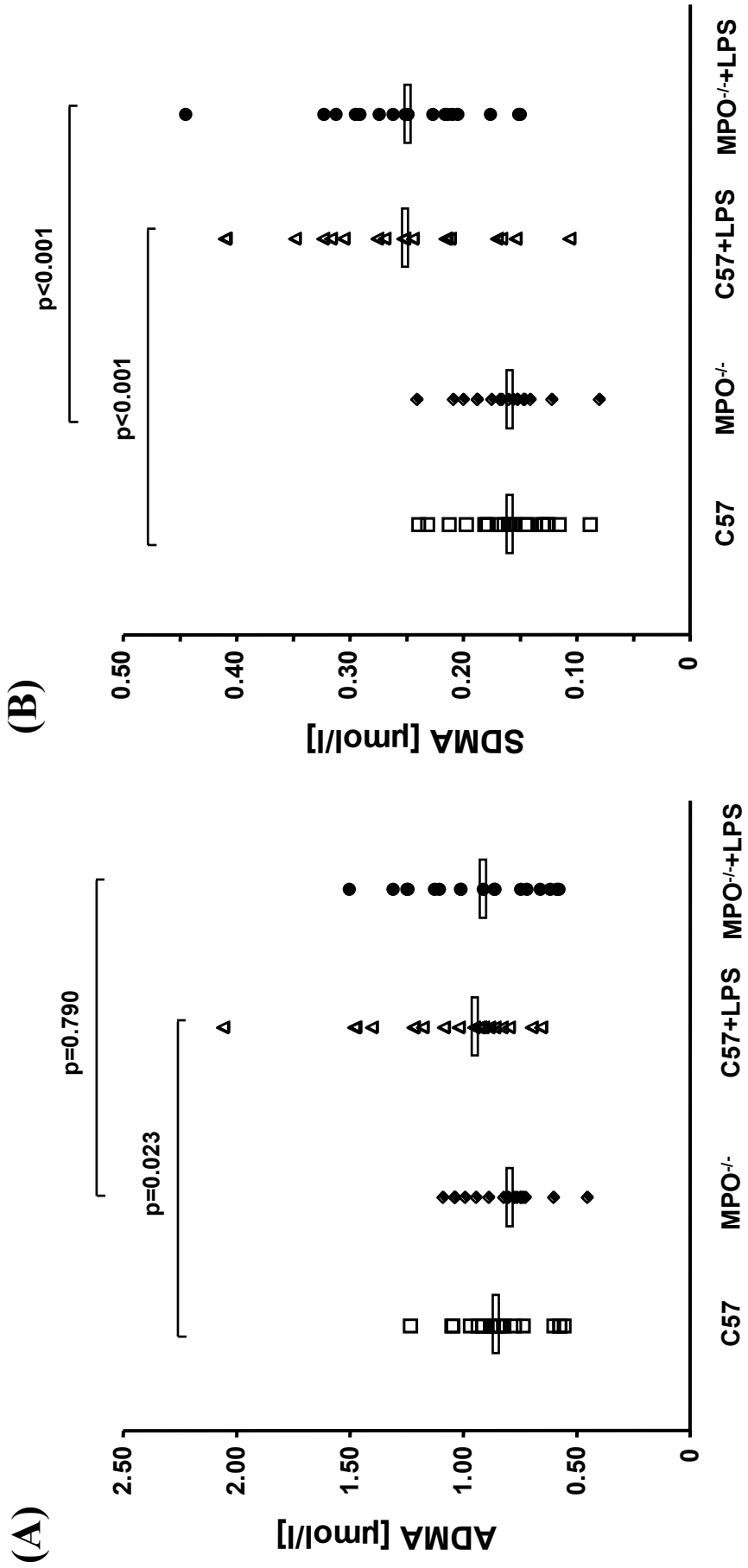
ADMA-treated WT mice showed a significant increase in MPO concentrations at day 28 when compared with saline-treated WT mice (WT: median at baseline vs. day 28, 99.4 vs. 176.5 ng/ml; 3A), whereas hDDAH1 overexpression was able to attenuate the ADMA-induced increase of MPO (hDDAH1: median at baseline vs. day 28, 125.8 vs. 113.9 ng/ml; 3B). *MPO:* WT+saline: N=4, WT+ADMA: N=5, hDDAH1+saline: N=2, hDDAH+ADMA: N=7.

Four hours after LPS stimulation, ADMA plasma concentrations in WT mice (WT: median at baseline vs. LPS, 0.67 vs. 1.00 µmol/l; 3B) were significantly elevated, whereas hDDAH1 mice showed no significant increase in ADMA concentrations (hDDAH1: median at baseline vs. LPS, 0.47 vs. 0.52 µmol/l). Creatinine concentrations revealed no significant increase after LPS challenge in all 4 groups and no significant difference between the two groups. *ADMA*: WT: N=9, hDDAH1: N=9, WT+LPS: N=9, hDDAH1+LPS: N=9.

Supplemental figure 1: von Leitner/Klinke et al.



Supplemental figure 2: von Leitner/Klinke et al.



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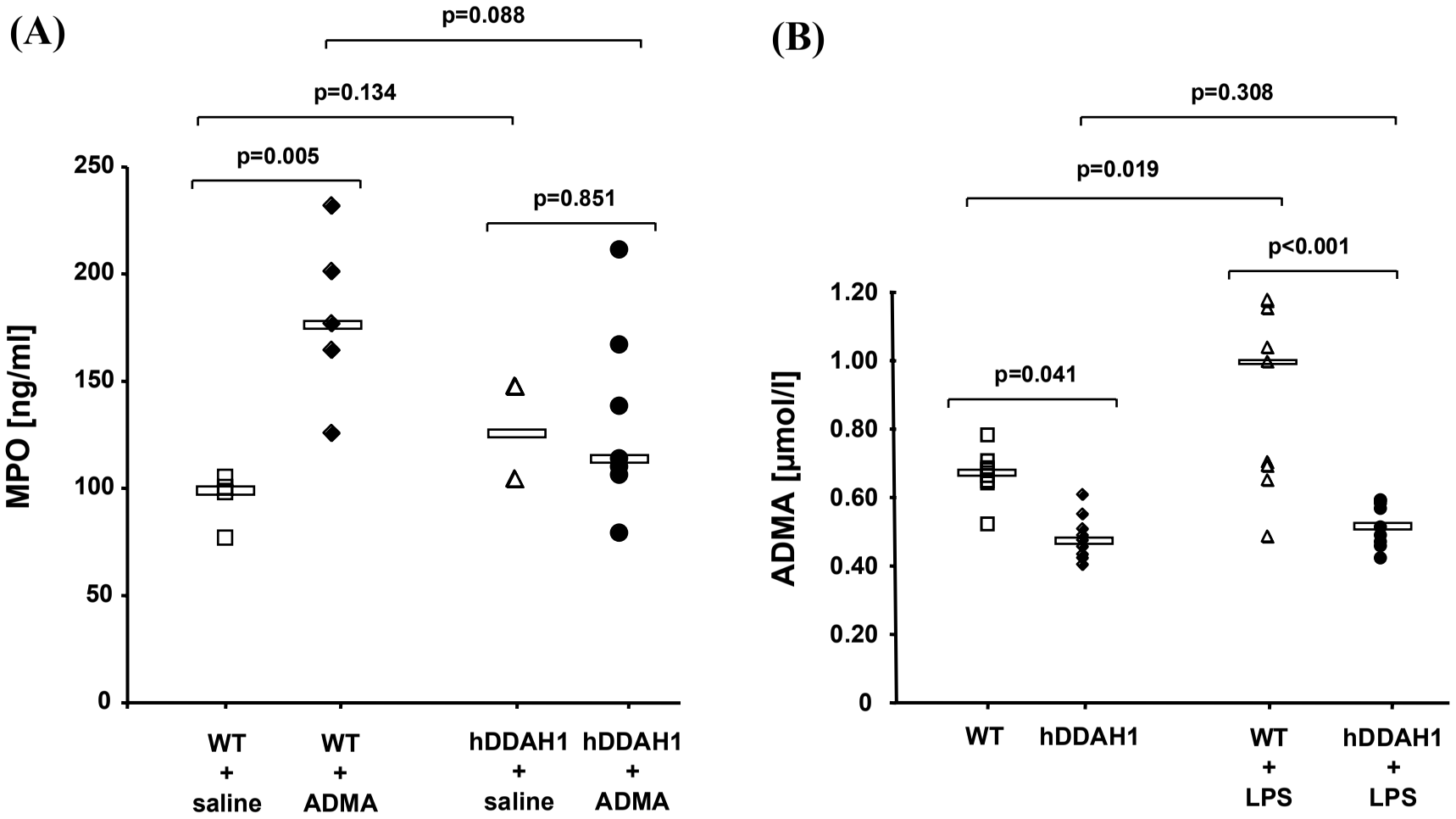

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Review Article

Modulation of Endothelial Glycocalyx Structure under Inflammatory Conditions

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The glycocalyx of the endothelium is an intravascular compartment that creates a barrier between circulating blood and the vessel wall. The glycocalyx is suggested to play an important role in numerous physiological processes including the regulation of vascular permeability, the prevention of the margination of blood cells to the vessel wall, and the transmission of shear stress. Various theoretical models and experimental approaches provide data about changes to the structure and functions of the glycocalyx under various types of inflammatory conditions. These alterations are suggested to promote inflammatory processes in vessels and contribute to the pathogenesis of number of diseases. In this review we summarize current knowledge about the modulation of the glycocalyx under inflammatory conditions and the consequences for the course of inflammation in vessels. The structure and functions of endothelial glycocalyx are briefly discussed in the context of methodological approaches regarding the determination of endothelial glycocalyx and the uncertainty and challenges involved in glycocalyx structure determination. In addition, the modulation of glycocalyx structure under inflammatory conditions and the possible consequences for pathogenesis of selected diseases and medical conditions (in particular, diabetes, atherosclerosis, ischemia/reperfusion, and sepsis) are summarized. Finally, therapeutic strategies to ameliorate glycocalyx dysfunction suggested by various authors are discussed.

1. Introduction

Based on theoretical models and experimental research over the past few decades, it has been shown that the glycocalyx is a multicomponent layer of proteoglycans and glycoproteins covering the luminar endothelium [1–4]. Over the past few decades there has been provided increasing evidence to indicate that the endothelial surface layer plays a considerable physiological role especially in relation to vascular permeability, the adhesion of leucocytes and platelets, the mediation of shear stress, and the modulation of inflammatory processes [2, 5–7]. The vasculoprotective functions of the vessel wall glycocalyx are suggested based on experimental data demonstrating that glycocalyx disruption is accompanied by enhanced sensitivity of the vasculature towards inflammatory and atherogenic stimuli [8, 9]. Detailed descriptions of biological systems and methodologies leading to these conclusions have been published and reviewed previously [2, 3, 7, 10, 11]; therefore, we provide only a brief overview of the glycocalyx functions associated with the modulation of haemostatic and inflammatory processes.

2. Structure of Endothelial Glycocalyx

The endothelial cell surface is covered by layer composed of membrane-bound proteoglycans and glycoproteins associated with adsorbed plasma components which are in

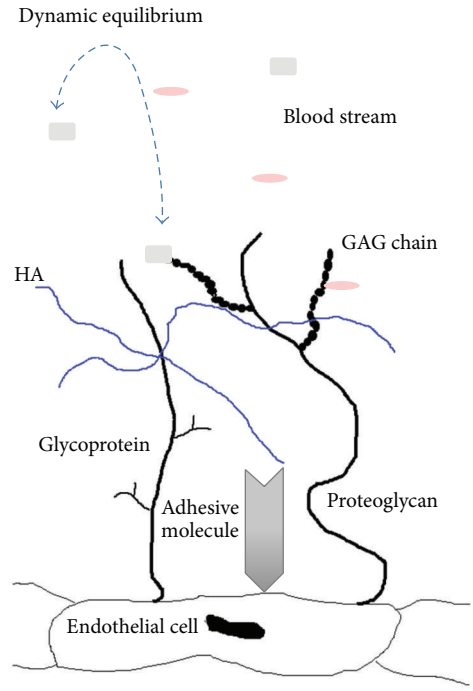

Figure 1: Structure of glycocalyx: the backbone molecules, glycoproteins and proteoglycans; GAG chains linked to core proteins; soluble molecules derived from plasma or endothelium bound to proteoglycans; intertwined HA molecules; sheltered adhesive molecules.

a dynamic equilibrium with flowing blood depending on the conditions of local microenvironment (Figure 1). Interaction between membrane-bound and soluble components of glycocalyx provides stability of this delicate layer; however, the composition of the glycocalyx is not static; there is a balance between biosynthesis and shedding of glycocalyx components. The backbone molecules of the glycocalyx are proteoglycans and glycoproteins that are mostly bound to endothelial cell surface [2]. On the basis of their structure, they belong to glycoproteins with core proteins of variable size characterized by a long (about 200 sugar residues) unbranched glycosaminoglycan (GAG) side chain linked through *O*-glycosyl linkage. A variety of saccharide motives, including galactosyl residues, as well as *N*-acetylglucosamine and *N*-acetylgalactosamine were demonstrated [7].

2.1. Proteoglycans. Among proteoglycans present in endothelial glycocalyx are syndecans, glypicans, mimecan (also known as osteoglycin), and biglycan. Syndecans are connected to the cell membrane via a single-span transmembrane domain. Further, they have a short cytoplasmic domain with conserved regions C1 (proximal to membrane) and C2 (distal to membrane) and a variable region V between C1 and C2, unique to the specific syndecan. The size of syndecans varies from 20 to 45 kDa. There are 4 subtypes of syndecans with an extracellular domain specific for each syndecan subtype which binds 3–5 heparan sulfate (HS) or chondroitin sulfate (CS) chains. This domain is variable depending on the structural diversity of the HS and CS chains resulting from a series of posttranslational modifications. The major syndecan in vascular endothelium is syndecan-2. Most of syndecans bind HS; the larger syndecans -1 and -3 can also bind CS. Interestingly, the syndecan ectodomains can be shed from cells and compete for cell surface binding [2, 12–14]. Glypicans have molecular weight around 60–70 kDa. They are connected to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor and, thus, can be released by phospholipase activity [7]. There are 6 glypican subtypes that all share a modular structure with the N-terminal signal sequence, a presumably globular domain containing a characteristic pattern of 14 cysteine residues, a domain with the GAG-attachment sites, and a hydrophobic C-terminal sequence that is involved in the formation of the GPI anchor structure. They bind exclusively HS close to the cell surface [15, 16]. Mimecan and biglycan belong to the small leucine-rich proteoglycan (SLRP) family which binds CS, dermatan sulfate (DS), and keratan sulphate (KS) [17, 18]. The size of SLRP proteins is up to 42 kDa and they are characterized by leucine-rich repeats (LRR) in their central domain. Each LRR has a conserved motif LXXLxLXXNxL, where L is leucine (substitution by isoleucine, valine, or other hydrophobic amino acid is possible) and x could be any amino acid. There are four cysteines with class-conserved domain at the N-terminus of all SLRPs; however, the N-terminus is variably modified in various SLRPs [18]. The SLRP family is divided into 5 classes. Mimecan belongs to class III. Biglycan is a class I member [18]. It is substituted with one or two covalently bound CS/DS chains which are attached to amino acids of the N-terminus [17, 19].

2.2. Glycosaminoglycans. GAGs are linear polymers of disaccharides that are composed of D-glucuronic acid, L-iduronic acid, or D-galactose linked to either D-*N*-acetylglucosamine or D-*N*-acetylgalactosamine. GAGs polymers differ in length and are modified by sulfation and/or (de)acetylation to a variable extent. Due to presence of carboxyl and sulfate groups GAGs are of negative charge under physiological conditions [11]. Glycocalyx GAGs except for hyaluronan (hyaluronic acid; HA) are covalently linked to core proteoglycans by coupling of their reducing end with core proteins [2, 20]. In general, GAGs are present in the glycocalyx in the following ratios: HS, more than 50% of the volume; HA forms more than 40%, and DS, around 10%. To a much lower extent, other GAGs such as KS are present in the glycocalyx.

HS is most abundant in the glycocalyx, since HS proteoglycans represent 50–90% of all proteoglycans in endothelial glycocalyx [2]. HS chains can vary with regard to disaccharide composition, domain arrangement, and size. In general, HS can range from 50 to 150 disaccharide units. HS structural diversity is based on a series of enzymatic reactions in the Golgi apparatus, which result in the removal of acetyl groups and sulfation, the epimerization of glucuronic acid to iduronic acid, and the sulfation of the C-6 and C-3 hydroxyl groups of glucosamine and the C-2-hydroxyl groups of uronic residues [21]. The HS chains bind primarily to syndecan ectodomains, often close to the N-terminus. They are connected to the core syndecan protein at a serine-glycine sequence lying within consensus regions rich in acidic residues through a tetrasaccharide linker of xylose-galactose-galactose-uronic acid [12, 22]. Structural diversity defines the functional properties of HS; fine structure appears to be a cell-specific signature, differing between proteoglycans from different sources but not between different proteoglycans from a single source [15].

CS is another abundant GAG in endothelial glycocalyx. The ratio of HS and CS is typically 4 : 1 in vascular endothelium. Type B CS, in which glucuronic acid can epimerize into iduronic acid and influence the functionality, is called dermatan sulfate (DS) and is sometimes classified as a unique class of GAGs [2]. CS is covalently linked to the core protein via the so-called GAG-protein linkage region (GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-O-Ser) [23]. The number of CS chains linked to the core protein can vary greatly and the MW of CS-GAG can reach up to 3000 kDa [24].

HA is a high-molecular weight not sulphated polymer (MW > 10^6 Da) that is the only GAG which is not linked to a core protein. It can be bound to the cell membrane and interacts with the cytoskeleton through hyaladherins (e.g., CD44 or RHAMM) [16]. HA is synthetized at the plasma membrane by HA synthases and pushed out of the membrane to the ECM. The configuration of the HA is a pseudorandom coil. HA is highly hygroscopic and, in its aqueous state, is highly viscous and elastic [25].

2.3. Glycoproteins. Glycoproteins are another group of "backbone" molecules connecting glycocalyx to the cell membrane. They are glycoconjugates with relatively small (5–12 sugar residues) and branched carbohydrate side chains [2, 7]. Among the most abundant of the functionally important glycocalyx glycoproteins are selectins, integrins, and other adhesion molecules with immunoglobulin structural domains [2, 26–28]. The expression of most of these adhesion molecules on endothelium varies considerably with cell activation or stimulation [2].

Selectins contain a small cytoplasmic tail, a transmembrane domain, several consensus repeats, and an EGF-like domain and a terminal lectin-like domain at the NH_2-terminus. The last mentioned is responsible for the binding of carbohydrate groups to glycosylated proteins or lipids. The EGF-like domain is involved in selectin-ligand recognition. On the vascular endothelium, E- (64 kDa - calculated from the sequence; different glycosylated forms vary between 100 and 115 kDa) and P- (140 kDa) selectins are expressed which differ in a number of consensus repeats: P-selectin has 9 and E-selectin 6 consensus repeats [2, 27, 28].

Integrins are heterodimeric integral membrane proteins composed of noncovalently bound type I transmembrane glycoprotein α (18 known in humans) and β subunits (8 known) creating 24 distinct heterodimers. Both of them have large extracellular domains, single-spanning transmembrane domains, and a short cytoplasmic tail [26, 29].

Other glycoproteins harbored within the glycocalyx are adhesion molecules like intercellular adhesion molecules 1 and 2, platelet/endothelial cell adhesion molecule 1, and vascular cell adhesion molecule 1, and glycoproteins acting in coagulation, fibrinolysis, and homeostasis, like Ib-IX-V complex [2].

2.4. Soluble Components. A wide range of other molecules are connected to endothelial glycocalyx, such as proteins and soluble proteoglycans that are either derived from the endothelium or from the bloodstream. From a functional view, endothelial glycocalyx harbors proteins involved in inflammation, coagulation, fibrinolysis, and haemostasis mostly connected with the vasculoprotective function of the glycocalyx [25]. They can be physically connected to the glycocalyx by different mechanisms. Firstly, these are receptors or enzymes (e.g., fibroblast growth factor receptor, lipoprotein lipase, and low-density lipoprotein (LDL)).

Another group is composed of plasma-derived molecules binding to the glycocalyx and leading to a concentration gradient (albumin, fibrinogen, orosomucoid). The glycocalyx sieves these soluble components of plasma, establishing a dynamic equilibrium between components in the blood and those retained within the glycocalyx [2, 25]. Finally, there is a group of molecules bound to the glycocalyx structure through interaction with GAGs. In particular, HS proteoglycans contain abundant binding sites for proteins by virtue of specific patterns of sulfation [4]. Among HS chain binding ligands are growth factors (e.g., fibroblast growth factor s, vascular endothelial growth factors, transforming growth factor-β, platelet-derived growth factors), extracellular matrix proteins (e.g., fibronectin, vitronectin, collagens, and thrombospondin-1), plasma proteins (e.g., extracellular superoxide dismutase (SOD)), and coagulation inhibitor factors (antithrombin-III, the protein C system, and tissue factor pathway inhibition) [2, 12–14, 30].

3. Endothelium Glycocalyx Dimension

Accurate assessment of the structural organization of normal or damaged endothelial glycocalyx requires reliable visualization techniques [2, 7]. Because of the structural composition of this highly fragile and unstable layer, it is especially challenging to visualize and measure its three-dimensional structure. Primary determination performed using transmission electron microscopy (TEM) suggested a thickness of the glycocalyx layer in the order of tens of nanometers [1, 2, 7, 31]. However, current analysis with the modified fixation of samples for TEM or other methods such as intravital microscopy and confocal microscopy suggested an apparent surface layer thickness of >5 μm [2, 10, 31]. This revealed that initial descriptions had significantly underestimated its actual dimension [5, 6]. Overall, methodological approaches employed to determine the thickness of the glycocalyx have produced widely varying results; thus, this review presents a summary of the various ways in which the endothelial glycocalyx has been visualized up to now.

3.1. Electron Microscopy. The first image of the endothelial glycocalyx was obtained by conventional TEM revealing a small layer with a dimension of approximately 20 nm in capillaries [32]. Since then, many subsequent approaches using TEM, along with varying perfusate contents or fixatives, have revealed stained structures on endothelial cell surfaces throughout vessels and cultured cells with large variations in dimension and appearance [8, 9]. In some studies [33, 34], ruthenium red staining in combination with glutaraldehyde/osmium tetroxide fixation was used. However, it is assumed that due to its relatively large molecular size ruthenium red may not gain entry to the entire glycocalyx and may affect glycocalyx geometry by modifying the electrostatic interactions between macromolecules presented on the membrane surface [7]. Interestingly, when specific fixation techniques were applied that stabilize negatively charged structures preventing the loss and/or collapse of these structures, such as lanthanum [34–37] or Alcian blue [35, 38, 39], evidence for a thick endothelial surface layer of up to approximately 800 nm in width was provided [39, 40].

Different results with regard to glycocalyx thickness and structure arising from the use of different TEM approaches may depend on whether the sample is fixed by perfusion or immersion. Chappell et al. documented a dramatic difference between perfusion-fixed and immersion-fixed umbilical vessels, where the latter did not exhibit a glycocalyx [36]. However, the perfusion method with protein free saline can cause shrinkage of the glycocalyx by a partial washout of proteins deposited inside the glycocalyx, which can explain the large discrepancy between *in vivo* measurements and those obtained from perfusion-fixed material [37]. Some studies point to artifacts caused by the presence of aqueous fixatives that may dissolve some structures of the glycocalyx layer. Nevertheless, to obviate this limitation, some groups [41, 42] modified the TEM staining protocol using nonaqueous fixatives based on osmium tetroxide dissolved in a fluorocarbon. This method compensates for techniques requiring washing procedures which can remove plasma proteins attached to glycocalyx structures [2, 7, 43].

However, in all these cases, dehydration by alcohol as a stepping stone towards the embedding of TEM specimens is required. This replacing of the water with organic solvents may lead to a considerable collapse of the hydrated and gel-like state of the glycocalyx [7, 31, 44]. To overcome this problem, Ebong et al., for example, employed the application of rapid freezing and the freeze substitution TEM technique, thus avoiding the use of conventional fixatives. These attempts to preserve the native state of the glycocalyx structure, which has a high water content, resulted in an apparent surface layer thickness of >5 μm [10, 31]. Moreover, this method discerned differences between glycocalyx thicknesses depending on cell type and culture environment [31]. In conclusion, TEM can provide information on the charge, composition, and structure of the glycocalyx; however, results vary greatly according to the fixation and staining methods employed and TEM cannot be used *in vivo* [45].

3.2. Intravital Microscopy. Several decades after the first TEM images were made, some studies showed differences between systemic and microvascular haematocrit on cremaster muscle suggesting a glycocalyx layer much thicker than had been indicated using original TEM approaches [1, 8, 9, 40, 46]. The intraluminal distribution of red blood cells (RBCs) in combination with a variety of fluorescently labelled tracer molecules (e.g., dextran) compared with the position of the endothelial wall in capillaries provided a more comprehensive understanding of the exclusion properties of the glycocalyx [2, 47, 48]. The permeation of inert macromolecules into the glycocalyx was determined by both the charge and size of macromolecules [46–49]. Using this methodological approach, the thickness of the glycocalyx in capillaries of hamster cremaster muscle *in vivo* was estimated to be ~0.4-0.5 μm [47].

Techniques measuring the thickness of the glycocalyx on the basis of the depth of infiltration of fluorescently labelled tracer molecules simultaneously enabled the development of other methods such as systemic infusion versus direct perfusion and the use of different species (hamster, mouse, rat) and/or different classes of microvessels (arterioles, capillaries, or venules) [2, 49–51]. One of the main limitations of these methods based on dye-exclusion *in vivo* is the required resolution that is close to the practical resolution limits of *in vivo* optical microscopy. This limits these determinations to microvessels less than 15 μm in diameter [52]. To address this limitation, some authors used high-resolution, near-wall, intravital fluorescent microparticle image velocimetry (μ-PIV). The μ-PIV technique allows the examination of the velocity profile near the vessel wall in microvessels more than 20 μm in diameter [53, 54].

Finally, the modern clinically applicable microscopic approaches that estimate individual capillary glycocalyx dimensions based on the change in capillary red cell column width following capillary leukocyte passage in microvasculature are orthogonal polarization spectral imaging (OPS, measured in the sublingual area) and its successor, side-stream

dark field imaging (SDF, measured on the nail fold) [16, 55–58]. However, these methods also have technical limitations such as the fact that sublingual glycocalyx measurement only gives information on capillary blood vessels [56].

3.3. Confocal Microscopy and other Methods. The poor spatial resolution of an intravital optical microscope allows the accurate measurement of glycocalyx thickness only on thin tissues that allow transillumination [55]. Advanced microscopic techniques bring significant improvements which allow the epi-illumination of thicker organ surfaces [2]. Direct visualization of the glycocalyx can be performed via several approaches, mostly using fluorescent labeled lectins that bind specific disaccharide moieties of glycosaminoglycan chains. Fluorescently labeled antibodies recognizing syndecan-1 and fluorescently labeled HA binding protein can be employed in a similar way. Confocal laser scanning microscopy also enables optical sectioning, allowing 3D reconstructions of the specimen. The application of confocal microscopy imaging has revealed a surface layer as thick as 2.5–4.5 μm depending on the size of vessels [2]. However, in general, confocal microscopy is still less suitable for imaging the glycocalyx in arteries due to limited penetration of light with a significant loss of resolution at greater depths (>40 μm) [2]. The arteries must be cut open longitudinally, which might compromise glycocalyx structure [43]. Further, similarly to TEM analysis, the major challenge is the fixation of samples. The preservation of samples using formaldehyde or glutaraldehyde can induce distortion of the glycocalyx because of aldehyde-induced cross-linking of the glycocalyx components and subsequent compression of the glycocalyx toward the cell surface [31]. To eliminate this problem, Barker et al. have applied confocal microscopy to living cells without destroying important functional features of the studied specimens, thereby demonstrating noninvasively the *in vitro* expression of the glycocalyx [59].

A promising technique to directly visualize larger vessels is two-photon laser scanning microscopy (TPLSM), a detailed description of which with respect to its advantages for glycocalyx determination is given elsewhere [2, 43, 60, 61]. Among the main advantages making TPLSM a suitable technique for directly visualizing the delicate endothelial glycocalyx are enhanced penetration depth, good resolution, optical sectioning, and low phototoxicity. When the glycocalyx was imaged with TPLSM in intact mouse carotid arteries, the glycocalyx thickness was found to be 4.5 μm [2].

The mechanical properties of the glycocalyx can be determined using other methods such as atomic force microscopy [62].

3.4. Uncertainties and Challenges with respect to Glycocalyx Structure Determination. As described above, there are several major difficulties limiting the demonstration of the three-dimensional structure of endothelial glycocalyx. The main uncertainties arise from comparing the glycocalyx determined using *in vitro* cultured endothelial cells versus *in vivo* vessels and further comparing various methodological approaches.

Concerning cell cultures, overall data suggest that there are more aspects that can modify the formation of the glycocalyx such as the density of cells, culture conditions, cell type, and shear stress [59, 63]. Some studies have demonstrated differences in the structure and composition of the glycocalyx in cells exposed to long-term shear when compared to cells grown under static conditions [10, 62, 64, 65]. Moreover, the comparison of TEM and μ-PIV techniques suggests that the thickness of the glycocalyx determined using *in vitro* cell-culture models may be drastically less than the glycocalyx thickness determined *in vivo* [34, 36, 53, 63] despite the fact that cultured cells have been demonstrated to produce some glycocalyx components [7, 66]. Since the validity of using cell culture experiments to study endothelial glycocalyx structure and function has recently been questioned, it remains to be determined whether the structure of the surface layer of cultured endothelium can be used as a model for that seen in *in vivo* conditions [10, 31, 45, 62, 64, 65].

From the viewpoint of methodological approaches, the fixation and/or dehydration of samples during their preparation for TEM analysis is one of the major drawbacks limiting the demonstration of the extent of an *in vitro* endothelial glycocalyx. In addition, the direct visualization of endothelial glycocalyx and the measurement of its dimensions and properties *in vivo* are also a challenge, mainly due to the fact that the endothelial glycocalyx is a very delicate structure depending critically on the presence of flowing plasma.

Interestingly, there are some methods used for the determination of the glycocalyx *in vivo* that are not based on visualization techniques. A generally employed approach to estimate the degradation of glycocalyx *in vivo* is the determination of glycocalyx constituents such as HS, syndecan-1, or HA in plasma [67, 68]. During numerous metabolic (diabetes), vascular (atherosclerosis, hypertension), and surgical (ischemia/reperfusion injury, trauma) disease states, it was shown that the plasma concentration of GAGs increases and that it correlates with the concentration of inflammatory markers [69, 70]. Another interesting method of indirectly comparing the thickness of the glycocalyx is comparing the difference between a noncirculating intravascular volume and a circulating volume using a glycocalyx permeable versus a glycocalyx impermeable tracer [5, 6, 71, 72]. The glycocalyx volume is estimated upon subtraction of these two volumes. Dextran 40 (MW 40 kDa) and indocyanine green are used as the glycocalyx-permeable tracer and a suitable impermeable tracer is a fluorescently labeled erythrocyte. Interestingly, the glycocalyx has been estimated to comprise as much as 25% of the total intravascular space in humans, which would yield an average glycocalyx thickness of up to 2 μm [30, 36, 56, 71–73]. However, the estimated systemic glycocalyx volume does not yield information about the heterogeneity of glycocalyx properties between organs and the technique is still subject to controversy [5, 6].

In conclusion, the heterogeneity of the thicknesses and structures of the glycocalyx reported by various authors arises in part from differences in the applied techniques; the use of *in vivo*, *ex vivo*, and *in vitro* systems/models; differences in sample preparation procedures; the heterogeneity of species and organs; differences in cultivation conditions (Figure 2).

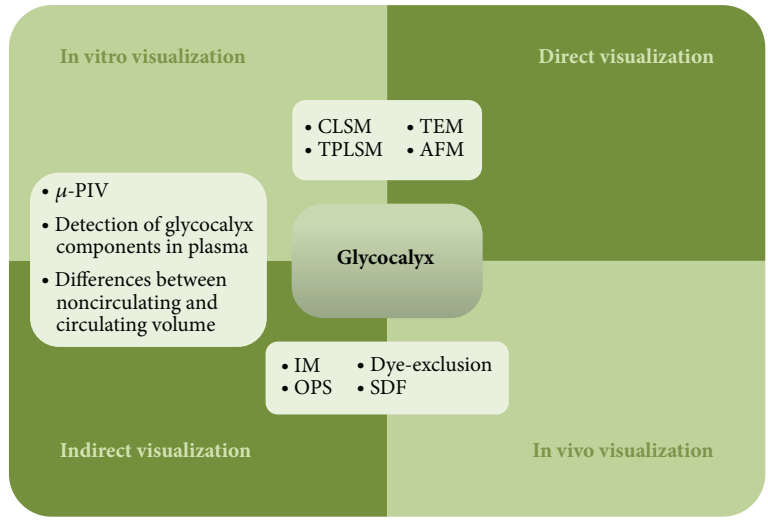

Figure 2: Summary of methods which are currently used in direct or indirect visualization of glycocalyx *in vivo* and *in vitro*. Confocal laser scanning microscopy (CLSM), two-photon laser scanning microscopy (TPLSM), transmission electron microscopy (TEM), atomic force microscopy (AFM), intravital microscopy (IM), microparticle image velocimetry (μ-PIV), orthogonal polarization spectral imaging (OPS), and side-stream dark field imaging (SDF).

4. Glycocalyx Physiological Functions

The glycocalyx layer consists of many highly sulfated GAGs chains providing negative charge for the endothelial surface layer. Due to these electrostatic properties, high molecular density glycocalyx can play a role in the regulation of vascular permeability and fluidic balance through restricting the entry of certain plasma molecules based on their charge, not only on the basis of size and steric hindrance [2, 5, 6, 8–10, 70, 74]. Disruption of glycocalyx can lead to the loss of permeability barrier function with subsequent edema formation. Further, the negative charge can also contribute to repulsion of red blood cells from the endothelium and thus influencing blood cell-vessel wall interaction. Besides this, intact glycocalyx serves as a barrier against the inadvertent adhesion of platelets and leukocytes to the vascular wall [2, 5, 6, 75]. The thickness of the glycocalyx, which is estimated to be around 0.5 μm, exceeds the dimensions of cellular adhesion molecules such as intercellular adhesion molecules 1, vascular cell adhesion molecule 1, and P- and L-selectins and, therefore, attenuating the interaction of blood cells with these molecules [5, 6, 10, 76]. Thus, shedding appears to be required for leukocyte adherence to the vessel wall, because, under normal conditions, leukocytes are supposed to be shielded from contact with their adhesion molecules by the glycocalyx [75, 77].

In recent years, several research studies have put forward the hypothesis that the glycocalyx plays an important role in mechanotransduction [3, 11, 78]. Glycocalyx structures transducing biochemical and mechanical forces into biochemical signals as a consequence of hemodynamic changes are responsible for conformational changes leading to changes in cellular responses. One of these changes is the upregulation of endothelial nitric oxide synthase and the increased production of nitric oxide (NO), which is an important determinant of vascular tone [2, 78, 79]. In any case, the mechanotransduction of glycocalyx is the result of cooperation of all different components.

Further, the importance of the glycocalyx in the protection of endothelial cells against damage by various mediators of oxidative stress was suggested. Under physiological conditions, the glycocalyx contributes to its vasculoprotective effect by docking major enzymatic systems. One of the most important enzymes is extracellular SOD bound to heparan sulphate (HS) proteoglycans, which contributes to a reduction in oxidative stress by quenching oxygen radicals and maintaining NO bioavailability [1, 2, 30].

The glycocalyx is suggested to play an important role in the regulation of coagulation, since a number of mediators involved in the regulation of coagulation pathways, such as antithrombin III, heparin cofactor II, thrombomodulin, and tissue factor pathway inhibitor, are bound within the glycocalyx structure.

Finally, the endothelial glycocalyx can also bind cytokines, which have profound effects on glycocalyx compound synthesis, or modulate inflammatory response by attenuating the binding of cytokines to cell surface receptors [2, 7–9, 30, 79].

All these physiological processes governed by the glycocalyx contribute to the nature of healthy endothelium and vascular homeostasis.

5. Inflammation Mediated Alternations of Endothelial Glycocalyx Functions

The vascular endothelium is one of the earliest sites of injury during inflammation. Rapid loss of glycocalyx functions

has been directly and indirectly evidenced under various systemic and local inflammatory responses such as diabetes, atherosclerosis, and surgical ischemia/reperfusion injury and sepsis [5, 6, 80]. As discussed above, the functions of glycocalyx are dependent on intact glycocalyx structure. Perturbation of the structure can range from deterioration to fundamental destruction of the glycocalyx layer. The loss of constituents of the endothelial glycocalyx, termed shedding, can encompass selective cleavage of HS and CS or major disturbance represented by removal of entire syndecan and glypican core proteins together with attached glycosaminoglycan side chains [5, 6]. The shedding of the glycocalyx in response to inflammatory mediators such as cytokines and chemoattractants was found to occur in arterioles, capillaries, and venules under various experimental models of inflammation [5, 6, 80]. Although at first sight, a reduction of the glycocalyx might appear favorable for nutrient supply, the microvascular changes associated with loss of the glycocalyx and impaired vascular protection could be negatively affect vessel functions [4]. For amplification of inflammation seems to be crucial the release of inflammatory mediators which could initiate the accessibility of leukocytes to adhesion molecules by degrading the enveloping glycocalyx, as was reviewed in Chappell et al. [80]. Based on mechanism of action, inflammatory mediators can directly affect endothelial cells that in response alter glycocalyx structures. Additionally, under inflammatory conditions, activated subsets of leukocytes such as polymorphonuclear leukocytes, macrophages, and mast cells degranulate enzymes which then can contribute to degradation of the glycocalyx [4, 81].

5.1. Mediators Released during Inflammation Contributing to Glycocalyx Destruction. Upon their activation, inflammatory cells release a wide range of enzymes and reactive species which can contribute to glycocalyx damage. In particular, activated neutrophil granulocytes, the most abundant blood leukocytes in humans, can induce glycocalyx damage by producing reactive oxygen and nitrogen species (ROS/RNSs) and releasing proteases from their storage granules [70]. Moreover, mast cells, a less abundant leukocyte subset, can release heparanase directly with a significant potential to disturb glycocalyx structure through the degradation of HS [5, 6, 79, 80, 82–84].

It is recognized that ROS/RNSs are capable of degrading HA, HS, and CS. Not all ROS/RNSs are a direct threat to the glycocalyx. The fragmentation of the glycocalyx is mediated by tertiary species, such as the hydroxyl radical or hypohalous acids, which are formed by the catalysis of neutrophil-derived MPO bound to the negatively charged GAG chains [70]. Further, it is supposed that ROS/RNSs are capable of attacking the glycocalyx generated in the direct vicinity of the endothelial lining [70]. The cleavage of HS after ROS/RNSs coupled with an increase in macromolecular passage follows a similar pattern seen after treatment with HS degrading enzymes [38, 85]. Moreover, the protein core of proteoglycans can also be subject to oxidation/nitrosation and these oxidative and nitrosative modifications at the level of proteoglycans could negatively affect glycocalyx integrity [70]. Further, ROS/RNSs not only pose a direct threat to the glycocalyx but additionally could potentiate the proteolysis of the glycocalyx via the activation of matrix metalloproteinases (MMPs) and inactivation of endogenous protease inhibitors [70].

Proteases are important mediators released and activated during inflammatory conditions that pose a significant threat to the glycocalyx [76, 86]. These include especially MMPs and neutrophil elastase. MMPs are stored within vesicles of phagocytes and the endothelium and after appropriate stimulation are released and activated [87]. The inhibition of MMP activity by doxycycline significantly reduced shedding of the glycocalyx [86–88]. It was suggested that cleavage of the entire syndecan ectodomain with the attached GAG branches by MMPs may be responsible for shedding of the glycocalyx [86, 87]. Interestingly, MMPs were shown to have a high affinity to HSs and this HS-mediated immobilization of MMPs on glycocalyx components underlines the destructive potential of MMPs [89]. Moreover, MMPs were suggested to directly cleave GAGs, HS proteoglycan syndecan-1, and the HA receptor CD44 [90–92].

Besides MMPs, the importance of neutrophil elastase in the degradation of endothelial glycocalyx under systemic or local inflammation conditions is suggested. Neutrophil elastase is released from the azurophilic granules of neutrophil granulocytes after activation. Neutrophil elastase can bind to the HS branches of syndecan probably via interaction with the sulfate moieties and is capable of syndecan degradation [70].

Overall, as described above, inflammatory mediator-mediated alterations of the glycocalyx structure or its shedding are best described as a result of the release of proteases and other degrading enzymes from different types of phagocytes and endothelial cells themselves [93].

5.2. Other Mediators. Further, glycocalyx degradation could be induced by low and/or turbulent shear stress or exposure of the endothelium to oxidized low density lipoprotein [94, 95]. It was also suggested that endothelial cells directly respond to inflammatory mediators such as tumor necrosis factor α (TNF-α) or bacterial lipopolysaccharide by shedding the glycocalyx [36, 67, 81, 93, 96]. This is possibly due to the activation or release of intracellular or membrane-bound degradation enzymes such as proteases [11, 76, 80].

6. Pathophysiological Implications of Glycocalyx Alternated Structure and Functions

As was summarized above, under physiological conditions the glycocalyx contributes to the regulation of vascular permeability and tone and serves as a barrier deterring leukocyte adhesion [70]. Thus alterations of these functions are associated with a wide range of pathophysiological consequences such as capillary leak syndrome, edema formation, accelerated inflammation, platelet hyperaggregation, hypercoagulation, and loss of vascular responsiveness [11]. In general, it is believed that vascular sites with diminished glycocalyx

are more vulnerable to proinflammatory and atherosclerotic consequences [1, 4–6] (Figure 3).

The importance of the loss of glycocalyx barrier functions leading to increased vascular permeability was suggested from experiments showing that glycocalyx degradation is associated with a reduction in the exclusion of anionic dextrans [49], with an increased protein permeability [97], with an increased glomerular clearance of albumin [98, 99], and with the formation of perivascular edema [39]. Further, it is suggested that the loss of the glycocalyx uncovers membrane surface adhesion molecules that potentiate leukocyte adhesion to the vessel wall [5, 6]. Thus, the increased adhesion of leukocytes and increased vascular permeability are the main experimentally confirmed consequences of damaged glycocalyx barrier function. Interestingly other mechanisms underlying this phenomenon of increased vascular permeability were suggested, including a loss of barrier function and induction of the rearrangement of intercellular endothelial junctions. In this case, signalling through syndecans integrates extracellular signals by their association with cytosolic effectors, leading to the rearrangement of cytoskeletal proteins and altering intercellular junctions, which become more permeable, allowing fluid extravasation [69].

Further, some of the pathological consequences are supposed to be due to the loss of different enzymes and signaling molecules stored in the glycocalyx structure, including SOD, antithrombin III, and thrombomodulin [8, 9, 79]. This perhaps contributes to an imbalance in enzymatic systems such as coagulation and antioxidant defense [79]. Finally, the pathological consequences of a loss of mechanotransduction functions due to damage of the glycocalyx structure are discussed by various authors [1, 3, 11, 79, 100, 101].

Focusing on inflammatory reaction, it should also be appreciated that degradation of the glycocalyx by inflammatory mediators and the release of its fragments into the circulation can significantly contribute to the potentiation of inflammatory processes, starting and maintaining a potentially destructive feed-back mechanism. The shed HS and HA fragments, which may be released with glycocalyx disruption, are suggested to act as pro-inflammatory molecules with, for example, significant chemotactical properties [16, 69, 102, 103].

7. Pathological Conditions Related to Altered Glycocalyx Structure

The alterations of functions of the endothelial glycocalyx layer are involved in many inflammation-based pathological states. Recent studies in humans revealed the degree of glycocalyx shedding to depend on the extent of the inflammatory state and that there are correlations between the severity of a disease and the level of glycocalyx components in blood [69, 70, 104]. The importance of glycocalyx changes in chronic inflammatory reactions was highlighted, suggesting that the vessel wall in patients with chronic inflammatory diseases is more sensitive to, for example, proatherogenic stimuli [79]. In this review we will discuss more deeply conditions such as diabetes, atherosclerosis, ischemia/reperfusion, and sepsis, in which inflammatory disorders take place.

7.1. Diabetes. Diabetes mellitus is a clinically well-defined metabolic disease connected with insulin absence or resistance and subsequent hyperglycemia. Patients with diabetes mellitus revealed a tendency to develop vascular complications, such as microalbuminuria, retino- and nephropathy, and elevated risks of atherothrombotic cardiovascular events [2, 74]. Fundamental pathogenic mechanisms in diabetes-associated vascular disease include accentuated vascular inflammation and increased oxidative stress [105]. These complications are suggested to be related to altered vascular functions such as enhanced endothelial permeability and impaired NO synthase function, indicating the compromised protective capacity of the vessel wall [71, 72, 74, 106–109]. Interestingly, it can be suggested that these typical pathological manifestations of impaired endothelium function in diabetes mellitus patients exhibit signs of glycocalyx degradation [71, 72, 110]. In the glomerulus, damage to the endothelial glycocalyx can alter the permeability of capillary beds, which is clinically apparent as albuminuria accompanied by increased systemic microvascular permeability [106]. It was shown that the changes in glomerular endothelial glycocalyx in early diabetic nephropathy are consistent with the early loss of charge selectivity that occurs in animal models of diabetic nephropathy and in individuals with type 1 and type 2 diabetes and microalbuminuria [98, 99, 111]. Hyperglycemia-induced glycocalyx degradation has been repeatedly demonstrated by a number of authors, who provided data that acute and long-term hyperglycemia is associated with the profound thinning of the glycocalyx and glycocalyx degradation as determined by various methods [71, 72, 112].

Several molecular mechanisms are suggested to be responsible for the reduction of glycocalyx structure in diabetic patients. Primarily, some authors speculate that it is directly caused by increased levels of glucose, since many of these effects can be experimentally induced in nondiabetic subjects as well. Interestingly, even short-term vessel perfusions with increased glucose above physiological levels were associated with vascular function alterations such as shear stress-induced arterial dilatation and vascular permeability, which could be connected with glycocalyx degradation [72, 74, 107–109]. It was suggested that glucose can influence glycocalyx structure because of the glycoproteinaceous nature of the glycocalyx with which glucose interacts [72]. Another suggested mechanism is connected with altered HS biosynthesis, since this was severely disrupted by the exposure of human glomerular endothelial cell monolayers to high glucose concentrations, these being associated with disrupted endothelial glycocalyx structure and the increased passage of albumin across the monolayer [113]. Finally, the relationship between HA metabolism alternations and hyperglycemia related vascular dysfunction was recently reviewed by Lennon and Singleton [25]. Plasma levels of HA and hyaluronidase were found to be elevated in patients with diabetes, reflecting the increased synthesis and shedding of HA under hyperglycemic conditions [71, 72, 112, 114].

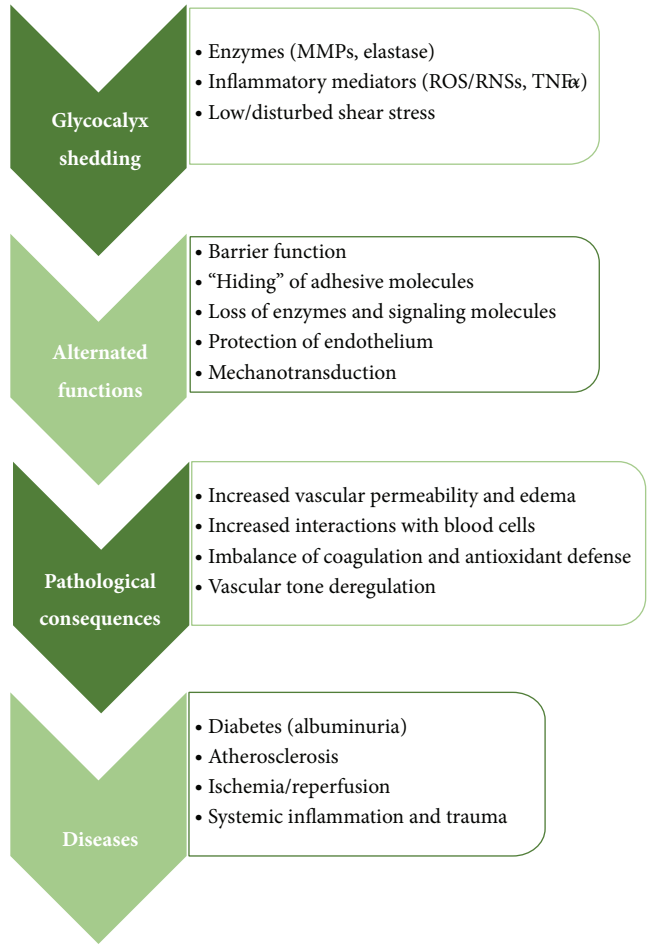

Figure 3: Overview of alternated glycocalyx functions as a result of its shedding which could lead to pathological states connected with various diseases. Matrix metalloproteinases (MMPs), reactive oxygen and nitrogen species (ROSs/RNSs), and tumor necrosis factor α (TNF-α).

Further, not directly related to glycocalyx structure alternations, inflammatory mediators can also increase endothelial cell-cell junction width and in some cases induce parajunctional transcellular holes, associated with increased permeability as was shown in both animal models and patients with diabetes [115]. Further, hyperglycemia-mediated damage to the podocytes may result in reduced vascular endothelial growth factor production, which leads to glomerular endothelial cell dysfunction and potentially also to glycocalyx disruption [116].

7.2. Atherosclerosis. Atherosclerosis is a large artery disease, of which the initiating step in pathogenesis is vascular endothelial barrier dysfunction. This is followed by the subendothelial retention of atherogenic lipoproteins, cholesterol, and monocytes forming a plaque that is marked by disturbed flow profiles. Subsequently, there is augmented endothelial barrier dysfunction and vascular smooth muscle cell proliferation, eventually leading to plaque rupture and thrombosis [25, 48, 95, 117, 118].

In general, the role of the endothelial glycocalyx in atherogenesis is, as yet, not well established, but there are some interesting observations which point at its involvement. The glycocalyx was visualized in large arteries in different animal models suggesting that the glycocalyx could add to the vasculoprotective properties of the vessel wall in the macrovasculature [30, 119]. The protective functions of the glycocalyx are suggested from research experimentally decreasing glycocalyx formation through the inhibition of HA synthesis, which led to the increased adhesion of leukocytes in the carotid artery of ApoE deficient mice and ultimately to increased atherosclerosis [117]. Interestingly, the thickness of the glycocalyx decreases in high-risk regions of the murine carotid artery (sites within the arterial system which have low or disturbed shear rates) compared with low-risk regions, supporting the potential role of glycocalyx disruption in rendering disturbed flow regions more susceptible to atherogenesis [30, 120]. Besides shear stress, another risk factor which has been shown to impair vascular glycocalyx is the ratio of oxidized lipoproteins to LDL [95]. Vink, van den Berg, and colleagues showed a disruption of the glycocalyx induced by a high-fat and high-cholesterol diet or by an administration of clinically relevant doses of ox-LDL evoking, for example, increased platelet adhesion

in hamster cremaster muscle microcirculation [95, 118]. In addition, exposure of endothelial cells to oxidized LDL *in vitro* decreases the amount of HS proteoglycans associated with the luminal cell surface. The loss of glycocalyx resulted also in the shedding of endogenous protective enzymes, such as extracellular SOD, and increased the oxidative stress on endothelial cells. Interestingly, the importance of ROSs in this process was underlined by the fact that SOD and catalases coinfusion annulled the effect of ox-LDL [95].

Overall, it can be suggested that the glycocalyx layer plays an important role in the protection of large vessels against atherothrombotic disease and that alterations to endothelial glycocalyx are involved in the initiation and progression of the atherosclerotic process.

7.3. Ischemia/Reperfusion. Ischemia/reperfusion injury leads to tissue damage caused by blood flow restoration to a tissue/organ after a period of disrupted blood flow inducing total or partial ischemia. Although the extent of the damage resulting from ischemia/reperfusion varies between tissues, a common component of this pathological process is microvascular dysfunction [2, 7, 93, 121]. In particular, in postcapillary venules, endothelial cells suffer from increased oxidative stress, leukocyte adherence and transmigration, and vascular permeability [2, 122]. The involvement of alterations to the endothelial glycocalyx in this process is supported by data showing that ischemia/reperfusion injury can stimulate shedding of the glycocalyx [7, 93, 121–123]. As was shown by Mulivor and Lipowsky, intestinal ischemia/reperfusion led to a significant reduction in glycocalyx thickness in rat mesenteric venules [93]. Similarly, Bruegger et al. showed that in an ischemia-reperfusion study using isolated guinea-pig hearts, the impairment of endothelium-derived vasodilation was paralleled by disruption of the endothelial glycocalyx [122]. In humans, glycocalyx shedding was demonstrated in patients undergoing major vascular surgery with global or regional ischemia [124]. Interestingly, the importance of oxidative stress in this phenomenon was also suggested from studies showing that the effects of ischemia/reperfusion on the glycocalyx could be attenuated by a blockade of xanthine-oxidoreductase, which is an endogenous ROS producing enzyme bound to HS domains in the glycocalyx [121]. Taken together, these data support a role for endothelial glycocalyx in the pathophysiology of inflammatory response connected with ischemia/reperfusion-induced tissue damage.

7.4. Systemic Inflammation and Trauma. Systemic inflammatory response to microbial infection or to extensive tissue damage leads to serious pathological conditions such as sepsis and multiple organ failure [80]. These conditions are accompanied by the disruption of vessel functions mediated by various factors including the deregulated synthesis of NO and the massive release of proinflammatory cytokines, such as TNF-α [80, 125]. Leakage due to defects in endothelial barrier functions is one of the major clinical problems facing critically ill patients [80, 104]. It leads to the severe disturbance of microcirculation and consequently the failure of systemic circulation. In a complex study, Schmidt et al. demonstrated that endotoxemia in mice rapidly induced pulmonary microvascular glycocalyx degradation via TNF-α-dependent mechanisms involving the activation of endothelial heparanase [125]. This pulmonary endothelial glycocalyx degradation was connected with neutrophil adherence and inflammation and potentially with sepsis-associated respiratory failure [125]. In previous studies, experimentally induced endotoxemia in rats elicited plasma HA release and a reduction in endothelial surface thickness, indicative of glycocalyx degradation [126]. Similarly, experimentally induced endotoxemia in mice led to increased syndecan-1 plasma levels suggesting glycocalyx degradation [127]. In clinical studies, a directly determined decrease in glycocalyx thickness in critically ill patients was shown by Donati et al., who observed a more profound decrease in thickness in more severely septic patients [128]. Other authors showed that increased levels of glycocalyx components such as HS or syndecan-1 appear in the blood of septic shock and trauma patients [104, 125, 129–131], and that such increased levels were even significantly higher in nonsurvivors or positively correlated with increased mortality [104, 131]. Further, significantly increased levels of HS and syndecan-1 were observed in patients after major postabdominal surgery even without systemic inflammatory response syndrome [130]. Significant destruction of the glycocalyx soon after trauma induction is also suggested from data obtained by Ostrowski et al. with respect to severely injured patients [132].

8. Therapeutic Strategies to Ameliorate Glycocalyx Dysfunction

As described above, under inflammatory conditions the integrity of the endothelial glycocalyx deteriorates to varying degrees particularly during generalized inflammatory responses of the body. To track the endogenous recovery of the glycocalyx *in vivo*, an interesting study was performed by Potter et al., who found that after acute enzymatic or cytokine-mediated degradation of the glycocalyx, 5 to 7 days were required for the glycocalyx to regain its original thickness. Thus the potentiation of this process should limit inflammatory processes in vessels. Positive effects of therapeutic strategies which would prevent shedding of glycocalyx components, prevent degradation of glycocalyx structure, and potentiate recovery of glycocalyx structure can be suggested for range of pathological conditions connected with inflammatory processes in the vessels mentioned above. Therapeutic strategies can be viewed from several perspectives. Principally, it is possible to differentiate between therapeutic strategies directly aimed at preserving, supporting, or reconstituting the glycocalyx structure and strategies with an indirect mechanism of action down regulating inflammatory processes which lead to glycocalyx structure damage.

8.1. Preservation of the Glycocalyx Structure by Exogenously Applied GAGs. The shedding of GAGs from the glycocalyx structure is observed both experimentally and in clinical trials. Thus, intravascular supplementation with sulfated

polysaccharides to support glycocalyx structure or to reconstitute the endothelial glycocalyx seems intuitively advantageous. It can be speculated that increased levels of precursors of the glycoproteins in the glycocalyx might induce these glycoproteins to be regenerated. *In vivo*, heparin applied by intravenous injection was shown to interact rapidly and specifically with the endothelium [133]. Further, a combination of two glycocalyx abundant GAGs, HA and CS, administered by infusion was shown to partially regenerate the capillary glycocalyx damaged by hyaluronidase in hamsters [49]. Interestingly, treatment with either molecule separately had no effect [49]. Interestingly, also *in vitro*, Potter and Damiano observed a surface-bound GAG layer on cultured human umbilical vein endothelium only after supplementation of the culture medium with GAGs, HA and CS [53]. Constantinescu et al. demonstrated that intravascular supplementation with HS and heparin, but not dextran sulfate, attenuated ox-LDL-induced leukocyte-endothelial cell adhesion in mouse cremaster venules after degradation of the endothelial glycocalyx by local microinjection of heparitinase [77]. Interestingly, they showed that fluorescently labeled HS and heparin, but not dextran sulfate, became attached to the venule luminal surface after ox-LDL administration. Similarly, the intravenous application of heparin inhibited the negative effects of ischemia/reperfusion which were determined by intravital-microscopy in mouse cremasteric microvessels as the macromolecule exclusion and intracapillary distribution of red blood cells [121]. However, this effect was suggested to be connected with a decrease in ROS production, not directly with the incorporation of exogenous heparin into the glycocalyx structure.

Interestingly, sulodexide, a mixture of glycocalyx GAG precursors consisting of heparin sulphate (80%) and dermatan sulphate (20%), can be included among compounds suggested as a possible treatment of dysfunctional glycocalyx. [112]. It was shown by Gambaro et al. that long-term administration of this GAG preparation prevents renal morphological and functional alterations and appears to revert established diabetic renal lesions in experimental diabetic nephropathy induced in rats [134]. In clinical trials, Broekhuizen et al. demonstrated that sulodexide administration for 2 months increased glycocalyx thickness, which was found to be primarily altered in patients with type 2 diabetes compared to healthy controls, as was estimated in two different vascular beds using sidestream dark field imaging and combined fluorescein and indocyanine green angiography for sublingual and retinal vessels, respectively [112]. Although the reversal of glycocalyx abnormalities in diabetes was only partial, it would appear that this approach is promising. In another study, sulodexide therapy for a period of one year reduced albuminuria in diabetic patients [135]. The drug appeared active in both type 1 and type 2 diabetes and in both micro- and macroalbuminuric patients. No change in metabolic control and no systemic side effects were reported [135]. Finally, sulodexide showed significant cardioprotective effects after myocardial revascularization in rabbits [136]. However, in none of these studies did the authors determine effects on glycocalyx properties.

In contrast to the abovementioned studies, intravenous heparin challenge was associated with increased vascular leakage of dextrans and impaired arteriolar vasodilation in mouse [101]. Further, the intravenous application of heparin led to the immediate release of eight proteins shown to localize in the glycocalyx with HS binding properties in patients with nephropathy [137]. The authors speculate that this was due to a previously described release of glycocalyx-associated proteins owing to their competitive binding to exogenous heparin [137], which then negatively affected glycocalyx barrier properties and the mechanotransduction of shear stress to the endothelium [101]. Overall, these authors conclude that a heparin challenge can have adverse effects on vascular homeostasis and suggest that a perturbation in the glycocalyx might be involved in this phenomenon. However, the effect of heparin on intact glycocalyx could differ from its effect on altered glycocalyx, which is the case of the suggested positive effects of the sequestration of exogenous heparin on vessel function.

8.2. Preservation of the Glycocalyx Structure by Natural Plasma Protein Levels. The simplest suggested way to protect the glycocalyx is to maintain a sufficiently high concentration of plasma proteins [5, 6]. From a theoretical standpoint, Becker et al. predict that poorer mechanical stability of a protein-denuded glycocalyx, heightened susceptibility to attack by proteases, as well as secondary damage to the vessel wall incurred by the greater adherence of inflammatory cells, could be expected [5, 6]. The protective effect of the albumin supplementation on glycocalyx preservation in a model of transplantation-induced ischemia/reperfusion glycocalyx damage was presented by Jacob et al. [138]. Albumin supplementation significantly attenuated pronounced shedding of the glycocalyx as well as interstitial edema and the increased adhesion of leukocytes observed after a cold ischemia. Interestingly, experimental studies suggest that concentrations of albumin significantly lower than the physiological value may be sufficient to protect vascular integrity [139].

8.3. Inhibitors or Scavengers of Glycocalyx Damaging Noxes. In this part direct inhibition of factors involved in glycocalyx degradation is discussed.

8.4. Antioxidants and NO. Antioxidants have been found to protect tissues and organs from ischemia/reperfusion damage in innumerable studies. This action might significantly involve protection of the glycocalyx [48, 95, 121, 140]. However, no clinical studies that focused on preserving vascular permeability have yet used this approach convincingly. NO can play a specific role. It is an important signaling molecule for vascular cells. At the same time, low levels of NO are suggested to prevent oxidative cell damage. An experimental study by Bruegger et al. described the protective effect of NO, applied only during reperfusion, on maintaining the glycocalyx and the permeability barrier in the face of redox stress [122]. This latter action was exerted only if the glycocalyx was not destroyed enzymatically beforehand;

thus, the direct radical scavenging action of NO was held responsible by the authors [122].

8.5. Inhibitors of Proteases. As discussed above, since proteases such as thrombin have been reported to support the cleavage of syndecan ectodomains, there could be an opening for the therapeutic use of protease inhibitors [36, 68, 141]. Among the tested inhibitors is doxycycline. In several studies, Mulivor and Lipowsky with coauthors demonstrated the inhibition of MMP activity by doxycycline that significantly reduced shedding of the glycocalyx and leukocyte adhesion to endothelial cells in response to inflammatory and ischemic stimuli [76, 86–88]. Another candidate is antithrombin III, a physiological inhibitor of numerous serine proteases naturally present in the glycocalyx, as described above. In several studies, Chappel at al. showed that antithrombin significantly protected the glycocalyx from TNF-α and ischemia/reperfusion-induced shedding in hearts [36, 67, 68, 142]. The glycocalyx protection was accompanied by reduced postischemic leukocyte adhesion in hearts, reduced vascular permeability, reduced coronary leak, and reduced interstitial edema [36, 67, 68, 142].

8.6. Approaches to Preserve Glycocalyx Structure with Indirect Mechanisms of Action (Modulating Processes Which Lead to Glycocalyx Structure Damage). Other approaches that could be suggested to protect glycocalyx are based on modulation of processes that lead to glycocalyx damage. In the context of the above described detrimental effects of inflammatory processes on glycocalyx integrity, the reduction of inflammation processes by various types of drugs can be suggested as highly potential therapeutic approaches.

8.7. TNF-α Signaling Inhibition. TNF-α is one of the key mediators in the development of acute and chronic inflammation. Interestingly, a clinically used inhibitor of TNF-α signaling, Etanercept, an analog of the TNF-α receptor, significantly reduced the shedding of glycocalyx constituents, coagulation activation, and functional vessel function disturbances induced by experimental endotoxin application in humans [57].

8.8. Glucocorticoids. Glucocorticoids are routinely applied in the prevention of interstitial edema and swelling due to the substantial reduction in vessel permeability for macromolecules [5, 6, 36, 67, 68, 83, 142, 143]. The importance of the preservation of the glycocalyx is supported by results from an isolated heart model, in which preconditioning with hydrocortisone significantly reduced glycocalyx shedding following both ischemia/reperfusion and TNF-α-induced inflammation [36, 67, 83, 142]. Interestingly, clinical data show that hydrocortisone significantly reduced inflammatory response and the need for circulatory and ventilatory support in cardiac surgical patients, which was suggested to be associated with sustaining vascular barrier function, that is, with the prevention of the shedding of the glycocalyx [143].

Despite the positive effects of glucocorticoids, the exact mode of their action remains unclear [83]. Apart from the direct effect of hydrocortisone on endothelial cells, an inhibitory effect on immune effector cells has also been noted. Some authors underline the importance of the glucocorticoidal stabilization of mast cells, which should prevent degranulation and consequently abrogate proteolytic damage to the glycocalyx and the potentiation of inflammation [5, 6].

8.9. Volatile Anesthetic Sevoflurane and Isoflurane. Volatile anesthetics, such as sevoflurane and isoflurane, were suggested to pose anti-inflammatory effects and to ameliorate endothelial glycocalyx destruction induced by inflammatory response mediated by ischemia/reperfusion [144–146]. Sevoflurane was shown to pose complex anti-inflammatory effects on blood cells, leukocytes, and platelets and provided endothelial protection against ischemia/reperfusion injury *in vivo* [147–149]. A direct effect on endothelial cells was shown in an *in vitro* study demonstrating the immediate and delayed protective effects of isoflurane pretreatment on cytokine-induced injury in human endothelial cells [150]. The sevoflurane-mediated protection of endothelial glycocalyx destruction was demonstrated in ischemia/reperfusion-induced degradation experiments with guinea pig hearts, with both preconditioning and rapid postconditioning being successful [144–146]. These authors suggested that the mechanism involved the attenuation of lysosomal cathepsin B release [145].

8.10. Reduction of Hyperglycemia or Hypercholesterolemia. Since glycocalyx volume is suggested to be significantly reduced during hyperglycemia or hypercholesterolemia, as discussed above, the therapeutic normalization of blood glucose levels or blood lipid status could be proposed to prevent these alterations to glycocalyx structure. However, to our knowledge, there are currently no more reports that would provide experimental or clinical data demonstrating direct improvements in glycocalyx structure or function after treatment with glucose lowering drugs such as insulin.

The reduction of hypercholesterolemia by statins can be suggested. Interestingly, a partial recovery of the systemic glycocalyx volume compared to healthy controls was observed in patients with familial hypercholesterolemia after treatment with rosuvastatin [151]. However, the study does not clarify whether this was a direct effect of the statin on endothelial cells, or more related to the improved lipid status.

9. Conclusion

A wide range of data, both experimental and clinical, demonstrates that the integrity of the endothelial glycocalyx deteriorates to varying degrees under inflammatory conditions, particularly during a generalized inflammatory response of the body. However, improvements in techniques that would allow valid studies of the microvascular glycocalyx to be made on isolated endothelial cells are required in order to definitively determine whether mechanotransduction is a

function of the endothelial cytoskeleton or of the arterial glycocalyx [78].

Interestingly, data from experimental and clinical studies suggest the possibility of glycocalyx protection or preservation by various means. However, despite some positive results in several studies, skepticism exists about their clinical use in the near future. Since the pathological process occurs early in the inflammatory process, this strategy is theoretically problematic. However, based on current data from various models any therapeutic approach that would improve the glycocalyx structure and function would have a good potential to prevent the pathological processes connected with vascular inflammation.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Vascular Medicine

Heparins Increase Endothelial Nitric Oxide Bioavailability by Liberating Vessel-Immobilized Myeloperoxidase

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Background—Neutrophils and monocytes are centrally linked to vascular inflammatory disease, and leukocyte-derived myeloperoxidase (MPO) has emerged as an important mechanistic participant in impaired vasomotor function. MPO binds to and transcytoses endothelial cells in a glycosaminoglycan-dependent manner, and MPO binding to the vessel wall is a prerequisite for MPO-dependent oxidation of endothelium-derived nitric oxide (NO) and impairment of endothelial function in animal models. In the present study, we investigated whether heparin mobilizes MPO from vascular compartments in humans and defined whether this translates into increased vascular NO bioavailability and function.

Methods and Results—Plasma MPO levels before and after heparin administration were assessed by ELISA in 109 patients undergoing coronary angiography. Whereas baseline plasma MPO levels did not differ between patients with or without angiographically detectable coronary artery disease (CAD), the increase in MPO plasma content on bolus heparin administration was higher in patients with CAD ($P=0.01$). Heparin treatment also improved endothelial NO bioavailability, as evidenced by flow-mediated dilation ($P<0.01$) and by acetylcholine-induced changes in forearm blood flow ($P<0.01$). The extent of heparin-induced MPO release was correlated with improvement in endothelial function ($r=0.69$, $P<0.01$). Moreover, and consistent with this tenet, ex vivo heparin treatment of extracellular matrix proteins, cultured endothelial cells, and saphenous vein graft specimens from CAD patients decreased MPO burden.

Conclusions—Mobilization of vessel-associated MPO may represent an important mechanism by which heparins exert antiinflammatory effects and increase vascular NO bioavailability. These data add to the growing body of evidence for a causal role of MPO in compromised vascular NO signaling in humans. **(*Circulation*. 2006;113:1871-1878.)**

Key Words: atherosclerosis ■ coronary disease ■ endothelium ■ inflammation ■ leukocytes

Recruitment and activation of leukocytes, in particular, neutrophils and monocytes, is a central event in acute and chronic vascular inflammatory disease.[1,2] One of the principal enzymes released from activated leukocytes is myeloperoxidase (MPO). This redox-activated hemoprotein is abundantly expressed in circulating neutrophils, monocytes, and tissue-associated macrophages and not only possesses potent bactericidal properties but also can be mechanistically linked to the underlying pathophysiology of chronic vascular inflammatory diseases, such as atherosclerosis and coronary artery disease (CAD).[3,4] For example, MPO, located in the atherosclerotic lesion,[5] mediates the oxidation of lipoproteins,[6] catalyzes the nitration of tyrosine residues, and has been shown to deplete endothelium-derived nitric oxide (NO) through its use as a substrate and by the generation of small radical intermediates.[7–12]

Clinical Perspective p 1878

A principal prerequisite for the NO-oxidizing properties of MPO is its sequestration into the subendothelial space. Studies involving cultured endothelial cells and rat aortic rings have revealed that MPO binds to the cell surface and is transcytosed toward the subendothelial matrix in a heparin glycosaminoglycan (GAG)-dependent manner.[10] So far, studies aiming to assess the relevance of MPO in mediating proinflammatory reactions in humans have been restricted to the analysis of circulating non–vessel-associated MPO.[13–15] Thus, it remains unclear whether luminal (plasma or serum)

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MPO levels reliably reflect MPO deposition within the vessel wall. Because the strategic localization of MPO in the subendothelial space has been demonstrated to be necessary for MPO to affect NO signaling pathways, the link between vessel wall–associated MPO and endothelial function in humans has remained elusive.

Because ex vivo studies have revealed that the binding of MPO to endothelial cells is prevented by heparins, we reasoned that systemic administration of heparins might release vessel wall–immobilized MPO in vivo. In the present study, we demonstrate that (1) patients with CAD, compared with control subjects, reveal an increased vascular deposition of MPO; (2) heparin administration improves endothelial NO bioavailability; and (3) the heparin-induced improvement of endothelial function is correlated with the extent of MPO liberation from the vessel wall into the luminal space.

Methods

Study Outline

The protocol was approved by the Hamburg medical board, and every patient gave written informed consent. One hundred nine consecutive patients who underwent coronary angiography at this institution and received a heparin bolus during intervention were included in the trial. CAD was defined by a history of myocardial infarction or coronary revascularization procedures or the presence of ≥50% luminal stenosis in at least 1 of the coronary arteries, as evidenced by coronary angiography. Patients with acute coronary syndromes within 1 month before study entry, those with congestive heart failure, those with impaired renal function (creatinine >2.0 mg/dL), and those with a history of long-acting antianginal medication were excluded. Blood samples were taken from each subject before and 15 minutes after administration of unfractionated heparin (70 U/kg body wt), and plasma was frozen at −80°C until further analysis.

With the use of venous occlusion plethysmography, 7 consecutive patients underwent assessment of acetylcholine (ACh)-induced changes in forearm blood flow in response to saline (NaCl), heparin, and N^G-monomethyl-L-arginine (L-NMMA). In addition, 27 subjects were randomized in a 2:1 fashion to either receive heparin (n=18) or NaCl (n=9), respectively, before and after the assessment of flow-mediated dilation (FMD), as described below.

Vascular Function Tests

Forearm Blood Flow in Response to ACh

To directly assess functional changes in vascular NO bioavailability and signaling, ACh-dependent increases in forearm blood flow were assessed by venous occlusion plethysmography, as described previously.[16] In brief, a 20-gauge polyethylene catheter was inserted into the brachial artery. The strain gauge was connected to an electronically calibrated plethysmograph. A wrist cuff was inflated to suprasystolic pressures 1 minute before and during each measurement to exclude hand circulation. A cuff placed on the upper arm was inflated to 40 mm Hg to occlude venous outflow from the extremity. Flow measurements were recorded for 5 seconds every 10 seconds; the mean flow value of 7 consecutive readings was used for analyses. Basal measurements were obtained during intra-arterial infusion of 0.9% saline at a rate of 1.66 mL/min. Endothelium-derived vasodilation was assessed by infusing ACh in increasing concentrations of 7.5, 15, and 30 μg/min into the brachial artery. Sodium nitroprusside (1, 3, and 10 μg/min) was administered at 30 μg/min. To evaluate the NO-specific effects of changes in forearm blood flow, patients subsequently received intra-arterial infusions of the NO synthase inhibitor L-NMMA (16 μmol/min), and measurements were repeated. After intravenous administration of heparin (70 U/μg body wt), the protocol was repeated as above.

Determination of FMD

Ultrasound determination of FMD was performed according to the guidelines of the American College of Cardiology. Briefly, a Siemens (Iselin, NJ) Sonoline G50 ultrasound system with a 12-MHz linear array transducer was used to record 2-dimensional cine sequences of the brachial artery over 4 seconds at baseline and 1 minute after the induction of reactive hyperemia by 5-minute cuff occlusion of the forearm. The velocity time integral of Doppler flow was assessed by pulsed-wave Doppler with correction of insonation angle at baseline and peak hyperemic flow to calculate the flow ratio. Flow-independent vasodilation was assessed after determination of brachial artery diameter before and 4 minutes after the administration of nitroglycerine (0.4 mg). Subjects were instructed not to eat, drink, or smoke within 12 hours before testing. Measurements were carried out at baseline and 15 minutes after the intravenous administration of heparin (70 U/kg body wt) or placebo, respectively. The second measurement was performed after a waiting period of 3 hours after the administration of nitroglycerine. Two-dimensional sequences were analyzed by use of edge-detection software (Brachial Analyzer, Medical Imaging Applications LLC, Coralville, Iowa). The operators were blinded to patients' treatments.

Determination of MPO Content in Matrix Proteins, Endothelial Cells, and Human Saphenous Veins

To assess the effect of heparin on the mobilization of MPO bound to extracellular matrices, fibronectin and collagen (13 μg/cm^2, Sigma, Sigma-Aldrich, Inc, St Louis, Mo) were exposed to MPO (13 nmol/L, 2 hours at room temperature in phosphate-buffered saline), washed once, and incubated with heparin (20 U/mL, 20 minutes). Subsequently, supernatants were analyzed for MPO by ELISA, and MPO bound to the matrices was detected by Western blotting with the use of a polyclonal anti-MPO antibody (1:10000, Calbiochem, EMD Biosciences, Inc, Merck KGaA, Darmstadt, Germany) and enhanced chemiluminescence for detection.

To determine the effect of heparin on the liberation of endothelium-bound MPO, cultured human umbilical vein endothelial cells (HUVECs) were grown to confluence, exposed to MPO (13 nmol/L, 2 hours; Planta Natural Products, Vienna, Austria), washed to remove nonadherent enzyme, and in some cases exposed to heparin (20 U/mL, 20 minutes). MPO content in the supernatant and cell lysates was determined by ELISA, as described below. MPO activity was assessed as described previously.[10] To test the effect of heparin on MPO mobilization in human vessels, nonheparinized specimens from saphenous vein grafts from patients undergoing coronary artery bypass surgery were liberated from adventitial tissue. Vessels were divided into equal parts and exposed to either heparin (20 U/mL) or saline (0.9%) for 30 minutes at 37°C. Subsequently, tissue was homogenized as previously described, and proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.[17] MPO protein content was determined by using a polyclonal anti-MPO antibody (1:10000; Calbiochem) and enhanced chemiluminescence for detection.

Assessment of Elastase, MPO, and Triglyceride Plasma Levels

MPO and elastase plasma levels were determined by ELISA according to the manufacturer's recommendations (Calbiochem and IBL Hamburg, Germany, respectively). All plasma samples were collected in heparinized tubes, with a final heparin concentration of 16 U/mL blood. In vitro supplementation of plasma samples with additional heparin (1 to 10 U/mL) did not affect MPO recovery by ELISA (data not shown). MPO recovery in heparinized plasma (as assessed by ex vivo addition of MPO to plasma from MPO-deficient individuals) was ≈0.5%. After MPO plasma content (n=50) was analyzed by ELISA, recovering >95% of plasma MPO (Prognostix, Cleveland, Ohio), a high linear correlation was observed between the 2 ELISAs (r=0.78, P<0.001). Triglyceride levels were determined with a Hitachi (Tokyo, Japan) Modular Analyzer 0303 GS by an enzymatic method.

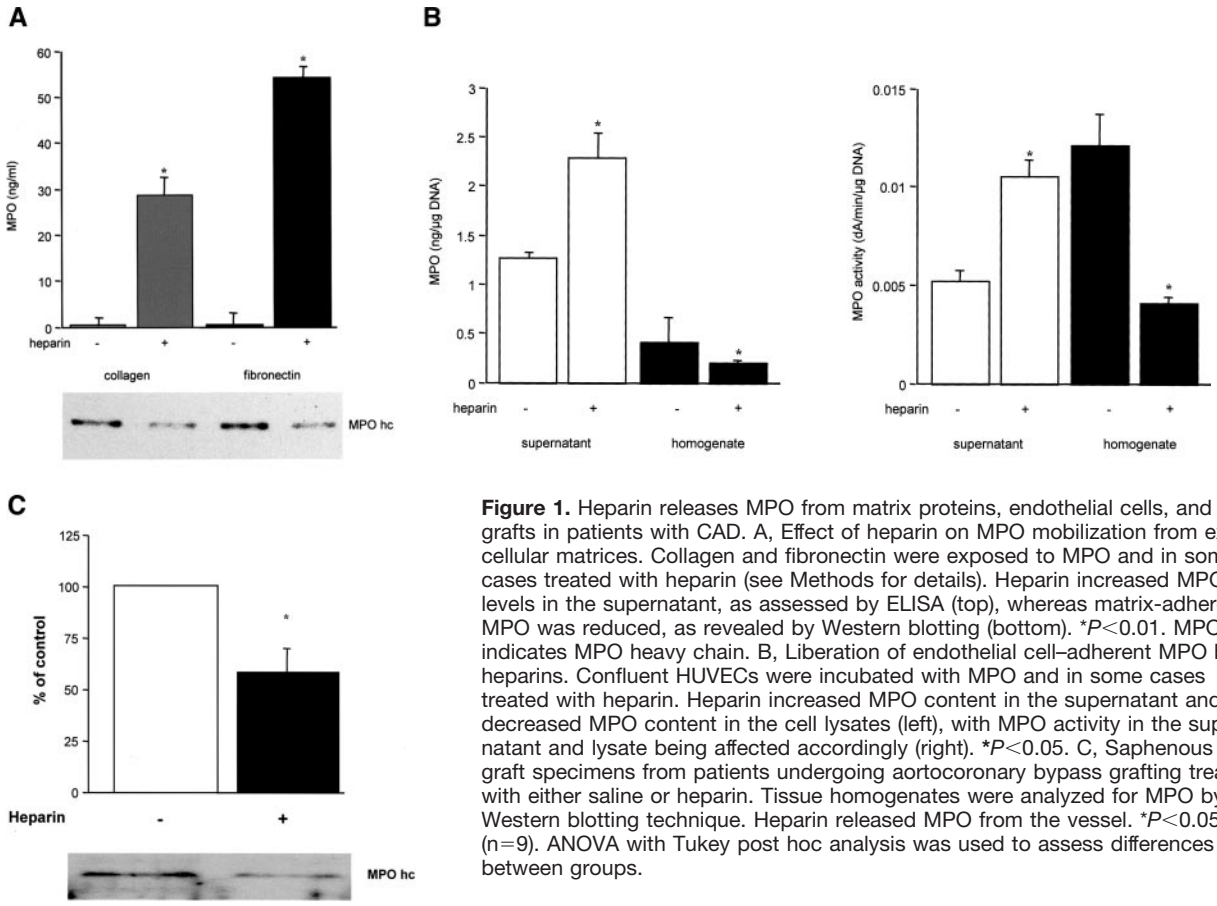

Figure 1. Heparin releases MPO from matrix proteins, endothelial cells, and vein grafts in patients with CAD. A, Effect of heparin on MPO mobilization from extracellular matrices. Collagen and fibronectin were exposed to MPO and in some cases treated with heparin (see Methods for details). Heparin increased MPO levels in the supernatant, as assessed by ELISA (top), whereas matrix-adherent MPO was reduced, as revealed by Western blotting (bottom). *$P<0.01$. MPO hc indicates MPO heavy chain. B, Liberation of endothelial cell–adherent MPO by heparins. Confluent HUVECs were incubated with MPO and in some cases treated with heparin. Heparin increased MPO content in the supernatant and decreased MPO content in the cell lysates (left), with MPO activity in the supernatant and lysate being affected accordingly (right). *$P<0.05$. C, Saphenous vein graft specimens from patients undergoing aortocoronary bypass grafting treated with either saline or heparin. Tissue homogenates were analyzed for MPO by the Western blotting technique. Heparin released MPO from the vessel. *$P<0.05$ (n=9). ANOVA with Tukey post hoc analysis was used to assess differences between groups.

Statistical Analysis

Categorical data are presented as frequencies and percentages and were compared by χ^2 test and the Fisher exact test. Continuous variables were tested for normal distribution by use of the Kolmogorov-Smirnov test. Data with normal distribution are presented as mean±SD; non-normally distributed data are presented as median and interquartile range (IR). For normally distributed data, Student paired and unpaired *t* tests were used. One-way ANOVA for repeated measures using the Bonferroni method for multiple comparisons was used for venous plethysmographic data. Comparisons for nonnormally distributed data were performed by the Mann-Whitney *U* test and Wilcoxon signed rank sum test. For assessment of the association between FMD and MPO, the Pearson correlation was applied. Because CAD patients and control subjects showed significant differences in baseline characteristics, multivariate ANOVA accounting for CAD, age, gender, diabetes, and intake of lipid-lowering medication on MPO increase was performed. Because MPO levels revealed a non-normal distribution, a log-transformation of data was performed before testing. A value of $P<0.05$ was considered statistically significant.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the article as written.

Results

Effect of Heparin on Vessel Wall–Immobilized MPO

Heparins have been previously demonstrated to prevent the binding of MPO to cultured endothelial cells and isolated rat aortic ring segments.[8,10] To test whether heparins also reverse MPO binding to the vessel wall, isolated extracellular matrix proteins, cultured endothelial cells, and saphenous vein graft specimens from patients undergoing aortocoronary bypass surgery were analyzed for MPO before and after treatment with heparin. MPO binding to fibronectin and collagen was markedly reversed in the presence of heparin (Figure 1A), as was MPO association with cultured HUVECs. Heparin treatment increased MPO content and MPO activity in the supernatant and concomitantly decreased MPO burden and activity in the cell lysates (Figure 1B). Compared with saline-treated rings, saphenous vein grafts that were treated ex vivo with heparin revealed significantly reduced MPO protein content (Figure 1C).

To assess the MPO-liberating effects of heparin in vivo, plasma samples from 109 consecutive patients before and after heparin administration were collected. Characteristics of the study population are presented in Table 1. Of the 109 patients, 78 were diagnosed with CAD, whereas 31 subjects did not display CAD. Compared with non-CAD subjects, patients with CAD were significantly older, a higher percentage of them were men, they revealed a higher incidence of hypertension or diabetes mellitus, and they were characterized by a higher intake of statins, resulting in lower low-density lipoprotein (LDL) cholesterol levels (Table 1). Multivariate ANOVA revealed no influence of any of these parameters on MPO plasma levels. Of the subgroup that underwent noninvasive endothelial function tests, 9 patients in the heparin group and 5 patients in the placebo group were diagnosed with CAD. The 2 groups did not differ significantly for any of the parameters listed in Table 1.

TABLE 1. Clinical Characteristics of Patients in Whom MPO and Elastase Plasma Levels Were Assessed Before and After Intravenous Heparin Administration

	CAD (n=78)	Non-CAD (n=31)	*P*
Gender			
Male	59 (75.6)	16 (51.6)	0.02
Female	19 (24.4)	15 (48.4)	
Age, y	66.4±8.5	58.9±10.1	0.01
Hypertension	70 (89.7)	16 (51.6)	0.01
Diabetes mellitus	27 (34.6)	3 (9.7)	0.01
Smoker	25 (32.1)	4 (12.9)	0.06
Family history	30 (38.5)	10 (32.3)	0.66
LDL	111.9±39.0	133.0±40.3	0.01
HDL	50.8±13.8	58.8±18.7	0.02
Triglycerides	149.0±91.2	115.7±59.8	0.06
Statins	48 (61.5)	4 (12.9)	0.01
ACE inhibitor	38 (48.7)	11 (35.5)	0.28
AT1 receptor blocker	8 (10.3)	1 (3.2)	0.44
MPO, ng/mL			
Before heparin	10.72 (7.89–13.10)	8.93 (7.66–10.95)	0.1
After heparin	17.06 (13.61–22.61)	13.57 (10.38–17.60)	0.03
Elastase, ng/mL			
Before heparin	84.96 (54.12–144.62)	59.68 (41.33–108.37)	0.06
After heparin	80.86 (50.83–113.64)	54.76 (43.30–122.36)	0.17

Values are given as n (%), mean±SD, or median (interquartile range). ACE indicates angiotensin-converting enzyme; AT1, angiotensin II type 1.

MPO and Elastase Plasma Levels

Whereas baseline MPO plasma levels tended to be slightly higher in CAD patients (10.72 [IR 7.89 to 13.10] ng/mL in CAD patients versus 8.93 [IR 7.66 to 10.95] ng/mL in non-CAD subjects, $P=0.1$), both groups revealed a significant increase in MPO plasma levels on heparin administration (7.05 [IR 4.17 to 9.27], $P<0.01$ for CAD patients; 4.09 [IR 1.52 to 7.44], $P<0.01$ for control subjects). The increase in MPO levels after heparin administration was significantly higher in CAD patients compared with control subjects (17.06 [IR 13.61 to 22.61] ng/mL versus 13.57 [IR 10.38 to 17.60] ng/mL, $P=0.01$). Moreover, there was a significant difference in MPO increase after heparin administration between both groups (Figure 2). To exclude the possibility that the increase in MPO was due to increased degranulation of leukocytes, circulating levels of elastase (which is stored in the same granules in polymorphonuclear leukocytes as it is in MPO) were measured. Elastase plasma levels did not increase after heparin treatment but decreased in both groups (Figure 2). This finding suggests that increased MPO plasma levels in response to heparin do not reflect increased activation/degranulation of leukocytes and supports the view that the increase in circulating MPO reflects liberation of vessel wall–associated MPO.

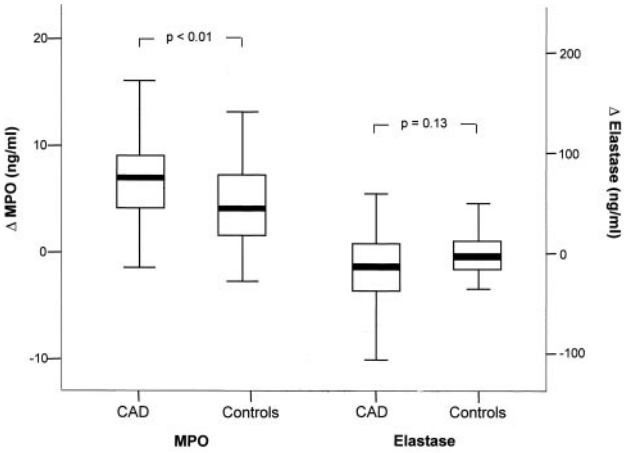

Figure 2. Effect of heparin on circulating MPO and elastase levels in patients with and without CAD. Heparin bolus addition increased MPO plasma levels in patients with and without angiographically documented CAD; however, the increase in circulating MPO on heparinization (ΔMPO) was significantly greater in CAD patients compared with patients without CAD. In contrast, no difference in elastase plasma levels was observed in CAD patients compared with patients without CAD after heparin treatment. Center line indicates median; box and whiskers, IR and 2.5 and 97.5 percentiles, respectively. Mann-Whitney *U* test assessed differences between groups.

MPO and NO Bioavailability

Given previous work identifying MPO as an enzyme capable of oxidizing endothelium-derived NO in vivo,[8] we tested the effect of vascular MPO liberation by heparin on vascular function in humans. Vascular function tests in patients were performed before and 15 minutes after the administration of heparin. ACh-induced changes in forearm blood flow, as assessed by venous occlusion plethysmography, were significantly increased after heparin injection, an effect that was completely inhibited in the presence of the NO synthase inhibitor L-NMMA (Figure 3). Endothelium-independent vasodilation in response to sodium nitroprusside was not altered in either group (not shown). The heparin-induced increase in vascular NO bioavailability and function was confirmed in conductance vessels: Forearm FMD in 18 patients (CAD, n=9; non-CAD, n=9) randomized to heparin was increased (from 6.55±4.50% to 9.15±4.03%, $P<0.01$), as opposed to those receiving NaCl (from 7.78±6.06% to 7.51±5.67%, $P=0.86$; Figure 4A). However, flow-independent dilation remained unchanged (Table 2). The increase in FMD after heparin administration was measured as a percentage and as absolute dilation and was observed in patients with and without CAD. The changes in FMD were significantly correlated with the changes in MPO plasma levels ($r=0.69$, $P<0.01$; Figure 4B and 4C, Table 3). Heparins also liberate endothelium-bound lipoprotein lipase, which increases triglyceride plasma levels and thus has an impact on the endothelial bioavailability of free fatty acids. Heparin treatment resulted in a significant decrease in triglyceride levels (for the heparin group, 90.13±33.39 mg/dL before versus 74.14±25.46 mg/dL after heparin administration; $P=0.01$). Partial correlation adjusting for changes in

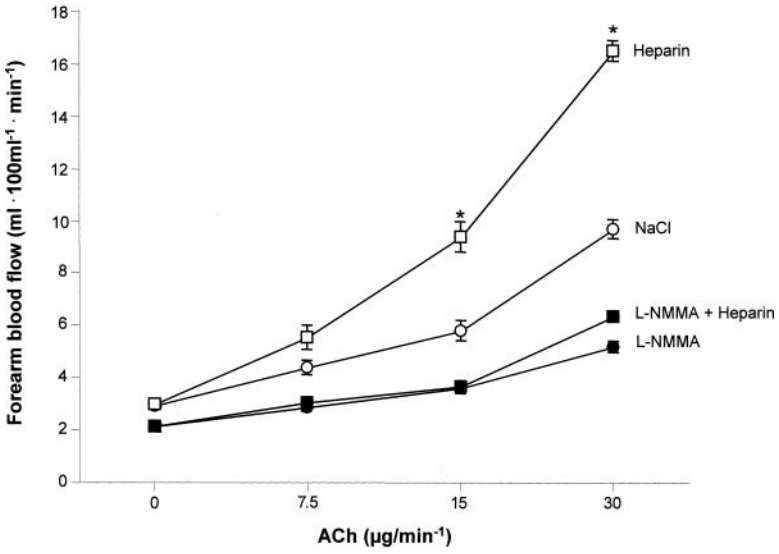

Figure 3. Heparin increases endothelial NO bioavailability. ACh-induced increase in forearm blood flow as measured by venous occlusion plethysmography was significantly increased after administration of heparin (70 U/kg body wt) *$P<0.01$ versus NaCl treatment. L-NMMA inhibited the heparin-induced increase in forearm blood flow. Data are presented as mean±SEM. To assess differences between groups, ANOVA for repeated measures with the Bonferroni method for multiple comparisons was used.

MPO plasma content excluded a significant inverse correlation between changes in triglyceride levels and changes in FMD ($r=-0.37$, $P=0.08$), whereas the correlation between changes in MPO plasma levels and vasomotor function remained significant after adjusting for triglyceride plasma levels ($r=0.59$, $P<0.01$).

Discussion

The principal findings reported in the present study are as follows: (1) Heparin mobilizes MPO from vascular compartments, (2) heparin augments endothelial NO bioavailability and improves NO-dependent vascular relaxation in vivo, and

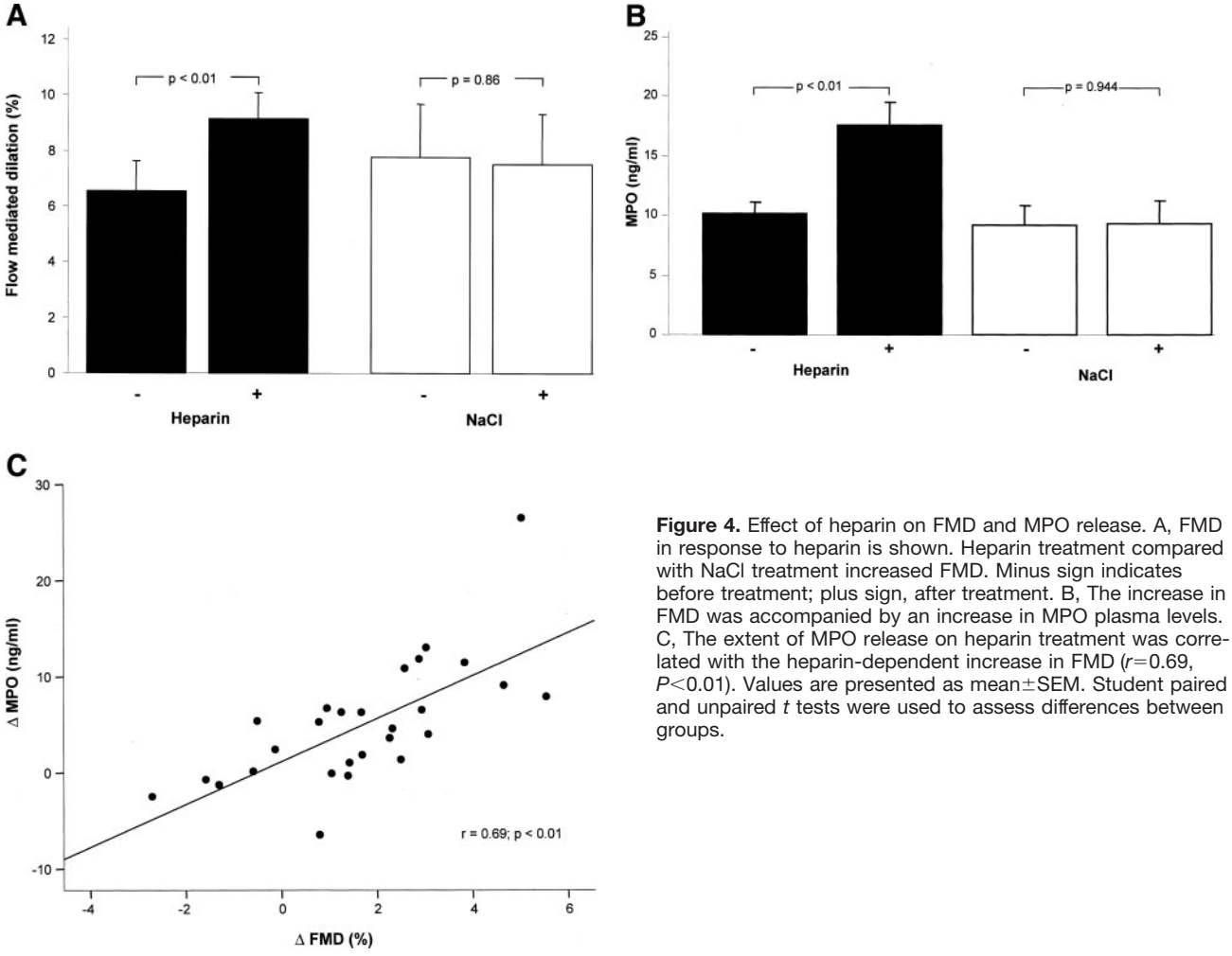

Figure 4. Effect of heparin on FMD and MPO release. A, FMD in response to heparin is shown. Heparin treatment compared with NaCl treatment increased FMD. Minus sign indicates before treatment; plus sign, after treatment. B, The increase in FMD was accompanied by an increase in MPO plasma levels. C, The extent of MPO release on heparin treatment was correlated with the heparin-dependent increase in FMD ($r=0.69$, $P<0.01$). Values are presented as mean±SEM. Student paired and unpaired *t* tests were used to assess differences between groups.

TABLE 2. MPO Plasma Levels and FMD

	Before	After	*P*
MPO, ng/mL			
Heparin	10.15±4.33	17.58±8.40	<0.01
NaCl	9.31±5.18	9.38±6.01	0.94
FMD, %			
Heparin	6.55±4.50	9.15±4.03	<0.01
NaCl	7.78±6.06	7.51±5.67	0.86
FMD, mm			
Heparin	0.28±0.18	0.39±0.15	<0.01
NaCl	0.33±0.24	0.34±0.25	0.41
Baseline diameter, mm			
Heparin	4.29±0.77	4.30±0.75	0.94
NaCl	4.60±0.66	4.69±0.64	0.05
Flow ratio			
Heparin	4.01 (2.50–5.61)	4.97 (2.04–7.43)	0.16
NaCl	4.92 (2.88–7.94)	5.41 (2.88–8.44)	0.37
NMD, %			
Heparin	12.43 (7.09–17.49)	14.40 (8.04–18.05)	0.17
NaCl	10.15 (7.92–16.55)	9.18 (7.47–16.41)	0.60
NMD, mm			
Heparin	0.49 (0.33–0.60)	0.61 (0.41–0.67)	0.14
NaCl	0.50 (0.42–0.62)	0.47 (0.36–0.63)	0.46

Values are n (%), mean±SD, or median (interquartile range).

FMD is shown in patients (n=27) who were randomized to receive either intravenous heparin or NaCl. NMD indicates nitroglycerin-mediated dilation.

(3) heparin-induced improvement in endothelial function is related to the extent of mobilized MPO.

MPO, until recently solely viewed a bactericidal enzyme, has emerged as a critical mediator of chronic inflammatory diseases, such as atherosclerosis. MPO has been shown to oxidize high-density lipoproteins (HDLs) and LDLs and to activate metalloproteinases, thereby affecting the composition and vulnerability of the atherosclerotic plaque.[11,12,18,19] MPO promotes tyrosine nitration, which has an adverse impact on the function of matrix proteins such as fibronectin, antiinflammatory enzymes such as superoxide dismutase (SOD), and coagulation factors such as fibrinogen.[20–22] Moreover, MPO exerts cytokine-like properties by modulating neutrophil activation states on binding to CD11b integrins.[23] Of importance, MPO has also been shown to oxidize endothelium-derived NO, thereby modulating redox-sensitive signaling pathways in the vessel wall[7,8] and its downstream effects on vascular reactivity. When all is considered, MPO stands out as a critical mediator affecting leukocyte, smooth muscle, and endothelial cell function. Oxidation of endothelium-derived NO by MPO has proven to be directly dependent on MPO sequestration into the subendothelial space[8] via apical to basolateral transcytosis across the endothelium. Heparins have previously been shown to prevent binding of MPO to the endothelium in cell culture studies and isolated rat aortic vessel segments.[8,10] The present study importantly adds to these findings, in that heparin not only prevented MPO binding to vascular compartments but also reversed vessel-immobilized MPO. That is, isolated matrix proteins, cultured endothelial cells, and entire vein grafts from patients with CAD revealed less MPO burden and MPO activity after exposure to heparin (Figure 1). This finding suggests deposition of MPO in a heparin-accessible compartment such as the subendothelial space, which is not only devoid of many of the competing substrates for MPO present in plasma but also particularly rich in low-molecular-weight substrates, which will enhance rather than inhibit MPO-driven NO catabolism.

Complementary to these observations, circulating plasma MPO levels in humans increased ≈1.6-fold after the administration of heparin (Figures 2 and 4B).Given the overall low recovery of MPO with the ELISA used, the extent of liberated MPO may be importantly underestimated. In contrast, plasma levels of elastase, a proteolytic enzyme, which is expressed in the same granules as MPO, did not increase on heparinization, further confirming that the elevation in MPO plasma levels reflects a release of vascular-bound MPO and is not a consequence of systemic neutrophil activation (Figure 2). Release of MPO from the vessel wall by heparin allowed for determination of the extent of vessel wall–immobilized MPO in stable CAD. The significantly higher MPO levels after heparin administration in CAD patients (Figure 2), despite nonsignificant differences in baseline plasma MPO levels between the CAD and non-CAD patients, further indicate that previous studies identifying MPO as a powerful marker of risk in CAD may even have underestimated the prognostic information obtained from this hemoprotein.[14,15] The fact that baseline MPO plasma levels only tended to be higher in CAD patients suggests that (despite increased MPO burden and MPO activity per neutrophil in patients with stable CAD[13]) activation and degranulation of MPO-containing leukocytes

TABLE 3. Association Between MPO and FMD

	Pearson Correlation Coefficient			
	MPO After Heparin	MPO Increase	FMD % Increase	FMD mm Increase
MPO after heparin	1	0.850 (<0.01)	0.618 (<0.01)	0.471 (0.01)
MPO increase	...	1	0.688 (<0.01)	0.526 (<0.01)
FMD % increase	...	...	1	0.708 (<0.01)
FMD mm increase	...	...	...	1

Values in parentheses indicate *P* values.

Changes in MPO plasma levels are positively correlated with percentage and absolute increase in flow-mediated dilation.

are not increased in patients with stable coronary disease but are restricted to patients with acute coronary syndromes.

Because MPO must be located within the vessel wall in order to interfere with NO-signaling cascades, the effect of heparin on NO-dependent vasomotor function was tested in conductance and resistance vessels of patients with and without CAD. Heparin improved microvascular function, yielding increased forearm blood flow in response to ACh, an effect that was NO specific, inasmuch as coinfusion with the NO synthase inhibitor L-NMMA blunted the effect of heparin (Figure 3). Also, heparin augmented flow in conductance vessels, as evidenced by the significant increase in FMD (Figure 4A). Inasmuch as endothelium-independent vasodilation remained unchanged, heparin may protect the endothelium from MPO-dependent NO oxidation, further supporting the pathophysiological significance of vessel-adherent MPO. In light of increased vascular deposition of MPO in patients with CAD, the association between endothelial NO bioavailability and MPO may have been thus far undervalued.[24,25]

Heparins have previously been demonstrated to exert antiinflammatory effects in vascular disease that are irrespective of their anticoagulative properties. Potential antiinflammatory properties include decreased endothelial cell activation, inhibition of platelet activation, and binding to chemokines.[26,27] Also, heparins are suggested to increase NO bioavailability in animal models of ischemia/reperfusion, sepsis, and balloon injury and in vessel segments from patients undergoing coronary artery bypass grafting; however, the underlying mechanism has remained elusive.[28–31] Liberation of NO-oxidizing enzymes such as MPO, as reported in the present study, may be an important pathophysiological link by which heparins exert antiinflammatory effects. Besides MPO, xanthine oxidase (XO) has also been shown to associate with the endothelium in a GAG-dependent manner,[32] and heparin treatment has been shown to result in increased circulating XO levels.[33] However, whereas MPO is shown to bind to heparan sulfate–based GAGs, XO predominantly binds to chondroitin sulfate–containing GAGs on the endothelial cell surface.[10,32] This suggests that heparin is more effective in releasing MPO from the vessel wall than heparin is in releasing XO; however, it is entirely possible that the 2 enzymes may function synergistically in consuming endothelium-derived NO. The ability of MPO to consume endothelium-derived NO is reinforced by the strong correlation between MPO release and improvement in endothelial function (Figure 4C), further supporting the tenet that MPO is a significant mediator of decreased vascular NO bioavailability under inflammatory conditions. The fact that changes in MPO plasma levels and improvement in endothelial function remained independent of changes in triglyceride levels further supports the tenet that the heparin-induced liberation of vessel-bound MPO reflects a critical mechanistic bond for the vasoactive properties of heparins.

Of significance, heparin also releases from the vessel wall extracellular SOD, an enzyme known to improve NO bioavailability by quenching levels of superoxide.[34] However, the robust increase in NO-mediated flow after heparin administration as reported in the present study advocates that the release of vessel wall–immobilized NO oxidases such as MPO and XO overrides the loss of NO-preserving enzyme systems such as SOD. SOD provides the principal substrate of MPO by reduction of superoxide to hydrogen peroxide (H_2O_2), whereas superoxide is known to retard the oxidation capacity of MPO; thus, the removal of SOD and depletion of an H_2O_2-generating source may in fact be beneficial and represent an additional explanation for the net increase in NO bioavailability after exposure to heparins.

The present results need to be interpreted with caution because the reported mechanism for the NO-preserving effects of heparins does not exclude additional actions of heparin yielding increased vascular NO. However, given (1) the heparin-induced liberation of endothelial and matrix-bound MPO, (2) evidence of increased MPO deposition in the vasculature of patients with CAD, and (3) the strong correlation between heparin-induced MPO release and improvement in NO-dependent vascular relaxation, mobilization of vessel wall-bound MPO appears to be an important causal link accounting for improved vascular NO bioavailability after heparin administration.

In light of the prognostic implications of impaired endothelial NO bioavailability and in the absence of any specific inhibitor for MPO to date, strategies aiming to specifically remove vessel wall–immobilized NO oxidases may represent a potential adjunct treatment strategy in patients with inflammatory vascular disease.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Myeloperoxidase (MPO), a heme enzyme abundantly expressed in neutrophils, monocytes, and macrophages, has long been solely viewed as an enzyme involved in host defense. However, recent data obtained in vitro and in animal models have revealed that MPO also modulates vascular tone by depleting the bioavailability of endothelium-derived nitric oxide (NO). A critical prerequisite for MPO to function in this regard is its binding to endothelial cells and its accumulation in the subendothelial space. In the present study, we show that heparins liberate vessel wall–immobilized MPO in humans. Compared with healthy control subjects, patients with stable coronary artery disease revealed increased vascular deposition of MPO, reflected by a higher degree of MPO mobilization after the administration of heparin. Heparin administration increased endothelial NO bioavailability in conductance and resistance vessels of patients with and without coronary disease. This heparin-dependent improvement in endothelial function was closely correlated with the extent of MPO liberation. In summary, these data underscore the significance of vessel wall–immobilized MPO for the regulation of endothelium-derived NO in patients with chronic inflammatory disease and point toward treatment strategies aiming to distract leukocyte-derived NO oxidases from the vascular bed.

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The potentiation of myeloperoxidase activity by the glycosaminoglycan-dependent binding of myeloperoxidase to proteins of the extracellular matrix

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ABSTRACT

Background: Myeloperoxidase (MPO) is an abundant hemoprotein expressed by neutrophil granulocytes that is recognized to play an important role in the development of vascular diseases. Upon degranulation from circulating neutrophil granulocytes, MPO binds to the surface of endothelial cells in an electrostatic-dependent manner and undergoes transcytotic migration to the underlying extracellular matrix (ECM). However, the mechanisms governing the binding of MPO to subendothelial ECM proteins, and whether this binding modulates its enzymatic functions are not well understood.
Methods: We investigated MPO binding to ECM derived from aortic endothelial cells, aortic smooth muscle cells, and fibroblasts, and to purified ECM proteins, and the modulation of these associations by glycosaminoglycans. The oxidizing and chlorinating potential of MPO upon binding to ECM proteins was tested.
Results: MPO binds to the ECM proteins collagen IV and fibronectin, and this association is enhanced by the pre-incubation of these proteins with glycosaminoglycans. Correspondingly, an excess of glycosaminoglycans in solution during incubation inhibits the binding of MPO to collagen IV and fibronectin. These observations were confirmed with cell-derived ECM. The oxidizing and chlorinating potential of MPO was preserved upon binding to collagen IV and fibronectin; even the potentiation of MPO activity in the presence of collagen IV and fibronectin was observed.
Conclusions: Collectively, the data reveal that MPO binds to ECM proteins on the basis of electrostatic interactions, and MPO chlorinating and oxidizing activity is potentiated upon association with these proteins.
General significance: Our findings provide new insights into the molecular mechanisms underlying the interaction of MPO with ECM proteins.

1. Introduction

Neutrophil granulocytes, the largest group of polymorphonuclear leukocytes, are among the key cells active in host defense against invading pathogens. However, they also present a potential risk to the host by increasing oxidative burden. Myeloperoxidase (MPO), a highly abundant hemoprotein expressed mainly by neutrophil granulocytes, is thought to play a primary role in host defense [1–3]. MPO is also perceived as a critical mediator of inflammatory tissue injury. The significant pathological importance of MPO is recognized in chronic vascular diseases such as atherosclerosis and coronary artery disease. This is based on the observation that MPO mediates oxidative damage within the vessel wall initiating and promoting vessel remodeling, the

Abbreviations: ABTS, 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; BAECs, bovine aortic endothelial cells; BSA, bovine serum albumin; CTAC, cetyltrimethylammonium chloride; DMF, N,N-dimethylformamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic) acid; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GAGs, glycosaminoglycans; HRP, horseradish peroxidase; MCD, monochlorodimedon; MPO, myeloperoxidase; OD, optical density; PBS, phosphate buffered saline; RASMCs, rat aortic smooth muscle cells; RT, room temperature; SEM, standard error of the mean; TMB, 3,3′,5,5′-tetramethylbenzidine; TNB, 5-thio-2-nitrobenzoic acid

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development of atherosclerotic lesions and endothelial dysfunction [1–4]. The most typically recognized mechanisms that are mediated by MPO and contribute to the development of these pathological processes are the catalysis of low-density lipoprotein oxidation, the oxidation of proteins altering their biological function, and the catabolism of nitric oxide contributing to endothelial dysfunction [2,3,5–7]. The particular importance of MPO in the impairment of vascular function is suggested for oxidative damage to vessel wall extracellular matrix [4,8–10]. Further, the pathological importance of MPO is supported by evidence that circulating levels of MPO are highly predictive of future vascular events associated with chest pain and acute coronary syndromes which correlate with overall patient outcome [1,3]. However, the overall picture of how MPO-driven pathological changes lead to the promotion of cardiovascular diseases is still incomplete.

During neutrophil granulocyte activation, MPO is released into the phagosome and the extracellular space where it can catalyze the production of a multitude of reactive species including hypochlorous acid, *N*-chloramines, reactive nitrogen species, and other oxidants [2–4,6,7]. Because MPO and markers of its enzymatic activity are generally increased at inflammatory foci, this pathway is thought to contribute to oxidant-dependent alterations in vascular and organ function during inflammation. For decades, an accumulation of MPO at inflammatory foci has been considered to be an index of inflammation, with increased tissue MPO activity thought to reflect neutrophil extravasation. However, the alternative process of MPO deposition within vascular tissue, independent of neutrophil diapedesis, was also shown [11–16]. Following the intraluminal release of highly cationic MPO, electrostatic interactions with the negatively charged endothelial plasma membrane are suggested to facilitate the binding of MPO with the vascular endothelium. Further, MPO can undergo apical to basolateral transcytosis across the vascular endothelium [8,11,14,15]. Immunohistochemical analysis of vascular MPO distribution revealed endothelial-associated MPO immunoreactivity on the endothelial surface, within endothelial cells, and in the extracellular matrix (ECM) of intima that was localized between the endothelial layer and the smooth muscle cell composed medial layer [11,17]. This ECM is synthesized by overlying endothelial cells forming a "basement membrane", a highly complex structure composed of various structural proteins, glycoproteins and proteoglycans including more abundantly present components such as collagen IV, laminins, nidogen/entactin, and perlecan, and less abundant components such as fibronectin [4,8,10,18]. The importance of MPO oxidative modification of the basement membrane structure was suggested by various authors particularly by authors Rees and Davies with their colleagues, who reported the MPO-dependent oxidation of extracellular matrix components particularly glycosaminoglycans (GAGs) [4,8–11,19–23].

The interaction of MPO with various types of plasma proteins including ceruloplasmin, apolipoproteins, and albumin has been demonstrated [14,24–28]. Interestingly, interactions of MPO with the ECM proteins perlecan and fibronectin were also reported [8,10]. The interaction of highly basic (cationic) MPO with other proteins has been suggested to be mostly dependent on electrostatic interactions, similar to the interaction of MPO with the negatively charged surfaces of endothelial cells or polymorphonuclear leukocytes [11–13,15,29]. In the case of MPO binding to the endothelium, the interaction was found to be dependent upon surface GAGs, since the exposure of MPO to an excess of external GAGs such as heparin, or a reduction in the surface presence of heparan profoundly reduced MPO binding to the endothelium and the consequent MPO transcytosis [11–13,15]. Interestingly, some proteins of subendothelial ECM or vessel wall ECM are, in general, proteoglycans or glycoproteins since a part of their molecules are sulfated anionic polysaccharides such as heparan sulfate [4,8,10,18].

It is well documented that MPO interaction with other biomolecules can modulate MPO activity. For example, the interaction of MPO with the plasma protein ceruloplasmin significantly decreases MPO activity [24,27,28]. Similarly, a decrease in MPO activity was reported after interaction with unfractioned and low molecular weight heparin [13]. In general, there is evidence that enzymatic activity of MPO sequestered in the subendothelial layer is preserved since products of MPO-dependent oxidation can be detected after transcytosis of MPO [8,11,13,17]. On the other side, studies evaluating the effects of MPO-derived oxidants on the proteoglycan perlecan and glycoprotein fibronectin reported significant modulation of these molecules by MPO [8,10]. However, the effects of MPO interaction with components of ECM on MPO activity are still not fully understood.

Overall, the fate of MPO sequestered from circulation by endothelial cells remains poorly understood in spite of clinical evidence of MPO localization within the subendothelial space and the suggested pathological consequences of oxidative damage mediated by MPO within the vessel wall. Further, since denudation of endothelial cells can occur as a consequence of acute inflammatory processes in the vessels, and the concomitant secretion of MPO along with an increase in plasma MPO levels can be expected, the exposed sub-endothelial matrix may be a likely direct target for MPO interaction. Thus, the aim of this study was to clarify the association of MPO with ECM proteins and to determine whether this association modulates MPO enzymatic activity.

2. Materials and methods

2.1. Chemicals and reagents

M199 and DMEM cell culture media were from Gibco (Invitrogen Corporation, CA, USA). Super Calf Serum and Fetal Bovine Serum were from Gemini Bio-Products (CA, USA). Purified MPO from human leukocytes was from Merck/Calbiochem (CA, USA) and from Planta Natural Products (Vienna, Austria). Chondroitin sulfate A from bovine trachea and polyclonal rabbit anti-human antibodies against MPO were from Merck/Calbiochem. Monoclonal mouse anti-human MPO antibodies were from Biomeda (CA, USA). Collagen type IV was from BD Biosciences (MA, USA). Trypsin/EDTA, penicillin/streptomycin, fibronectin from human plasma, heparin sodium salt from porcine intestine mucosa (207 IU/mg), alkaline phosphatase-conjugated anti-rabbit IgG antibodies, and mouse anti-fibronectin antibodies were from Sigma Chemical Co. (MO, USA). All other reagents were from Sigma Chemical Co. (MO, USA) or Fisher Scientific (CA, USA).

2.2. Cell cultures

Human aortic endothelial cells (HAECs) (Lonza, Germany) were maintained in complete Endothelial Cell Growth Medium-2 medium (Lonza). Bovine aortic endothelial cells (BAECs) from the European Collection of Cell Cultures (Health Protection Agency Culture Collections, UK) were maintained in medium 199 with 5% fetal bovine serum, 5% super calf serum, 25 U/ml penicillin, and 25 mg/ml streptomycin. Murine 3T3 fibroblasts and rat aortic smooth muscle cells A10 (RASMCs) were obtained from the American Type Culture Collection (USA) and were grown in DMEM containing 10% fetal bovine serum, 25 U/ml penicillin, and 25 mg/ml streptomycin. Cells were maintained at 37 °C in 5% CO_2 and the culture medium was changed every 2 days.

2.3. Isolation of cell-derived ECM proteins

Confluent cell cultures were maintained in microtiter plates for 6–12 days, with fresh medium being added every 2 days. Cells were washed with phosphate buffered saline (pH 7.4) (PBS) and gently lysed with PBS buffer containing Triton X-100 (0.5% v/v) and ethylenediaminetetraacetic acid (EDTA) (10 mM) at room temperature (RT) for 20 min. The extent of cell lysis was monitored periodically by microscopy, which revealed an immediate and complete loss of cell structure. Upon lysis of the cells, the remaining ECM was washed 5 times with PBS. Finally, the isolated matrix proteins were

blocked with PBS containing 5% bovine serum albumin (BSA) for 1 h. After a further four washes with PBS, the plates were immediately used for binding assays.

2.4. Binding of MPO to ECM

ECM proteins, isolated as described above, were exposed to various concentrations of purified human MPO (100 μl of 0.8–13.3 nM MPO in PBS) at RT for 1 h. Then, the wells were washed four times with Tris buffer containing 0.05% (v/v) Tween 20 (Tris/Tween) to remove unbound MPO. In some experiments, various concentrations of sodium heparin and chondroitin sulfate (100 μl of 0.05–10 mg/ml in PBS) were pre-incubated with the isolated ECM for 1 h at RT prior to the addition of MPO (100 μl of 6.6 nM MPO in PBS). In some cases, MPO (50 μl of 13.3 nM MPO in PBS) was incubated in the wells together with various concentrations of sodium heparin and chondroitin sulfate (50 μl of 0.1–20 mg/ml in PBS). ECM was exposed to these compounds after blocking with 5% BSA in PBS.

2.5. Binding of MPO to fibronectin and collagen IV by modified ELISA

Microtiter plates were coated with various concentrations of fibronectin and collagen IV (50 μl of 0.425–42.5 μg/ml, approx. 2.3–236.1 nM collagen IV and approx. 0.9–94.4 nM fibronectin) overnight. The wells were washed with PBS and were blocked with 1% BSA in PBS for 1 h at RT. After further four washes with PBS, the plates were immediately used for binding assays. Fibronectin and collagen IV coated plates were exposed to various concentrations of MPO (50 μl of 0.33–6.6 nM MPO in PBS). In some cases, coated wells were incubated with MPO isolated from leukocytes (50 μl of 6.6 nM), recombinant MPO (R&D Systems, USA) (50 μl of 6.6 nM), and 50 μl of conditioned buffer obtained from isolated human polymorphonuclear leukocytes (isolated as described previously [31]) activated by opsonized zymosan (OZP) (prepared as described previously [30]) with final MPO concentration 5.4 nM, which was determined by ELISA (CardioMPO kit, PrognostiX, USA). The binding of MPO was allowed to proceed for 1 h at RT.

In some cases, MPO (50 μl of 6.6 nM MPO in PBS) was incubated in the fibronectin (4.72 pmol per well) or collagen IV (11.8 pmol per well) coated wells together with various concentrations of sodium heparin and chondroitin sulfate (50 μl of 0.1–20 mg/ml in PBS). In some cases, various concentrations of sodium heparin and chondroitin sulfate (100 μl of 0.05–10 mg/ml in PBS) were pre-incubated in fibronectin (4.72 pmol per well) or collagen IV (11.8 pmol per well) coated wells prior to the addition of MPO (100 μl of 3.3 nM MPO per well). In some cases, plates coated with fibronectin (4.72 pmol per well) or collagen IV (11.8 pmol per well) and blocked with 5% BSA were incubated with MPO (100 μl of 3.3 nM MPO) for 1 h at RT. The wells were then washed and exposed to various concentrations of sodium heparin and chondroitin sulfate (100 μl of 0.05–10 mg/ml in PBS) for 1 h at RT.

After the MPO binding experiments, MPO protein levels were determined by modified ELISA based on an optimized protocol used for the determination of MPO in human plasma as described previously [32]. Plates were washed four times with Tris/Tween, and an antibody to MPO was added in Tris/Tween buffer with 1% BSA (Ab dilution 1:500) for 2 h at RT. Next, the plates were washed four times with Tris/Tween and incubated with alkaline phosphatase-conjugated anti-rabbit IgG in Tris/Tween buffer with 1% BSA (Ab dilution 1:2500) for 1 h at RT. The plates were then developed with p-nitrophenyl phosphate for 20–40 min, the stop solution added (1 mM EDTA in 1 M NaOH) and the absorbance read at 405 nm using a PowerWave$_x$ UV–vis plate reader (Bio-Tek Instruments, USA).

In parallel, to confirm the coating efficiency by collagen IV and fibronectin, total protein concentrations in wells were determined by means of micro BCA protein assay kit (Roche, Germany) before the blocking with BSA. The adsorbed proteins were extracted with 50 μl of PBS containing 0.5% (w/v) SDS under rapid shaking at RT for 30 min. Extracts from wells were pooled to collect a sufficient volume for the assay. Diluted collagen IV and fibronectin were used for the calibration curve. Controls to determine the reliability of the assay (background signal and protein recovery) were carried out in parallel. Quantification limits were 0.5 μg/ml.

2.6. Association of collagen IV and fibronectin with MPO detected by native gel electrophoresis

The associations of MPO with collagen IV and fibronectin and with protamine and BSA as controls were determined by means of an imidazole–HEPES continuous native gel at pH 7.4 with in-gel peroxidase staining according to Salavej with slight modifications [26]. Similarly, the effects of cetyltrimethylammonium chloride (CTAC) on the complex formation of MPO with collagen IV were determined. Briefly, MPO (10 μl of 1.33 μM MPO) was incubated with selected combinations of 10 μl of collagen IV (1.2, 2.0, 3.0 μM) or 10 μl of fibronectin (0.48, 0.80, 1.20 μM) or 10 μl of protamine (36 μM) or BSA (4.1 μM) or 10 μl of CTAC (0.4 mM) and for 20 min at RT. Then, samples were loaded onto 7% imidazole–HEPES gels (pH 7.4) and run for 3 h at 100 V. The gels were incubated for 10 min in buffer A (10 mM sodium citrate and 10 mM EDTA, pH 5.0) and for 15 min in buffer B (buffer A containing 10% dextran sulfate) at RT. The gels were washed three times with buffer A for 5 min each time. Next, the gels were incubated in buffer C [buffer A containing stabilized commercial 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sdt-reagents, Germany) 1:1] for 30 min at RT to detect peroxidase activity. The developed gel staining was recorded by a scanner (Canon Scan 8800F, Canon).

2.7. MPO-catalyzed oxidation of TMB or guaiacol

MPO-dependent oxidizing activity was determined by measuring the rate of H_2O_2-dependent oxidation of TMB or guaiacol. Reaction mixtures of MPO (50 μl of 13.3 nM MPO) with fibronectin or collagen IV (50 μl of 0.5–50 μg/ml) in PBS were incubated for 1 h at RT prior to the determination of MPO oxidation activity. A reaction mixture containing only the highest concentration of fibronectin or collagen IV (50 μg/ml) without MPO was also included.

The reaction mixtures were then mixed with sodium acetate buffer (300 mM, pH 5.6, containing 1.2 mM TMB prepared in N, N-dimethylformamide (DMF)) and 300 μM H_2O_2. The rate of TMB oxidation was monitored at 655 nm over 2 min at RT on a PowerWave$_x$ UV–vis plate reader. Rates are expressed as the ΔOD at 655 nm/min. In the case of guaiacol, the samples were mixed with PBS containing guaiacol (30 mM) and H_2O_2 (100 μM) and the rate of guaiacol oxidation was monitored at 470 nm over 2 min at RT on a PowerWave$_x$ UV–vis plate reader. Rates are expressed as the ΔOD at 470 nm/min.

2.8. MPO-catalyzed chlorination activity

Monochlorodimedon (MCD) or taurine in conjunction with 5-thio-2-nitrobenzoic acid (TNB) was used to determine the chlorination activity of MPO.

Chlorination of MCD results in dichlorodimedon formation and causes a change in absorbance at 290 nm [33]. Reaction mixtures of MPO (50 μl of 13 nM MPO) with fibronectin or collagen IV (50 μl of 0.5–50 μg/ml) in PBS were incubated for 1 h prior to the determination of MPO oxidation activity. MCD (40 μM) was dissolved in PBS and mixed with 100 μM H_2O_2. Chlorination reactions were initiated by the addition of this mixture to MPO-containing samples, and the rate of chlorination was monitored as a decrease of OD at 290 nm over 2 min on a PowerWave$_x$ UV–vis plate reader. Rates are expressed as ΔOD at 290 nm/min.

The chlorination of taurine gives rise to taurine chloramine, which is in turn capable of oxidizing TNB. This is accompanied by the loss of OD at 412 nm [33]. TNB was prepared by reduction of 5,5′-dithiobis(2-nitrobenzoic) acid (DNTB) with mercaptoethanol [34]. As above, MPO was incubated with 50 μl of fibronectin (67 nM) or collagen IV (167 nM) in PBS for 1 h. Then, a solution of taurine in PBS (80 μl 12.5 mM) was added and the reaction was started with H_2O_2 (20 μl of 800 μM). After 3 min the reaction was stopped by the addition of 200 μg of catalase and by placing the mixture into an ice bath. TNB (20 μl of 2.5 mM) was added and the absorbance at 412 nm was read. To calculate the HOCl production, a stoichiometry 1:2 of HOCl to TNB was assumed [33].

2.9. MPO-catalyzed degradation of H_2O_2

The MPO-dependent degradation of H_2O_2 was monitored using an electrochemical H_2O_2 selective sensor (ISO-HPO-2, WPI). A mixture of MPO (5 nM) and collagen IV (167 nM) was incubated in a total volume of 100 μl of PBS at RT for 1 h prior to the determination of MPO-dependent H_2O_2 degradation. PBS and diluted hydrochloric acid served as control treatments. The mixtures were then combined with 80 μl of 1.25 mM methionine in sodium acetate buffer (300 mM, pH 5.6). The mixtures were stirred and kept at 25 °C in a water bath. Reactions were started by the addition of 20 μl of 800 μM H_2O_2 in sodium acetate buffer (300 mM, pH 5.6) The degradation of H_2O_2 was monitored after 60 s for 200 s.

2.10. Kinetic parameters of collagen IV-mediated modulation of MPO peroxidation activity

The oxidation of 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was employed to determine the effect of collagen IV on the peroxidase activity of MPO or horseradish peroxidase (HRP). MPO (10 nM) or HRP (10 nM) was mixed with collagen IV (17, 56, and 167 nM) in a total volume of 100 μl of PBS and incubated at RT for 1 h. PBS and diluted hydrochloric acid served as control treatments. At the end of the incubation, ABTS (1 mM, in 300 mM sodium acetate buffer, pH 5.6 or in PBS buffer, pH 7.4) was added. The reaction was started by the addition of 50 μl of 320 μM H_2O_2 in 300 mM sodium acetate buffer or in PBS buffer. ABTS oxidation was monitored at 422 nm with a reference at 480 nm in a microplate reader (Infinite M200, Tecan) at 25 °C. Reaction rates were determined over a 145 s interval. Kinetic parameters were calculated using Hanes–Woolf plots.

2.11. MPO binding to rat aortic wall

Descending thoracic aortas were excised from rats. In some cases, the endothelium was removed (denuded) by cotton swab before vessel exposure to MPO. Both normal and denuded aortic segments were infused with MPO (13 nM) in PBS or PBS alone (control) for 30 min or 1 h at RT. Unbound MPO was removed by thoroughly washing the rings 5 times in PBS. Aortic segments were further used for the determination of MPO distribution combined with determination of MPO activity. For the determination of the MPO catalyzed formation of nitrotyrosine the aortic rings were incubated with $NaNO_2$ (100 μM) and H_2O_2 (50 μM) in PBS for 120 min at 37 °C, washed and frozen. For determination of MPO catalyzed dityrosine formation, aortic segments were incubated with H_2O_2 and Alexa 594 labeled tyramide for 30 min in amplification buffer according to the manufacturer's protocol for the Tyramide Signal Amplification Kit (Molecular Probes, Inc., Eugene, Oregon, USA), washed and frozen. Frozen aortas were sectioned. Sections were fixed in methanol and blocked in 10% goat serum in PBS for 2 h at 4 °C.

MPO distribution was visualized using rabbit polyclonal anti-human MPO or mouse monoclonal anti-human MPO antibodies. Fibronectin and nitrotyrosine distribution was visualized with mouse anti-fibronectin and mouse anti-nitrotyrosine antibodies, respectively. Antibodies were diluted 1:500 in PBS/10% goat serum and incubated on slides for 1 h at 4 °C. The secondary antibodies were Alexa 488 or Alexa 594 conjugated goat anti-rabbit or anti-mouse antibodies (Invitrogen, USA) diluted 1:1000 in PBS/10% goat serum.

Nuclei were counterstained with propidium iodide (0.1 μg/ml) or DAPI (1 μg/ml). Images of MPO and nitrotyrosine localization were acquired on a Zeiss LSM 5 Pa Laser Scanning Confocal Microscope (Carl Zeiss Inc., Oberkochen, Germany). Dityrosine localization was acquired using an Axiocam HRC digital camera on an Axiovert 2000 M epifluorescent microscope (Carl Zeiss Inc.).

3. Results

3.1. Sequestration of MPO by ECM derived from sub-endothelial vessel matrix and cultured cells

Primarily, to confirm the suggested phenomenon that MPO can bind to vascular endothelium the isolated rat aortas were incubated with MPO. Immunohistochemical analysis confirmed the presence of MPO within the endothelium and underlying layers (Fig. 1B upper panel) in aortic tissues incubated with MPO in contrast to aortic tissues incubated under identical conditions in the absence of MPO (Fig. 1A upper panel). Further, the determination of fibronectin (Fig. 1A and B middle panels) also confirmed previously reported [11,17] and similar localization of fibronectin and transcytosed MPO in aortic wall endothelium (Fig. 1B lower panel).

Since denudation of the vessel from endothelial cells can occur concomitantly with MPO secretion during inflammatory processes, the exposed sub-endothelial matrix may be a likely target for MPO. Thus, we first examined the binding of MPO to endothelium-denuded rat aortic vascular tissue. The immunohistochemical analysis revealed that MPO binds avidly to the luminal aspect of endothelium-denuded aortic tissue within the sub-endothelial matrix (Fig. 2A, lower panel). Staining for MPO was absent in endothelium-denuded aortic tissues incubated under identical conditions in the absence of added MPO (Fig. 2A, upper panel). As shown in Fig. 2B, MPO bound to endothelium-denuded aortic vessels colocalizes with the ECM protein fibronectin, further demonstrating its colocalization with the subendothelial matrix.

To further confirm that MPO binds to ECM proteins produced by cells from the vessel wall, experiments were conducted with ECM isolated from various vessel wall cell types. Primarily, it was shown that MPO isolated from human leukocytes binds dose dependently to ECM isolated from HAECs (Fig. 3A). To exclude the possibility that this can be an artifact of MPO isolated from leukocytes, similar experiments were performed employing human recombinant MPO, which showed dose dependent binding to ECM isolated from HAECs (Fig. 3A). Finally, the conditioned buffer obtained from OZP activated human polymorphonuclear leukocytes (MPO concentration at 5.4 nM determined by ELISA) was tested and confirmed extensive binding of MPO to isolated ECM (OD at 405 nm; 0.858 ± 0.029 compared to control without MPO 0.108 ± 0.015; mean ± SEM) (compared to Fig. 3A). Further, MPO also bound in a concentration-dependent manner to ECM isolated from other cell types such as BAECs, NIH 3T3 mouse fibroblasts, and RASMCs suggesting that MPO binding to ECM is not specific to ECM isolated from HAECs (Fig. 3B). In all cases, wells coated with BSA as a blocking agent served as a control and did not considerably bind MPO. These data demonstrate that ECM in the sub-endothelial matrix of vascular tissues and ECM derived from cultured cells can sequester MPO. Data suggest that this phenomenon is not specific to ECM derived from aortic endothelium; such MPO binding can be general for ECM produced by various cell types contributing to the ECM synthesis of vessel walls.

3.2. Role of GAGs in the binding of MPO by ECM

Since many proteins deposited within the ECM are attached to GAGs, we hypothesized that GAGs may facilitate the binding of MPO

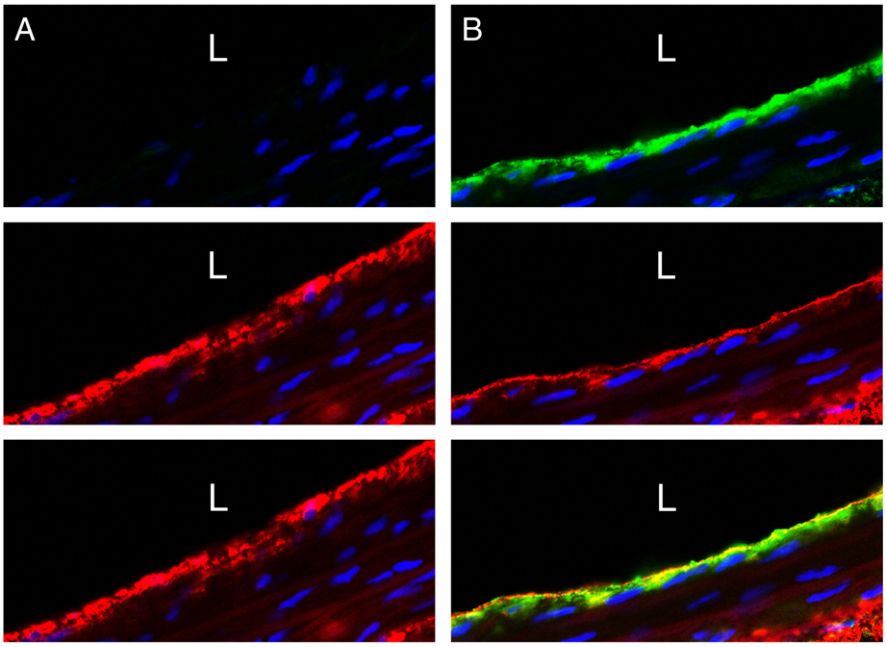

Fig. 1. MPO association with vascular endothelium. Intact rat aortic segments were infused with PBS as a control (A) or with MPO (13 nM in PBS) (B), incubated for 1 h at RT and processed for immunohistochemical staining. MPO (green signal, upper panel) in vessel segments was determined together with fibronectin (red, middle panel). Nuclei were counterstained with DAPI (blue). Colocalization of the signals for MPO and fibronectin is shown in the lower panel. "L" depicts the blood vessel lumen. Images were obtained with objective 63×. The figures represent typical pictures from at least 3 independent repeats.

to ECM. To test this hypothesis, ECM derived from BAECs, RASMCs, and NIH 3T3 fibroblasts was coated with sodium heparin or chondroitin sulfate prior to the addition of MPO. Sodium heparin and chondroitin sulfate were chosen as readily available model GAGs. Under these conditions, sodium heparin markedly increased the quantity of MPO sequestered by cell-derived ECM (Fig. 4A). In contrast, cell-derived ECM coated with chondroitin sulfate did not substantially affect the capacity of cell-derived ECM to sequester MPO (Fig. 4B). To determine whether an excess of sodium heparin or chondroitin sulfate could negatively influence MPO binding to ECM, MPO was incubated simultaneously with increasing concentrations of sodium heparin or chondroitin sulfate and ECM. An excess of sodium heparin or chondroitin sulfate substantially decreased the quantity of MPO bound to cell-derived ECM (Fig. 4C and D). These results establish that the binding of MPO to cell-derived ECM proteins can be modulated by GAGs sodium heparin and to a lesser extent chondroitin sulfate.

3.3. Binding of MPO to purified fibronectin and collagen IV

To further understand the nature of MPO binding to ECM proteins, the binding of MPO to the ECM proteins fibronectin and collagen IV was examined. MPO bound in a dose-dependent manner to both fibronectin- and collagen-coated microplate wells (Fig. 5A and B). To exclude the possibility that this could be an artifact of MPO isolated from leukocytes, similar experiments were performed to compare the binding of human MPO isolated from leukocytes, human recombinant MPO, and the conditioned buffer obtained from activated human polymorphonuclear leukocytes. The results confirmed the binding of MPO from all tested sources to fibronectin and collagen IV (Fig. 5C and D).

The interaction of MPO with fibronectin and collagen IV was confirmed by native gel electrophoresis, where a significant reduction in MPO mobility was observed in the presence of fibronectin and collagen IV (Fig. 6A). Interestingly, in this case, BSA revealed a similar effect. As a negative control, positively charged protamine was employed, which even potentiated the migration of MPO (Fig. 6A). CTAC, a positively charged detergent, inhibited complex formation between MPO and collagen IV (Fig. 6B).

Consistent with studies using cell-derived ECM, incubation of fibronectin or collagen IV coated wells with sodium heparin considerably increased MPO binding to these proteins (Fig. 7A). In contrast to isolated cell-derived ECM (Fig. 4B), chondroitin sulfate also extensively increased MPO binding to coated fibronectin or collagen IV (Fig. 7B). In addition, and consistent with results obtained with cell-derived ECM, excess sodium heparin or chondroitin sulfate in the buffer extensively decreased the amount of MPO bound to both fibronectin or collagen IV (Fig. 7C and D).

To gain an insight into the relative affinity with which MPO binds to cell-derived ECM, we determined whether MPO can be displaced from ECM by GAGs. As shown in Fig. 8A and B, pre-bound MPO was displaced from fibronectin or collagen IV-coated wells by both sodium heparin and chondroitin sulfate in a dose-dependent manner.

Similar results using GAGs were observed with ECM-derived from cultured cells (data not shown). These results further support the view that the binding of MPO to prototypical ECM proteins may be dependent upon, and modulated by negatively charged GAGs.

3.4. Binding of MPO to fibronectin or collagen IV increases its oxidizing and chlorinating activity

Next we explored whether the binding of MPO to ECM proteins modulates its enzymatic activity. Firstly, the incubation of MPO in solution with collagen IV or fibronectin dose dependently increased its capacity to oxidize classical heme peroxidase substrates in an acidic environment (pH 5.6) as revealed with TMB (Fig. 9A) as well as under physiological conditions (pH 7.4), which was demonstrated with guaiacol (Fig. 9B). In all cases for all substrates, the reaction mixtures containing only fibronectin or collagen IV (50 μg/ml) without MPO did not reveal any measurable change in OD during the measured period (data not shown). Collagen IV and fibronectin also marginally increased MPO-catalyzed chlorination reactions determined with monochlorodimedon (MCD) under physiological conditions (pH 7.4) (Fig. 9C). The assay for chlorination activity by means of the taurine/TNB method under physiological conditions (pH 7.4) showed similar potentiation of MPO catalytic activity, since the reaction rates of reactions in the presence of collagen IV (2.266 ±

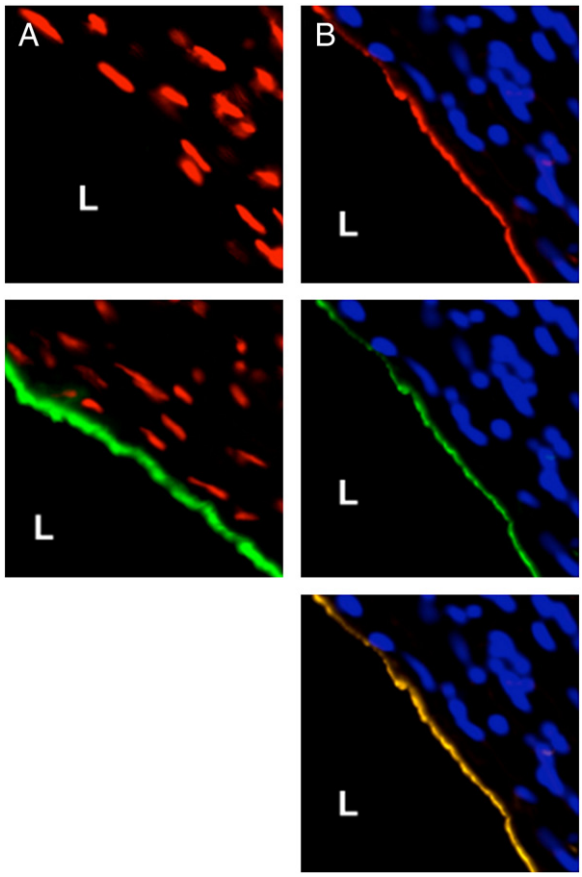

Fig. 2. MPO association with ECM in denuded aortic tissue. Denuded rat aortic segments were infused with PBS with or without MPO (13 nM), incubated for 30 min at RT and processed for immunohistochemical staining. (A) Endothelium-denuded vessels incubated without MPO (the upper panel) and with MPO (the lower panel) are shown. MPO is depicted as a green signal; cell nuclei are counterstained with propidium iodide (red). (B) MPO (red, upper panel) in endothelium-denuded vessel segments incubated with MPO was detected together with fibronectin (green, middle panel) and signals were colocalized (lower panel). Nuclei were counterstained with DAPI (blue). "L" depicts the blood vessel lumen. Images were obtained with objective 63×. The figures represent typical pictures from at least 4 independent repeats.

0.046 nmol HOCl/min) and in the presence of fibronectin (2.109 ± 0.063 nmol HOCl/min) were both significantly faster compared to the control MPO reaction in the absence of these proteins (1.852 ± 0.062 nmol HOCl/min) (mean ± SEM, n = 9). Further, the determination of direct hydrogen peroxide consumption detected by electrochemical methods also confirmed the increased rate of degradation of hydrogen peroxide in the presence of collagen IV (Fig. 9D).

To analyze this interesting effect in more detail, the reaction rate, Km values, and Vmax were calculated for the oxidation of ABTS in the presence of collagen IV. In an acidic environment (pH 5.6), the capacity of MPO to oxidize ABTS (the reaction rate) was increased in the presence of collagen IV (Fig. 10A), which was connected with an increase in Vmax (Fig. 10B). Interestingly, Km values for this reaction were also increased in presence of collagen IV (Fig. 10C). Next, the same reactions were carried out under physiological conditions (pH 7.4). In this case, the presence of collagen IV increased the capacity of MPO to oxidize ABTS as well (Fig. 10D). However, the increases in the Vmax and Km of the reactions were observed only with lower concentrations of collagen IV and did not reach statistical significance (Fig. 10E and F). Control samples with hydrochloric acid diluted in PBS showed essentially the same results as PBS (not shown). Similar experiments employing HRP did not reveal any significant effects of the presence of collagen IV on HRP catalyzed oxidation of ABTS (neither Vmax nor Km) (data not shown).

3.5. MPO sequestered by the sub-endothelial matrix catalyzes the nitration and oxidative dimerization of tyrosine

Having observed that MPO binds avidly to ECM proteins in endothelium-denuded rat aortic tissues and that the enzymatic activity of MPO is retained when sequestered within ECM proteins, we next asked whether MPO could locally catalyze protein nitration and oxidation reactions within the subendothelial ECM of rat aortic tissue denuded of endothelium. Endothelial-denuded vessels were pre-incubated with MPO and then with nitrite or tyramide in the presence of H_2O_2 to observe the formation of nitrotyrosine and protein-associated tyramide. The products of peroxidase catalyzed oxidation and nitration reactions *i.e.* nitrotyrosine (Fig. 11B) and protein-associated tyramine (Fig. 11D) were detectable only in the case of denuded aortic segments incubated with MPO that was proven by immunohistochemical analysis.

4. Discussion

The data presented here indicate that MPO is sequestered within the ECM in a manner modulated by GAGs. This was shown particularly for two ECM proteins, fibronectin and collagen IV. MPO enzymatic activity is preserved when localized within the proteins of the

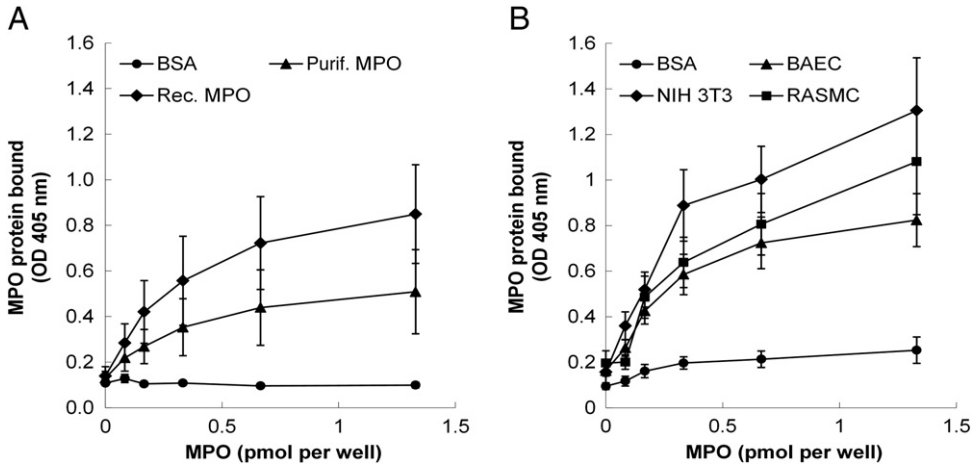

Fig. 3. MPO associates with ECM derived from cultured cells *in vitro*. (A) Binding of MPO isolated from leukocytes and of recombinant MPO to isolated ECM derived from HAECs. (B) Binding of MPO isolated from leukocytes to ECM derived from BAECs, NIH 3 T3 fibroblasts, and RASMCs. In all cases, isolated ECM was incubated with MPO (0.08–1.3 pmol per well) in PBS for 1 h at RT and MPO content was determined by modified ELISA after extensive washing. Wells coated with BSA served as a control. Values represent the mean ± SEM (n = 4).

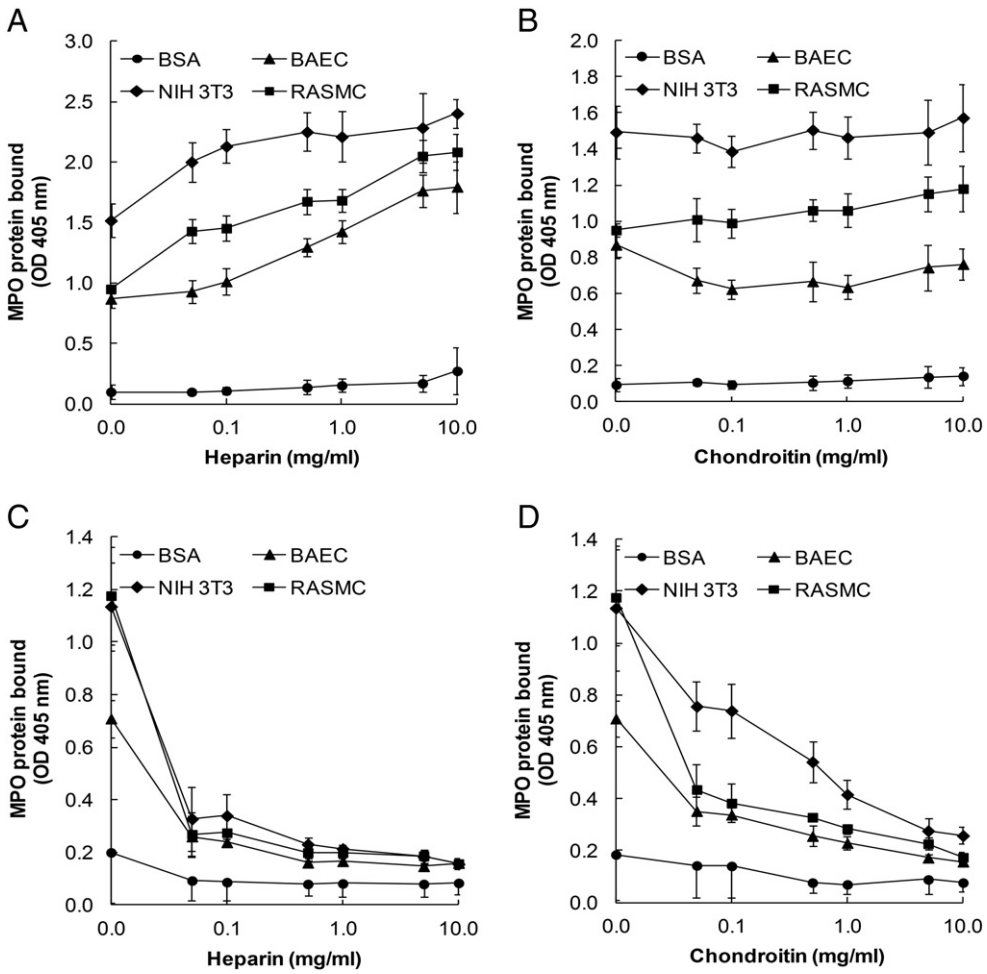

Fig. 4. Modulation of MPO binding to ECM derived from cultured cells *in vitro* by the pre-coating of ECM with sodium heparin (A) or chondroitin sulfate (B) and by the excess of sodium heparin (C) or chondroitin sulfate (D) during incubation. (A, B) Isolated ECM in microtiter plate wells was blocked with BSA and treated with sodium heparin or chondroitin sulfate (0.1 ml per well of 0.05–10 mg/ml in PBS) for 1 h at RT. Plates were washed to remove unbound GAGs and incubated with MPO (0.66 pmol per well) for 1 h at RT. (C, D) Isolated ECM in microtiter plate wells was blocked with BSA and incubated with MPO (0.66 nmol per well) together with sodium heparin or chondroitin sulfate (50 μl per well of 0.1–20 mg/ml of sodium heparin or chondroitin sulfate in PBS) for 1 h at RT. In all cases, bound MPO was determined by modified ELISA. Wells coated only by BSA were used as a control. Values represent the mean ± SEM (n = 5).

subendothelial ECM. These findings support the theoretical construct that MPO can play a regulatory role in this biological compartment after being released from neutrophil granulocytes and sequestered within ECM.

Present evidence indicates that MPO binds to the ECM isolated from various cell types and similarly to both chosen representatives of ECM, purified collagen IV and fibronectin. The fact that the binding occurred at a physiological salt concentration and at a pH of 7.4 makes it likely that the binding also occurs *in vivo*. The localization of MPO within the subendothelial matrix was previously documented by various authors on the basis of the immunohistochemical localization of MPO in this space [8,11,13,17]. As representative ECM proteins, collagen IV and fibronectin were selected to show MPO binding to these purified ECM proteins. Interestingly, MPO binding to fibronectin and to the other major component of subendothelial ECM, perlecan, was shown also by Rees et al. [8,10]. Primarily, the interaction of MPO with isolated ECM and purified collagen IV and fibronectin was shown by employing microtiter plate binding assays, a modified ELISA method. In all cases, BSA was used as an efficient blocking agent. This suggests a higher affinity of MPO to both collagen IV and fibronectin compared to BSA. However, native gel electrophoresis revealed a similar level of retardation of MPO migration by BSA, collagen IV and fibronectin, suggesting also the formation of an MPO albumin complex. In general, most of the previously reported interactions between MPO and a binding partner were thought to be due to the highly cationic nature of MPO (pI > 10) [1,2]. MPO was shown to interact strongly with polyanionic polysaccharide molecules. Daphna et al. reported the retardation of MPO migration towards the cathode by sodium heparin also suggesting the formation of a complex between MPO and this negatively charged GAG [15]. Further, in several studies, Rees and colleagues extensively characterized MPO interactions with various GAGs including heparin sulfate and chondroitin sulfate [4,10,21,23]. Interestingly, MPO interactions with glycoproteins and proteoglycans are not dependent only on the presence of glycan chains. In the case of perlecan, Rees at al. clearly showed that despite the MPO binding to perlecan was largely dependent on the presence of GAGs, the perlecan protein core was still able to bind MPO after treatment with heparinase [10]. In the case of the formation of the MPO and human serum albumin complex, the MPO-heavy chain residues 425–454 exhibited higher-affinity binding to negatively charged human serum albumin [14].

Thus, in the case of interaction between MPO and the anionic glycoprotein fibronectin (pI 5.6–6.1) [35] the major importance of the electrostatic interaction between MPO and glycan chains attached to the protein core can be suggested. In contrast, the interaction of collagen IV with MPO cannot be explained on the basis of total protein charge, as collagen IV has a rather cationic nature at physiological pH (pI 8.4–9) [36]. However, the association may occur as a result

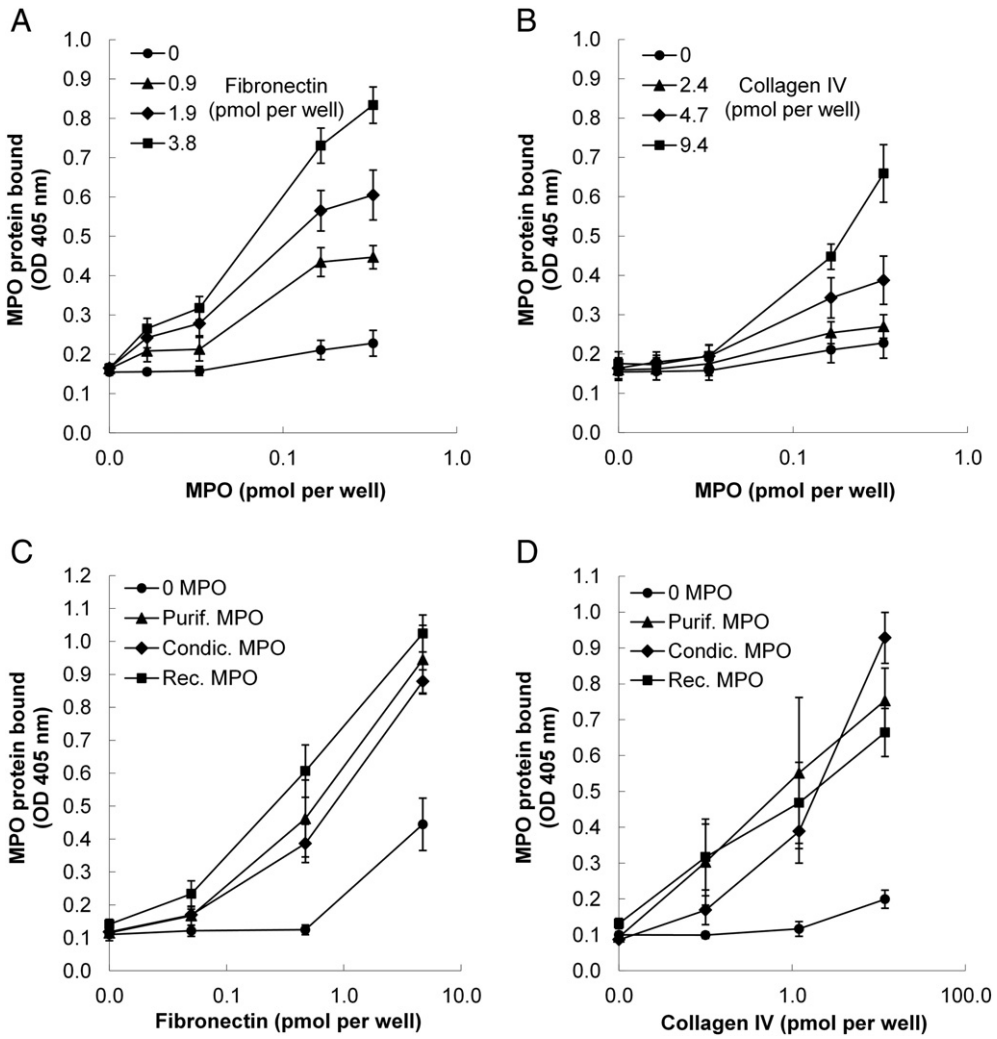

Fig. 5. MPO binding to purified fibronectin (A, C) or collagen IV (B, D). (A, B) Microtiter plates were coated with purified fibronectin (0.9–3.8 pmol per well) or collagen IV (2.4–9.4 pmol per well), washed, blocked with 0.1% BSA in PBS, and incubated with MPO isolated from leukocytes (0.03–0.33 pmol MPO per well) for 1 h at RT. (C, D) Microtiter plates were coated with purified fibronectin (0.05–4.72 pmol per well) or collagen IV (0.1–11.8 pmol per well), washed, blocked with 0.1% BSA in PBS, and incubated with MPO isolated from leukocytes (0.33 pmol per well), recombinant MPO (0.33 pmol MPO per well), and conditioned buffer obtained from OZP activated human polymorphonuclear leukocytes (MPO concentration 5.4 nM determined by ELISA e.g. 0.27 pmol per well) for 1 h at RT. In all cases, the bound MPO was determined by modified ELISA. Wells coated with only BSA instead of ECM proteins (18.9–75.8 pmol per well) served as a control. Values represent the mean ± SEM (n = 5).

of the local interaction of individual parts of collagen IV with MPO. The electrostatic nature of this interaction is supported by the potentiation of the interaction of fibronectin and collagen IV with MPO by the coating of these proteins with GAGs and by the disruption of such interactions with an excess of GAGs in the incubation mixture. Both collagen IV and fibronectin were shown to strongly interact with GAGs such as heparin employed in our model [37,38]. Interestingly, Rees et al. showed GAGs of perlecan as the primary binding partner for MPO [10]. In general, it can be suggested that despite the fact that the core proteins can bind MPO, the GAGs associated with these ECM proteins can be the main target of MPO interactions. This can be particularly significant under *in vivo* conditions, since several ECM proteins are reported to be heavily glycosylated under *in vivo* conditions [18].

It would be interesting to exactly determine the capacities of fibronectin and collagen IV to bind MPO. However, in our experimental set up, an exact calculation of this ratio was not possible. The binding assay was based on the determination of MPO bound to ECM protein-coated wells which were extensively washed throughout the experimental procedure. Thus, it is unclear what exact amounts of these proteins remained bound to the well surface and were available for interaction with MPO. Attempts to quantify the amounts of these proteins sequestered on the surface were limited by the sensitivity of currently available methodological approaches of protein quantification. Generally, the assay showed a linear relationship between amounts of coating and bound protein; however, except for data for the highest coating concentration, the amount of bound protein determined was under the quantification limit of the assay (data not shown). Protein determination revealed the amounts of deposited collagen IV and fibronectin to be 0.37 ± 0.07 μg per well (2.06 pmol) and 0.48 ± 0.07 μg per well (1.07 pmol), respectively (mean ± SEM, n = 3), in the wells coated with the highest protein concentration used for the coating (1.9 μg per well). Thus, it can be suggested that approximately one quarter of the amount of protein used for coating was left deposited on the microtiter plate wells, with a linear relation over the range of employed concentrations for coating: 0.02–1.91 μg per well.

Our data suggest that the interaction of MPO with collagen IV or fibronectin does not decrease the peroxidase activity of MPO. In contrast, data suggest mild, though significant, potentiation of MPO activity. The analysis of MPO kinetics upon MPO interaction with collagen IV using ABTS as a substrate for peroxidation indicates an increase in both Vmax and Km. Assuming that collagen IV binding to MPO is not likely to change the reaction mechanism, Vmax is largely dependent

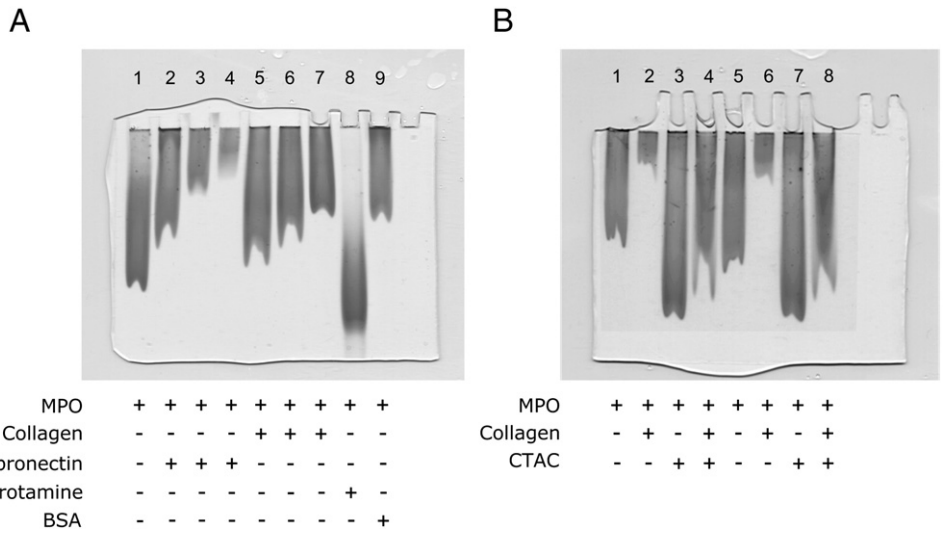

Fig. 6. Formation of MPO complexes with fibronectin and collagen IV as well as with BSA (A) and the effect of CTAC (B). The associations of MPO with fibronectin, collagen IV, protamine (negative control) and BSA and the ability of CTAC to inhibit MPO association with collagen IV were determined by native gel electrophoresis. (A) MPO (13.3 pmol) was incubated with fibronectin (4.8, 8.0, 12.0 pmol) or collagen IV (12, 20, 30 pmol) or protamine (360 pmol) or BSA (41 pmol) in a total volume 20 μl for 20 min at RT. (B) MPO (13.3 pmol) was incubated with collagen IV (30 pmol) or CTAC (4 nmol) or a combination of collagen IV and CTAC in a total volume 20 μl for 20 min at RT. Samples were loaded onto gels (in the case of gel B in duplicates), run and stained with TMB substrate as described in materials and methods. The gel is representative of 4 independent repeats.

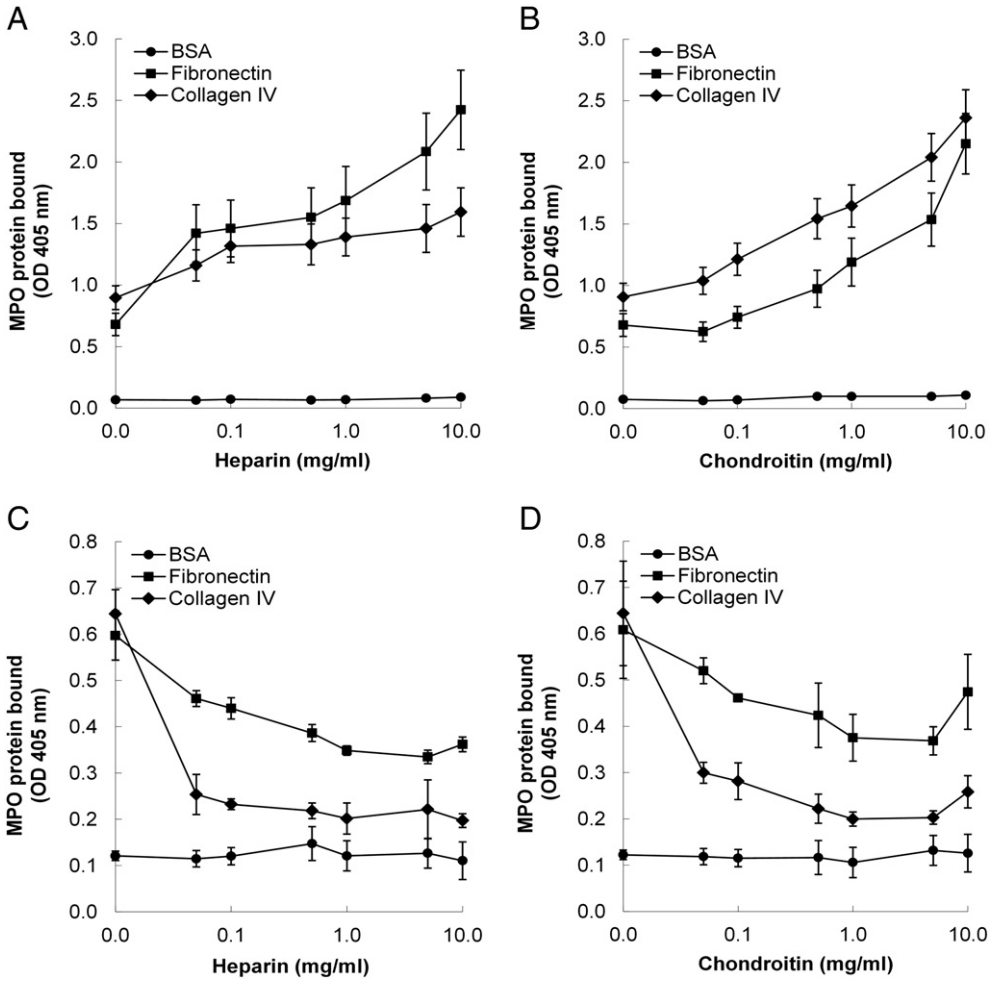

Fig. 7. Effects of sodium heparin (A) and chondroitin sulfate (B) pre-treatment and excess of sodium heparin (C) or chondroitin sulfate (D) on MPO binding to fibronectin and collagen IV. (A,B) Microtiter plates were coated with fibronectin (3.8 pmol per well) or collagen IV (9.4 pmol per well), washed and blocked with BSA. Sodium heparin or chondroitin sulfate (0.1 ml per well of 0.05–10 mg/ml of sodium heparin or chondroitin sulfate in PBS) was added for 1 h. Plates were washed and incubated with MPO (0.33 pmol per well) for 1 h at RT. (C,D) Plates were coated with fibronectin (3.8 pmol per well) or collagen IV (9.4 pmol per well), washed, blocked with BSA, and treated with sodium heparin or chondroitin sulfate (50 μl per well of 0.1–20 mg/ml of sodium heparin or chondroitin sulfate in PBS) together with MPO (0.33 pmol per well) for 1 h at RT. In all cases, bound MPO was determined by modified ELISA. Wells blocked with BSA were used as a control. Values represent the mean ± SEM (n = 5).

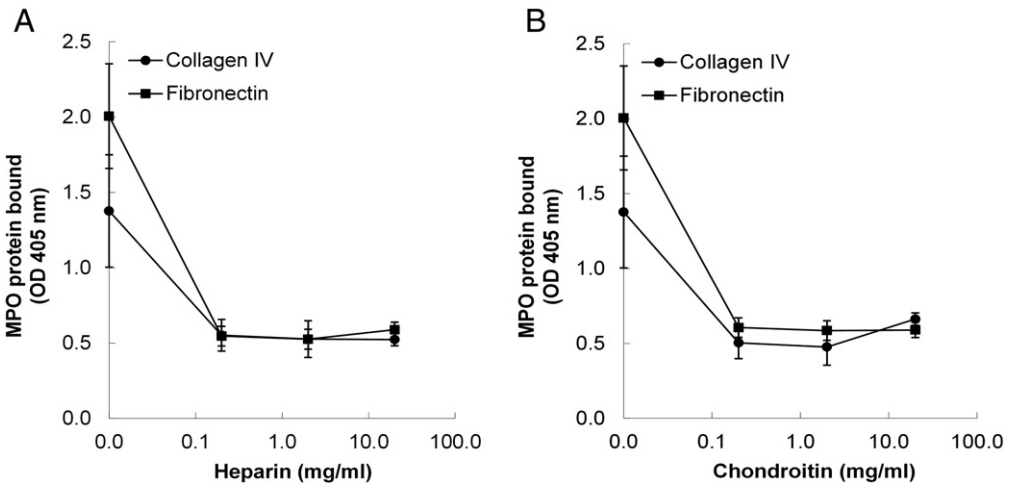

Fig. 8. Release of MPO bound to fibronectin or collagen IV by an excess of sodium heparin (A) and chondroitin sulfate (B). Microtiter plates were coated with fibronectin (3.8 pmol per well) or collagen IV (9.4 pmol per well), washed, blocked with BSA, washed, incubated with MPO (0.33 pmol per well) for 1 h at RT, washed, and treated with sodium heparin or chondroitin sulfate (0.1 ml per well of 0.05–10 mg/ml of sodium heparin or chondroitin sulfate in PBS) for 1 h at RT. Bound MPO was determined by modified ELISA. Wells coated with BSA were used as a control. Values represent the mean ± SEM (n = 5).

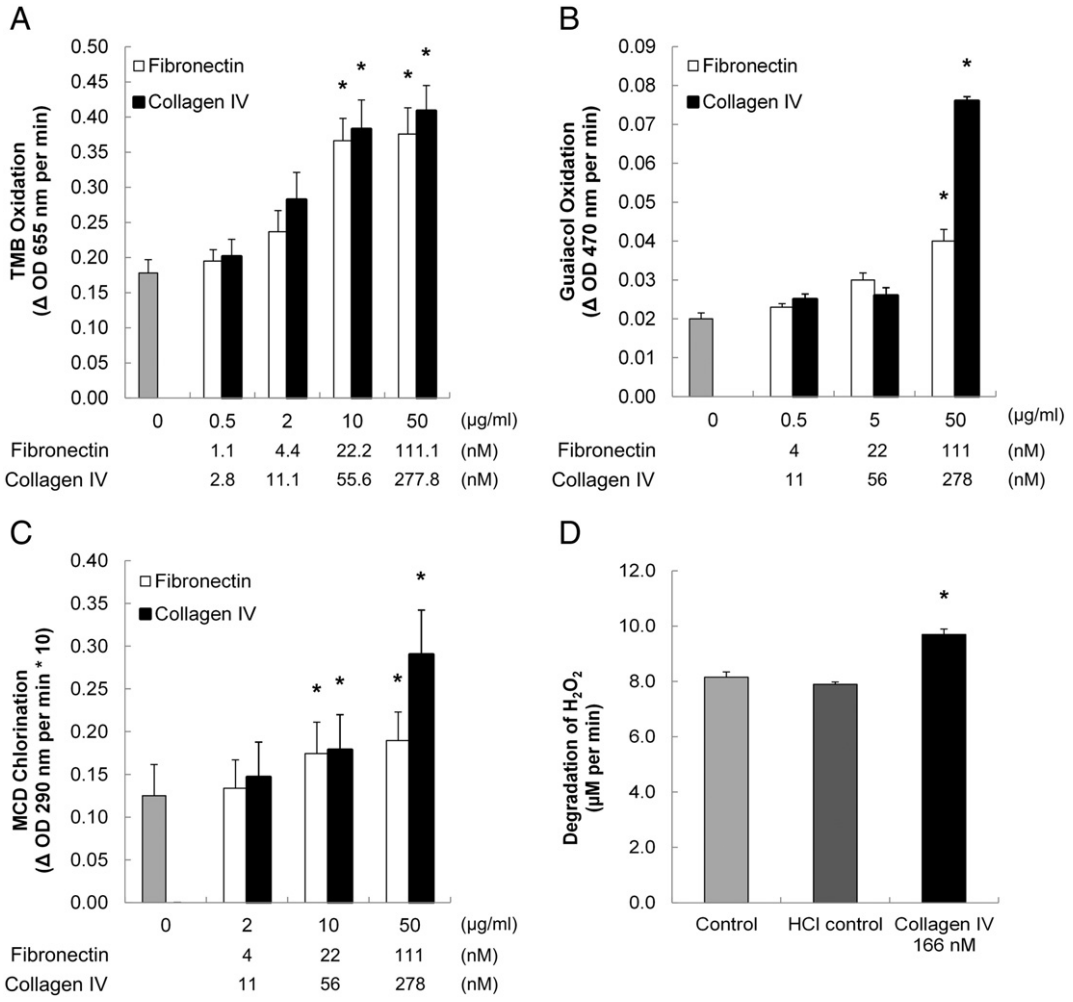

Fig. 9. Fibronectin and collagen IV enhance the MPO-catalyzed oxidation of TMB (A), the oxidation of guaiacol (B), the chlorination of MCD (C), and the catabolism of H_2O_2 (D). Fibronectin (1.1–111.1 nM) or collagen IV (2.8–277.8 nM) were incubated together with MPO (13 nM) in PBS for 1 h at RT. (A) The MPO oxidation of TMB was determined after the addition of 1.2 mM TMB and 300 µM H_2O_2 in acetate buffer. Absorbance kinetics were assessed at 655 nm at RT, and rates are expressed as the ΔOD at 655 nm/min. (B) The MPO oxidation of guaiacol was determined after the addition of 100 µM guaiacol and 100 µM H_2O_2 in PBS. Absorbance kinetics was assessed at 470 nm at RT, and rates are expressed as the ΔOD at 470 nm/min. (C) The MPO chlorination of MCD was determined after the addition of 40 µM MCD and 100 µM H_2O_2 in PBS. Absorbance kinetics were assessed at 290 nm at RT, and rates are expressed as the ΔOD at 290 nm/min. (D) To determine the catabolism of H_2O_2 by MPO, MPO (2.5 nM) was incubated with collagen IV in PBS for 1 h at RT and the decay of H_2O_2 was determined using an electrochemical sensor after the addition of H_2O_2 (80 µM) in acetate buffer at RT. PBS or hydrochloric acid appropriately diluted with PBS served as control. The degradation of hydrogen peroxide was monitored for 200 s after an initial 60 s interval. Data are expressed as mean ± SEM (n = 3). For multiple comparisons, one-way ANOVA followed by the Dunnett's post hoc test was used. The difference from the control group was considered statistically significant when the p-value was equal to, or lower than 0.05 and is marked with asterisks.

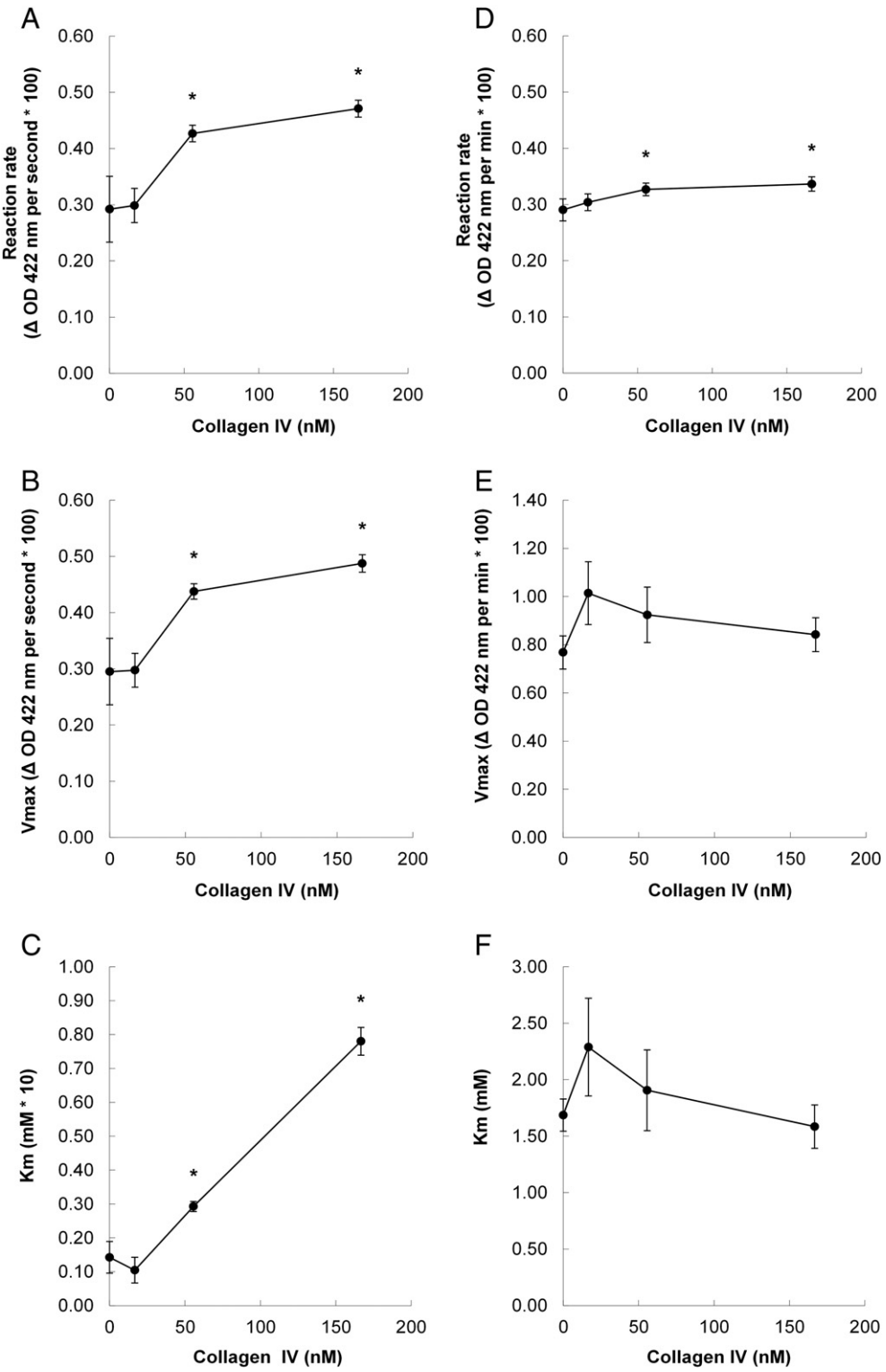

Fig. 10. The kinetic parameters of MPO catalyzed ABTS oxidation in the presence of collagen IV. (A, D) The reaction rate, (B, E) the maximal reaction rate (Vmax), and (C, F) Michaelis constant (Km) of MPO-catalyzed ABTS oxidation were determined in 300 mM sodium acetate buffer at pH 5.6, which is optimal for this reaction (A, B, C), and in PBS under physiological pH 7.4 (E, F, G). MPO (10 nM) was incubated with collagen IV (16.6–166.6 nM) in PBS for 1 h. The rate of ABTS oxidation was determined after the addition of H_2O_2 (80 μM) and ABTS (1 mM) by reading the increase in OD at 422 nm. Data are expressed as mean ± SEM (n = 3–4). For multiple comparisons, one-way ANOVA followed by the Dunnett's post hoc test was used. The difference from the control group was considered statistically significant when the p-value was equal to, or lower than 0.05 and is marked with asterisks.

on the existence of a diffusion barrier to the active site. Thus, it could be speculated that collagen IV binding could open the entrance to the active site of MPO. On the other hand, such a rearrangement of the 3D structure of MPO could result in a decreased affinity to ABTS, which would result in a higher Km. The reaction catalyzed by MPO is highly complicated, influenced by, for example, pH, halide concentrations, reactive oxygen species, pO_2, and substrate concentrations. This complexity of the reaction makes it difficult to predict the effect of interactions between collagen IV or fibronectin and MPO *in vivo*. Interestingly, it is well documented that MPO interactions with proteins

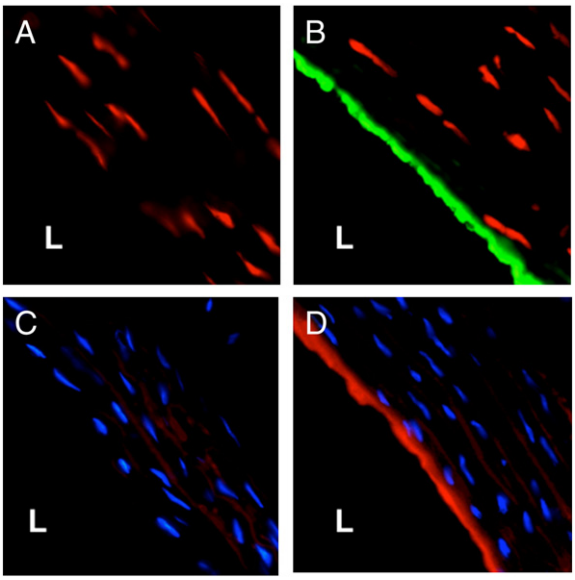

Fig. 11. MPO sequestered by vascular tissue ECM is enzymatically active and catalyzes the nitration (A, B) and oxidation of ECM proteins (C, D). Endothelium denuded rat aortic segments were exposed to MPO (13 nM) in PBS for 30 min at RT. Aortic segments were then washed extensively with PBS to remove unbound MPO. To determine the nitration of ECM protein (A, B), aortic segments were incubated with nitrite (100 μM) and H_2O_2 (50 μM) in PBS for 120 min at 37 °C. The distribution of nitrotyrosine-containing proteins was detected using polyclonal nitrotyrosine antibodies and visualized with fluorescence microscopy (green); nuclei were counterstained with propidium iodide (red). To determine the oxidation of ECM proteins (C, D), aortic segments were incubated with H_2O_2 (10 μM) and tyramide in amplification buffer according to the manufacturer's protocol. Protein-associated tyramide (as a covalent adduct with protein tyrosine residues) was detected by its inherent fluorescence and visualized by immunofluorescence microscopy (red); nuclei were counterstained with DAPI (blue). "L" depicts the vessel lumen. Images are shown at 40× magnification. Figures A and C represent controls without MPO and figures B and D represent samples treated with MPO. The figures represent typical pictures from at least 4 independent repeats.

such as ceruloplasmin result in the inhibition of peroxidase activity [24,27,28]. Studies on the enzymatic properties of MPO show that ceruloplasmin behaves as a competitive inhibitor impeding the binding of aromatic substrates to the active center of MPO [28]. Interestingly, the interaction of MPO with GAGs such as sodium heparin was also reported to decrease MPO activity [13]. In contrast, other MPO interactions with plasma proteins including albumin did not exhibit a significant inhibitory effect [27]. Modulatory effects on enzymatic activity based on electrostatic interactions with GAGs have been reported previously. Heparin was shown to have a hyperbolic and competitive inhibitory effect on elastase and cathepsin G, two serine cationic proteases present in PMNs [39,40]. In these cases, the authors suggest that the inhibitory effects of heparin on MPO are not specific but linked to electrostatic interactions and are dependent on a similar competitive inhibitory mechanism: at a high concentration, the inhibitory capacity of low molecular weight heparin would be greater than that of high molecular weight heparin; the access of high molecular weight heparin to MPO would be limited by steric effects.

Collectively, the data reveal that MPO binds to ECM proteins probably in an electrostatic-dependent manner, and that MPO activity is not decreased upon association with these proteins. Even, data suggest a potentiation of MPO activity in a presence of proteins such as collagen IV or fibronectin. However, the complexity of MPO-catalyzed reactions makes it challenging to predict the exact mechanisms responsible for modulation of MPO enzymatic activity by these interactions. Further, effects of interactions between MPO and collagen IV or fibronectin on MPO enzymatic activity *in vivo* is difficult to predict. This will require further analysis and it is beyond the scope of this study.

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Conflict of interest

The authors declare no competing financial interests.

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Plasma levels of myeloperoxidase are not elevated in patients with stable coronary artery disease

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ABSTRACT

Background: Plasma and serum levels of myeloperoxidase (MPO), a redox-active hemoprotein released by polymorphonuclear neutrophils (PMN) upon activation, is now recognized as a powerful prognostic determinant of myocardial infarction in patients suffering acute coronary syndromes. However, there is limited information on whether systemic MPO levels are also elevated and of discriminating value in patients with stable coronary artery disease (CAD) representing different ethnic groups.
Methods: Plasma levels of MPO and traditional CAD risk factors were quantified in African American and Caucasian patients ($n=557$) undergoing elective coronary angiography.
Results: MPO levels did not differ significantly between patients with or without CAD [421 pM (321, 533) vs. 412 pM (326, 500), $p>0.05$]. MPO levels were similar across ethnicity and gender, and correlated positively with CRP and fibrinogen levels ($r=0.132$, $p=0.002$ and $r=0.106$, $p=0.011$, respectively).
Conclusion: In conclusion, plasma MPO levels were not elevated in patients with stable CAD, suggesting that systemic release of MPO is not a characteristic feature of asymptomatic CAD.

1. Introduction

Atherosclerosis in general and coronary artery disease (CAD) in particular are considered to have an inflammatory component [1,2]. *Clinically* the presentation of CAD ranges from asymptomatic patients with stable CAD to patients with chest pain at rest experiencing acute coronary syndromes. *Pathophysiologically*, acute coronary disease is not only reflected by increased plaque vulnerability and elevated platelet activation, but also by recruitment and activation of leukocytes [3]. Notably, polymorphonuclear neutrophils (PMN), a cell type previously considered less relevant in coronary disease, were found to undergo site specific activation and degranulation in patients with unstable CAD and were localized to culprit lesions [4–6].

One of the principal enzymes released upon PMN activation is myeloperoxidase (MPO), a highly abundant redox-active hemoprotein [2,7,8]. MPO and its products display a diversity of pro-inflammatory and pro-atherogenic properties including catalytic consumption of endothelium-derived nitric oxide, LDL oxidation, modulation of metalloproteinase activities, and activation of PMN in a cytokine-like manner independent of the catalytic activity [7–10]. The significance of MPO in the development of CAD has been demonstrated in studies showing association of systemic MPO level and expression of MPO with the prevalence of CAD or with chronic heart failure [9,11]. Interestingly, MPO serum and plasma levels are markedly elevated in patients with acute coronary disease, forming a firm mechanistic link between PMN activation, MPO release, and compromised vascular reactivity [12–16].

Given the diversity of the clinical presentations of patients with coronary disease we sought to evaluate whether systemic release of MPO is a characteristic feature in patients with stable CAD in the absence of acute events representing different ethnic groups. In the present study, we investigated MPO plasma levels in Caucasian and African American patients without acute coronary syndromes undergoing elective angiography who were participating in the Harlem–Bassett study undertaken to evaluate novel cardiovascular risk factors across ethnicity [17]. We have previously reported that other CAD risk factors, including metabolic syndrome components, differ across ethnicity among these high-risk patients [18]. In addition to characterizing MPO levels and their relation to disease, we explored the relationship between MPO levels and traditional coronary disease risk factors.

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2. Methods

2.1. Subjects

Subjects were recruited from a patient population scheduled for diagnostic coronary arteriography either at Harlem Hospital Center in New York City or at the Mary Imogene Bassett Hospital in Cooperstown, NY. A total of 648 consecutive subjects, 401 men and 247 women, ethnically self-identified as African Americans (n=232), Caucasians (n=344) or Other (n=72) were recruited from 1993–1997 as described in detail elsewhere [17,19]. In the present study, results were available for 557 of the 576 African American and Caucasian subjects [211 Caucasian men (C/M), 120 Caucasian women (C/F), 127 African American men (AA/M), and 99 African American women (AA/F)]. Exclusion criteria were: age >70 years, recent (within 6 months) myocardial infarction or thrombolysis, a history of percutaneous transluminal coronary angioplasty, surgery during the previous 6 weeks, a known communicable disease such as hepatitis or AIDS, or current lipid-lowering medication. Information on diabetes mellitus, hypertension and smoking was obtained by a standardized questionnaire upon entry into the study. The study was approved by the Institutional Review Boards at Harlem Hospital, Bassett Healthcare, Columbia University College of Physicians and Surgeons, and the University of California, Davis, and informed consent was obtained from all participants.

2.2. Angiographic definition of CAD

Coronary angiograms were analyzed by 2 experienced investigators blinded to the patient's identity, clinical diagnosis, and MPO levels [20]. A total of 15 pre-defined coronary artery segments were evaluated in each individual for degree of stenosis. Diagnosis of coronary artery disease was defined as a luminal narrowing of at least 50% of a vessel diameter in any of the analyzed 15 coronary artery segments. In patients defined as not having CAD, the majority (81%) had a maximum stenosis of less than 25%, whereas in 81% of the patients defined as having CAD, the luminal narrowing was at least 75%. A composite cardiovascular score (0–75) was calculated based on determination of presence of stenosis on a scale of 0–5 of the 15 predetermined coronary artery segments.

2.3. Analytical procedures

Plasma samples with ethylenediaminetetraacetic acid as an anticoagulant were drawn from every patient after an overnight fast and prior to the angiographic procedure. The blood samples were centrifuged for 20 min at 1300 g at 4 °C, and the supernatant separated and aliquotted into storage vials. The vials were immediately frozen and stored at −80 °C until further analyzed. All analytical procedures were performed by investigators blinded to the sample identity. Serum triglycerides, total and HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), Lipoprotein (a) [Lp(a)], glucose, insulin, high sensitivity C-reactive protein (hs-CRP), fibrinogen, factor VII were measured by standard techniques as described previously [17].

2.4. Plasma MPO quantification

For determination of MPO in plasma samples an ELISA procedure was used. In brief, Maxisorb 96-well microtiter plates (Nalge Nunc International; Rochester, NY, USA) were coated with 100 µl purified monoclonal mouse anti-human MPO (Biomeda, Foster City, CA, USA) diluted 1:499 (final concentration 0.6 µg/ml) in carbonate buffer, pH 9.6 overnight at 4 °C. The plates were washed three times with washing buffer (0.01 M Tris buffer with 0.05% Tween, pH 7.4), blocked with 4% BSA in washing buffer for 2 h at 25 °C, and again washed three times. Plasma samples diluted 1:4 in PBS containing 0.5% bovine serum albumin (BSA) were added to the wells (100 µl) and incubated for 2 h at 25 °C. All samples were measured in duplicates. After washing 4 times, a purified polyclonal rabbit anti-human MPO antibody (Calbiochem, San Diego, CA, USA) diluted 1:399 in washing buffer containing 0.5% BSA was added to the wells (100 µl) and incubated for 2 h at 25 °C, followed by washing 4 times. Bound rabbit IgG was detected using an alkaline phosphatase conjugated goat anti-rabbit IgG antibody (Zymed-Invitrogen, Carlsbad, CA, USA). Initially, serial dilutions of primary and both secondary Ab were carried out using washing buffer containing 0.5% BSA to determine saturation kinetics. Concentrations of both antibodies were carefully optimized to give linear signal in the range of expected values. Plasma dilution was selected based on the best ratio between a background signal and a signal of plasma spiked with 330 pM/L of MPO. The average recovery was over 20%. After washing the plates four times, 4-nitrophenyl phosphate (Sigma-Aldrich Corp.; St. Louis, MO, USA) (1.5 g/l) in diethanolamine buffer (1 M) was added, the plates developed for 20 min and the absorbance read at 405 nm in PowerWavex UV–Vis plate reader (Bio-Tek Instruments, Winooski, VT, USA). Values were related to a set of purified human MPO (Calbiochem) standards to quantify the values in picomoles (pM) per liter. The linearity of the assay was in the range 70–2000 pM MPO in plasma. Average intra-assay variability was 10.4% and the inter-assay CV was 11.9%. The obtained over all average of MPO plasma level 416 pM (155 pM and 1064 pM minimum and maximum, respectively) was in good agreement with MPO levels in peripheral circulation reported in many other studies, including MPO levels observed by Vita et al. in control subjects and subjects with endothelial dysfunction 298 pM MPO (154.1 pM and 638.1 pM interquartile range) in serum [21], by Brennan et al. in patients presenting with chest pain 198 pM MPO (119 pM and 394 pM interquartile range) in plasma [13], by Hoy et al. in healthy individuals 5.4–141.6 µg/l MPO in serum [22], by Rudolph et al. in patients with impaired left ventricular function and controls 8.9–54 µg/l MPO in plasma [23], and by Baldus et al. in patients with and without CAD 7.66–13.10 µg/l MPO in plasma [14]. However, MPO levels reported herein were lower compared to levels reported by Baldus et al. in patients with acute coronary syndrome 287 µg/l (range 1.5–1112 µg/l) in plasma [12].

2.5. Statistical analysis

Data are described as mean±SD or as median and interquartile range as appropriate. Levels of MPO, triglycerides, insulin, hs-CRP, fibrinogen and HOMA were log transformed, and adiponectin levels were square root transformed to achieve normal distributions prior to statistical analysis. Comparisons of means between groups were made by Student's t-test. Univariate relationships were described by Pearson's correlation coefficients. Multiple logistic regression was used to assess the association of MPO with the various known and potential risk factors. Contingency table analysis was performed to assess differences in patients' characteristics across MPO plasma level quartiles. All statistical analyses were done using SAS software (SAS Institute, Cary, NC). Statistical significance was set at $p<0.05$.

3. Results

The demographic and clinical characteristics of the populations studied are summarized in Table 1. Patients with CAD were significantly older and had significantly higher levels of clinical and inflammatory markers associated with CAD risk such as the waist/hip ratio, glucose, HOMA, cholesterol, triglyceride, LDL-C, HDL-C, Factor VII, fibrinogen, and adiponectin. No significant difference was detected for body mass index (BMI) and insulin levels between those with and without CAD.

MPO was detectable in plasma samples of all study subjects, and the frequency distribution was skewed. For all subjects, the median MPO level was 416 pM (323 pM upper and 512 pM lower quartile). There was no significant difference in the MPO plasma levels between subjects with and without stable CAD [421 pM (321 pM, 533 pM) vs. 412 pM (326 pM, 500 pM) in CAD vs controls, respectively $p>0.05$] (Fig. 1A). We then explored MPO levels across ethnicity and gender. As shown in Fig. 1B, there was no difference in plasma MPO levels between the four ethnicity/gender groups; African American and Caucasian men and women. Table 2 illustrates patients characteristics stratified according plasma MPO quartiles. Corresponding to other analysis, any significant difference was not observed across MPO quartiles (Table 2). Further, there was no significant relationship between MPO levels and CAD, expressed as cardiovascular score, in either Caucasians or African Americans. Thus, our results suggest that the plasma level of MPO did not identify patients with stable CAD and was also independent of gender and ethnicity.

Table 1
Demographic and clinical characteristics of subjects with and without CAD

	No CAD (n=268)	CAD (n=289)	P
Age (years)	52.3±10.3	58.8±8.6	<0.001
Waist/hip ratio	0.94±0.08	0.96±0.07	0.004
BMI (kg/m^2)	29.3±6.5	29.1±5.8	n.s
Insulin (mU/l)	13.7 (8.8–22.9)	15.2 (9.5–26.2)	n.s.
Glucose (mM/l)	6.5±2.6	7.4±3.7	0.003
HOMA	0.62±0.44	0.73±0.46	0.011
Cholesterol (mg/dl)	190.0±40.3	204.1±42.6	<0.0001
Triglyceride (mg/dl)	115.5 (85.3–165.0)	143.0 (105.0–211.0)	<0.0001
LDL-C (mg/dl)	117.1±34.6	129.3±39.9	<0.0001
HDL-C (mg/dl)	46.1±15.9	41.9±13.8	0.001
Factor VII (%)	100.2±30.6	105.4±31.3	<0.05
Fibrinogen (mg/dl)	324.5 (279.8–385.8)	342.0 (291.0–408.8)	<0.05
Adiponectin (µg/ml)	9.6 (6.1–14.2)	7.9 (5.0–11.7)	<0.0001
hs-CRP (mg/l)	3.1 (1.4–7.5)	3.7 (1.8–11.5)	n.s

Results are expressed as means±SD, or for non-normally distributed variables as median (interquartile range).

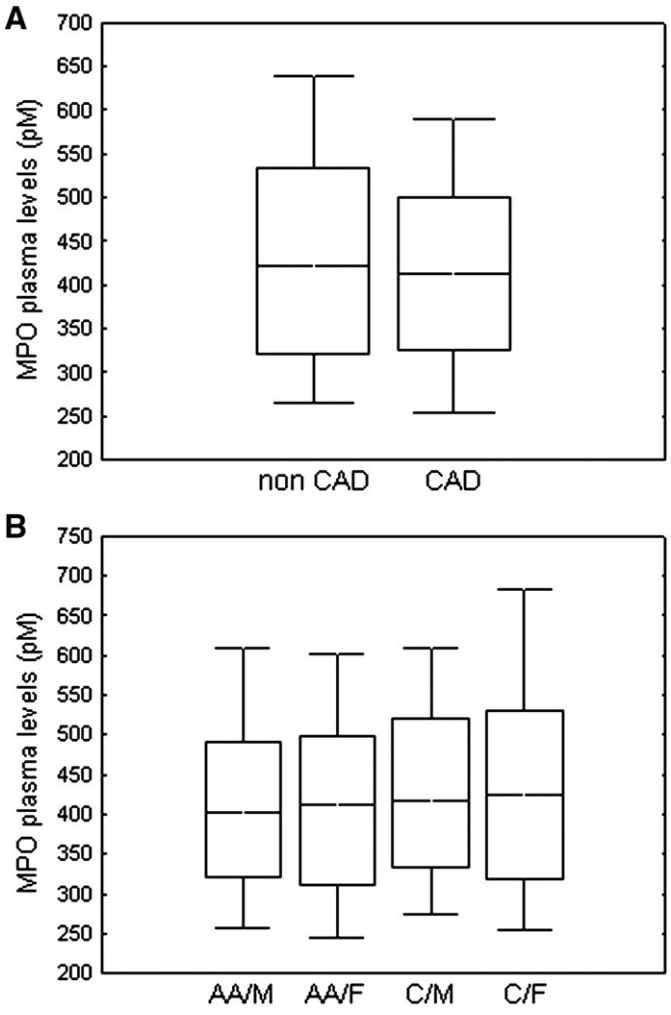

Fig. 1. Comparison of MPO plasma levels between patients with and without CAD (A); Comparison of MPO plasma levels in four gender/ethnicity groups (AA/M — African American men, C/M — Caucasian men, AA/F — African American women, and C/F — Caucasian women) (B). The top, bottom, and line through the middle of the box correspond to the 75th percentile (top quartile), 25th percentile (bottom quartile), and 50th percentile (median) respectively. The whiskers on the bottom extend from the 10th percentile (bottom decile) and top 90th percentile (top decile).

Further, we considered whether the plasma MPO level was associated with other markers of stable CAD. Significant associations were found between MPO levels and the following markers in univariate analysis: factor VII ($r=-0.251$, $p<0.0001$); adiponectin ($r=0.115$, $p=0.01$); glucose ($r=0.095$, $p=0.023$); triglyceride ($r=-0.186$, $p<0.0001$); cholesterol ($r=-0.081$, $p=0.05$); hs-CRP ($r=0.132$, $p=0.002$); fibrinogen ($r=0.106$, $p=0.011$). There was no significant correlation between MPO plasma levels and age, BMI, waist/hip ratio, insulin, LDL-C, or HDL-C. Interestingly, Factor VII and adiponectin were significant predictors of MPO levels in a multiple regression model [Factor VII ($r=-0.205$, $p<0.001$) and adiponectin ($r=0.119$, $p=0.021$)]. In contrast, multiple regression analysis did not reveal levels of glucose, triglyceride, cholesterol, hs-CRP, and fibrinogen to be significant predictors of MPO plasma levels.

4. Discussion

In the present study, we have aimed to determine whether MPO plasma levels identify patients with stable CAD. The principal finding of our study is that plasma MPO levels are not elevated in patients with stable CAD compared to non-CAD patients, and does not discriminate these cohorts of patients.

CAD remains a heterogeneous disease with a wide range of clinical presentations and outcomes. Various systemic markers of inflammation have been investigated and linked to identify patients at risk of CAD and predict future cardiovascular events [1]. Recently, the importance of PMN degranulation of MPO in the coronary circulation is illustrated by the fact that systemic MPO plasma and serum are markedly elevated and have emerged as powerful predictors of adverse outcome in patients with acute CAD [12–16]. As opposed to these studies evaluating circulating MPO in symptomatic and unstable coronary disease we evaluated MPO levels in asymptomatic individuals: none of the patients, who underwent elective coronary angiography, were hospitalized because of symptomatic chest pain at rest, and none had evidence for ongoing myocardial ischemia or necrosis, respectively. Other studies showed that patients with stable CAD have significantly lower or no evidence of PMN recruitment and activation, as evidenced by unchanged CD11b expression and MPO content in PMN [4,6]. Moreover, in patients with resolving unstable angina the decreased MPO content in PMN returned to levels similar to that in patients with chronic stable angina or subjects lacking coronary disease [6].

Whereas our data suggest that release of MPO is not a primary event in patients with stable CAD, it cannot be excluded that assessment of circulating MPO undervalues the extent of MPO sequestered into the vessel wall. There is now a large body of evidence revealing avid binding of MPO to heparan-sulfated glycosaminoglycans (GAGs) and subendothelial deposition of MPO in the vessel wall [8]. Heparinization with concomitant release of vessel-immobilized MPO revealed increased MPO burden in patients with CAD as compared to non diseased individuals, suggesting increased sequestration of leukocyte derived-MPO into the vessel wall [14]. This would also explain decreased MPO content in PMN in both acute and stable CAD patients compared to controls [5,6,9].

Despite MPO being equally distributed among patients with and without stable CAD, we observed weak but significant associations between plasma MPO levels and inflammatory markers known to indicate risk for CAD including CRP and fibrinogen. Similarly, Vita et al. observed that serum MPO levels correlated with cardiovascular risk factors including hypertension, HDL cholesterol, C-reactive protein, serum triglycerides and smoking [21]. Recently, Meuwese et al. observed that elevated MPO plasma levels significantly predicted risk of future CAD in over all 3375 apparently healthy individuals [24]. However, the difference in medians of MPO plasma levels between controls and case subjects was around 10%. Authors pointed out that relationship between MPO and CAD in these individuals was weaker than was reported in patients with acute CAD [24]. Lipid-lowering drugs statins downregulated MPO systemic levels in patients with acute coronary syndrome [25]. Interestingly, there are emerging data also linking MPO plasma levels with presence of heart failure [11,16] and left ventricular dysfunction [23,26]. However, no difference in MPO levels was observed between patients with and without CAD who had normal left ventricular function [23]. Furthermore, MPO plasma levels were neither related to intima media thickness, nor to progression of intima media thickness in 122 familiar hypercholesterolemia patients [27].

Table 2
Patient baseline characteristics across quartiles of MPO plasma levels

MPO quartile (pM)	155–323	324–416	417–511	512–1064
African Americans	76 (13.6)	80 (14.4)	83 (14.9)	92 (16.5)
Caucasians	63 (11.3)	59 (10.6)	56 (10.1)	48 (8.6)
Male	79 (14.2)	96 (17.2)	79 (14.2)	84 (15.1)
Female	60 (10.8)	43 (7.7)	60 (10.8)	56 (10.1)
Cases with CAD	71 (12.7)	79 (14.2)	75 (13.5)	64 (11.5)

Categorical data are presented as frequencies and percentages.

We acknowledge some of the limitations of this study. Subjects in our study were recruited from patients scheduled for coronary angiography and are likely more typical of a high-risk patient group than the healthy population at large. This may explain the relatively high levels of CRP and fibrinogen among our subjects. However, none of the patients had a history of acute coronary symptoms or surgical intervention within 6 months, arguing against any secondary increase in inflammatory parameters due to an acute CAD. Although the number of subjects representing each gender–ethnicity group was limited, the number of subjects, both Caucasians and African Americans was considerably higher than in some previous multi-ethnic studies [15]. Further, clinical and laboratory parameters were in agreement with differences generally observed between healthy African American and Caucasian populations from other studies [28,29].

In conclusion, the present study reveals that plasma MPO levels do not identify patients with stable CAD. This is in contrast to determination of systemic MPO levels as emerging powerful and rapidly detectable markers of unstable CAD [12–16]. Our findings further support the concept that a robust release of MPO from activated PMN would unmask a state of acute inflammation in the coronary circulation preceding myocardial injury and this cannot be extended to stable cardiovascular disease using traditional blood drawing techniques. Further studies aiming to determine the pathophysiological role of MPO in acute coronary disease and addressing the pathophysiological heterogeneity of the different clinical presentations of coronary artery disease are needed.

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Original Contribution

A myeloperoxidase promoter polymorphism is independently associated with mortality in patients with impaired left ventricular function

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ABSTRACT

Circulating levels of myeloperoxidase (MPO), a heme enzyme released upon activation of polymorphonuclear neutrophils, predict adverse outcome in patients with impaired left ventricular (LV) function. The MPO −463 G/A promoter polymorphism (rs 2333227) regulates MPO transcription, with the G allele being linked to increased protein expression. The aim of this study was to assess the prognostic information derived from the −463 G/A MPO polymorphism on outcomes of patients with impaired LV function. The −463 G/A promoter MPO genotype as well as MPO plasma levels were determined in 116 patients with impaired LV function. Patients were prospectively followed for a median of 1050 days. The GG genotype was associated with a decrease in overall survival (χ^2 5.80; $p=0.016$). This association remained after multivariate adjustment for plasma levels of NT-proBNP, creatinine, hsCRP, and MPO; leukocyte count; and LV function (hazard ratio 3.16 (95% CI 1.17–8.53), $p=0.024$) and for classical cardiovascular risk factors (hazard ratio 2.88 (95% CI 1.13–7.33), $p=0.026$). Interestingly, we observed no association of the MPO polymorphism with total MPO protein concentration or MPO activity in plasma. The −463 G/A MPO polymorphism is linked to adverse clinical outcome of patients with impaired LV function. Further studies are needed to elucidate the value of this polymorphism for risk stratification.

Introduction

Myeloperoxidase (MPO) is a heme enzyme predominately expressed by polymorphonuclear neutrophils (PMN) and to a lesser extent by monocytes and macrophages [1]. Because of its capacity to generate potent oxidants, such as hypochlorous acid, it was traditionally viewed as a bactericidal protein exclusively involved in host defense [2]. However, over the past decade MPO has been put forward as an enzyme linked to various other disease processes [3]. Most recently, circulating levels of MPO have been found elevated in patients with heart failure of both ischemic and nonischemic origin [4–6]. There is accumulating evidence from ex vivo and animal models suggesting that MPO is also mechanistically involved in the pathophysiology of heart failure: MPO adversely affects myocardial perfusion by inducing endothelial dysfunction and arteriosclerotic lesion development. Upon its secretion from azurophilic granules of PMN, MPO accumulates in the subendothelial matrix to catalytically consume nitric oxide ($^{\bullet}$NO) [7,8]. Additionally, MPO leads to oxidation of plasma lipoproteins thereby facilitating foam-cell formation and plaque instability [9]. Moreover, MPO oxidizes redox-sensitive enzymes localized in the myocardium, such as plasminogen activator inhibitor, thereby affecting the function of these enzymes [10,11].

In support of the proposed tight mechanistic link between MPO and impairment of left ventricular (LV) function, circulating MPO plasma and serum levels provide powerful diagnostic and prognostic information in patients with heart failure: not only are MPO plasma levels elevated in patients with symptomatic heart failure independent of its origin, but also circulating levels of MPO provide prognostic information, which complements established biomarkers of LV dysfunction such as brain natriuretic peptide (NT-proBNP) and high-sensitive C-reactive protein (hsCRP) [6,12,13].

In contrast to the prognostic value of circulating MPO levels, the association of the −463 G/A polymorphism of MPO (rs 2333227) with the clinical outcome of these patients remains elusive. This allelic polymorphism affects the promoter region of the MPO gene, which is located 463 bp upstream of the gene. The GG genotype, which is present in about two-thirds of the Western population, is associated with a higher expression of MPO and consequently results in higher

Abbreviations: MPO, myeloperoxidase; PMN, polymorphonuclear neutrophil; $^{\bullet}$NO, nitric oxide; NT-proBNP, N-terminal pro-brain natriuretic peptide; hsCRP, high-sensitive C-reactive protein; LV, left ventricular; EF, ejection fraction.

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MPO concentrations in PMN after culturing compared to the AA or AG genotype [14].

Herein, we sought to investigate the association of the −463 G/A MPO promoter polymorphism with the long-term clinical outcome of patients with LV function impairment by employing a genotypic model in which the GG genotype was compared to the AA and AG genotypes and an allelic model that compared the G and A alleles.

Methods

Study outline

The study was approved by the local ethics committee of the University of Hamburg and was conducted in accordance with the Declaration of Helsinki. LV function was assessed either by LV angiography or by echocardiography; patients with an ejection fraction (EF) below 60% were considered eligible. Between May 2004 and November 2005 consecutive patients with impaired LV function of ischemic and nonischemic origin were included in the study. The presence or absence of coronary artery disease was determined by coronary angiography performed in all study participants. Patients with acute coronary syndromes within 1 month before inclusion, as well as patients with plasma creatinine levels above 3.5 mg/dl and those with acute infectious disease, malignancies, or autoimmune disorders at presentation, were excluded. After inclusion blood samples were collected for MPO, NT-proBNP, and hsCRP levels; white blood cell count; and determination of the MPO promoter polymorphism. During follow-up the patients were contacted by telephone to answer a standardized questionnaire. Confirmation of death, cause of death, and hospitalization were obtained from the individual's general practitioner or the hospital to which the patient was last admitted. Overall hospitalizations were defined as hospitalizations for any reason not scheduled before the time of inclusion and were further subclassified as cardiovascular and noncardiovascular hospitalizations.

Biochemical analysis

Plasma levels of MPO (Biosite, San Diego, CA, USA) and NT-proBNP (chemiluminescence immunoassay; Roche, Mannheim, Germany) were determined by ELISA according to the manufacturer's recommendations. All other laboratory variables were obtained by certified routine clinical chemistry procedures. MPO activity as determined by $^{\bullet}$NO consumption was determined as previously described [15].

Determination of the MPO −463 G/A polymorphism

After isolation of DNA from EDTA whole blood, fragments with the polymorphic site at −463 of the MPO gene were amplified by polymerase chain reaction (PCR) according to standard protocols. Listed primer sequences were as follows: −463 MPO forward, AGCGATTGTTTGAGCAGGTCA; −463 MPO backward, AGTTTCCTCGCCAATTTCAGG. The PCR product was digested with AciI restriction endonuclease (New England Biolabs, Beverly, MA, USA). Fragments were analyzed by agarose gel electrophoresis as previously described [16].

Statistical analysis

Sample size calculation was carried out in accordance with the method proposed by Freedman [17] for determination of the number of patients in clinical trials using the log-rank test and defining overall mortality as the primary end-point. Assuming a 3-year survival rate of 70% for our patients and expecting a difference in survival rate of an absolute percentage of 20%, the number of required patients was 109 and the number of required observed events was 21 to achieve a power $(1-\beta)$ of 80% with an α of 5%. The study population was tested for Hardy–Weinberg equilibrium using a goodness-of-fit χ^2 test [18]. To test which genetical model would provide the best fit to the data χ^2 statistics were employed. The genotypic model GG vs AG/AA (χ^2 4.74) was superior to the model AA vs AG/GG (χ^2 1.16) and was thus used for further analysis. Moreover, following an allelic model analyses were performed to compare the alleles A and G. Categorical data are presented as frequencies and percentages and were compared by χ^2 test or Fisher's exact test where appropriate. Continuous variables were tested for normal distribution using the Kolmogorov–Smirnov test. Normally distributed variables are presented as means ± standard deviation; for nonnormally distributed data the median and interquartile range are given. For comparison between groups Student's unpaired *t* test was used in the case of normal distribution; in the case of nonnormal distribution the Mann–Whitney *U* test was employed. Survival analyses were carried out to compare clinical outcomes of patients with GG genotype versus those with GA and AA genotype, to compare outcomes of patients within the lowest tertile of plasma MPO levels (<16.9 ng/ml) versus those in the two highest tertiles and to compare the G with the A allele. Kaplan–Meier curves were calculated from baseline to time of death, cardiovascular death, hospitalization, or cardiovascular hospitalization. Cardiovascular death was defined as death due to arrhythmias, cardiac arrest, acute coronary syndrome, cardiac pump failure, and pulmonary edema. Total mortality assessed by log-rank test using the genotypic model was defined as primary analysis. For univariate and multivariate Cox proportional hazard analyses the same end-points as for Kaplan–Meier curves were used. Two multivariate models were

Table 1
Baseline characteristics

	GG ($n=66$)	AA/AG ($n=50$)	*p*
Age, years	62.1 ± 12.7	59.6 ± 13.0	0.30
Gender, female	8 (12.1)	5 (10.0)	0.72
Body mass index	27.3 ± 5.4	26.7 ± 3.9	0.58
Coronary artery disease	43 (65.2)	27 (54.0)	0.22
LV function impairment			
Mild	16 (24.2)	17 (34.0)	0.21
Moderate	36 (54.5)	19 (38.0)	
Severe	14 (21.2)	14 (28.0)	
NT-proBNP, ng/L	712.0 [206.6–1454.0]	525.9 [147.4–1023.0]	0.21
Creatinine, mg/dl	1.03 [0.83–1.19]	1.07 [0.90–1.32]	0.11
hsCRP, mg/L	2.91 [1.42–7.60]	2.88 [1.85–8.46]	0.47
MPO, ng/ml	27.3 [14.7–48.6]	24.4 [12.9–48.6]	0.75
NO consumption, nM/s/ml	119.8 [63.4–230.0]	117.7 [75.0–180.3]	0.51
Blood count			
Hemoglobin, g/dl	13.9 ± 1.9	14.4 ± 1.6	0.14
Leukocytes, $\times 10^6$/ml	8.5 ± 2.0	7.7 ± 1.8	0.03
Platelets, $\times 10^6$/ml	236.2 ± 67.9	243.7 ± 81.2	0.59
Cardiovascular risk factors			
Hypertension	35 (53.0)	31 (62.0)	0.33
Hyperlipoproteinemia	40 (60.6)	33 (66.0)	0.55
Diabetes mellitus	20 (30.3)	13 (26.0)	0.61
Current smoking	17 (25.8)	10 (20.0)	0.47
Family history	6 (9.1)	9 (18.0)	0.16
Medication			
Acetylsalicylic acid	38 (57.6)	21 (42.0)	0.10
Clopidogrel	24 (36.4)	11 (22.0)	0.10
Beta blocker	54 (81.8)	41 (82.0)	0.98
ACE inhibitor	47 (71.2)	35 (70.0)	0.89
AT1 inhibitor	16 (24.2)	6 (12.0)	0.10
Statin	40 (60.6)	27 (54.0)	0.48
Loop diuretic	32 (48.5)	24 (48.0)	0.96
Thiazid diuretic	31 (47.0)	16 (32.0)	0.10
Spironolactone	29 (43.9)	22 (44.0)	0.99
Digitalis	25 (37.9)	20 (40.0)	0.82

ACE, angiotensin-converting enzyme, AT1, angiotensin II type 1 receptor. Values are displayed as means ± SD, median [interquartile range], or frequency (percentage).

employed. Model 1 included plasma levels of NT-proBNP, creatinine, hsCRP, and MPO and leukocyte count and LV function. Model 2 included the classical cardiovascular risk factors (hypertension, hyperlipoproteinemia, type 2 diabetes mellitus, nicotine abuse, family history) as well as age, gender, and BMI. For allelic analysis acetylsalicylic acid intake was added to Model 2 because the G allele was associated with a higher intake of acetylsalicylic acid at baseline. Univariate analyses were carried out for each covariate included in multivariate analyses. All statistical analyses were calculated using SPSS (version 13; SPSS, Chicago, IL, USA).

Results

One hundred sixteen consecutive patients with impaired LV function, who were electively admitted to this hospital (55.2%) or who electively presented to the outpatient clinic (44.8%), were included in the study. The median follow-up time was 1050 (interquartile range (IR) 991–1142) days. The genotypes of the study population were distributed according to Hardy–Weinberg equilibrium (χ^2 3.97; $p = 0.14$).

The mean age of the study population was 61 ± 13 years, and 11% of the included patients were female. Sixty-six patients (56.9%) were diagnosed with the GG genotype, 39 patients (33.6%) with the AG genotype, and 11 patients (9.5%) with the AA genotype. Seventy patients (60.3%) were diagnosed with coronary artery disease. Twenty-eight patients (24.1%) had mild left ventricular function impairment (EF 45–60%), 55 patients (47.4%) had moderate (EF 30–45%) and 33 patients (28.4%) displayed severely reduced LV function impairment (EF <30%). After the median follow-up time of 1050 (IR 991–1142) days, 25 patients (21.6%) had died, 20 (17.2%) had experienced cardiovascular death, 81 (69.8%) had been hospitalized, and 49 (42.2%) had been hospitalized for cardiovascular reasons. Myocardial infarction occurred in 1 patient of the study population; 15 patients underwent myocardial revascularization therapy, 15 patients had a stroke, and successful cardiopulmonary resuscitation was carried out in 2 patients.

Genotypic analyses

No significant differences between patients with the GG genotype and those with the GA or AA genotype were observed for baseline characteristics with the exception of leukocyte count. Patients exhibiting the GG genotype had a significantly elevated leukocyte count ($8.5 \pm 2.0 \times 10^6$/ml vs $7.7 \pm 1.8 \times 10^6$/ml; $p < 0.05$; see Table 1). Of the 66 patients with GG genotype, 19 (29%) died, 16 (24%) experienced cardiovascular death, 32 (70%) were rehospitalized, and 52 (79%) reached the combined end-point of death and hospitalization.

Log-rank test revealed that the GG genotype was associated with the overall survival of the patients included into the study (χ^2 5.80; $p = 0.016$). Moreover, the GG genotype was also associated with cardiovascular mortality (χ^2 6.18; $p = 0.013$), but not with hospitalization (χ^2 2.01; $p = 0.156$) or cardiovascular hospitalization (χ^2 2.20; $p = 0.138$). The corresponding Kaplan–Meier curves for these end-points are shown in Fig. 1.

Values for univariate Cox regression analyses are shown in Table 2. In addition to the GG genotype, creatinine, LV function, diabetes, and age could be identified as univariate predictors of death and cardiovascular death. Hyperlipoproteinemia was predictive of cardiovascular death, but not of overall death. Creatinine and male gender predicted hospitalization and cardiovascular hospitalization in univariate analysis. Diabetes was predictive of overall hospitalization but not of cardiovascular hospitalization. Multivariate Cox regression analyses revealed the GG genotype to be independently associated with all-cause mortality and cardiovascular mortality according to all

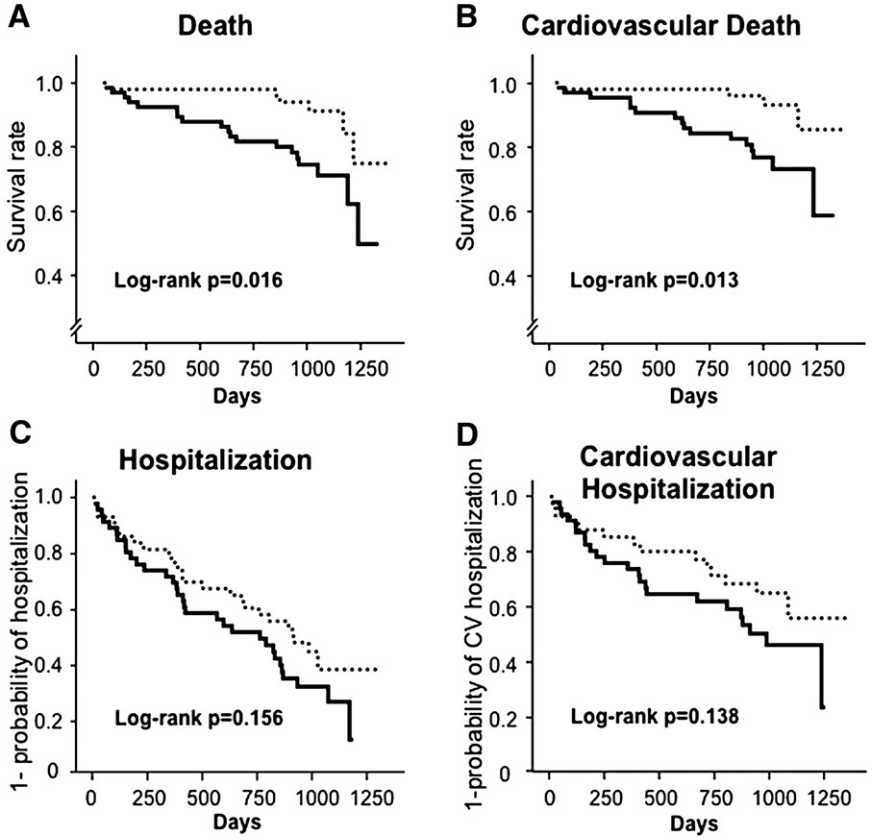

Fig. 1. Genotypic model. Kaplan–Meier analyses for death, cardiovascular death, hospitalization, and cardiovascular hospitalization. Solid line, GG genotype; dotted line, AA/AG genotype.

Table 2
Univariate analyses

	Death	Cardiovascular death	Hospitalization	Cardiovascular hospitalization
GG genotype	2.99 (1.19−7.50)*	3.74 (1.25−11.21)*	1.43 (0.84−2.43)	1.65 (1.03−2.64)*
NT-proBNP	1.00 (0.91−1.12)	1.00 (0.91−1.12)	1.00 (0.91−1.12)	1.00 (0.91−1.12)
Creatinine	1.96 (1.37−2.80)*	2.10 (1.44−3.05)*	2.06 (1.42−2.98)*	2.03 (1.33−3.08)*
hsCRP	1.00 (0.99−1.02)	1.01 (0.99−1.02)	1.01 (1.00−1.02)	1.00 (0.98−1.02)
MPO	1.00 (0.99−1.01)	1.00 (1.00−1.01)	1.00 (0.99−1.00)	1.00 (1.00−1.01)
Leukocytes	1.11 (0.96−1.27)	1.03 (0.87−1.21)	1.01 (0.97−1.17)	0.96 (0.85−1.09)
LV function impairment	2.33 (1.54−3.53)*	2.13 (1.36−3.36)*	0.79 (0.61−1.03)	0.80 (0.58−1.11)
Hypertension	1.10 (0.62−1.93)	0.75 (0.40−1.39)	1.12 (0.77−1.63)	1.24 (0.78−1.97)
Hyperlipoproteinemia	1.83 (0.99−3.41)	2.82 (1.29−6.14)*	1.32 (0.90−1.95)	1.31 (0.82−2.13)
Diabetes	2.10 (1.20−3.67)*	2.65 (1.42−4.93)*	1.78 (1.18−2.67)*	1.39 (0.83−2.33)
Current smoker	0.80 (0.40−1.60)	0.81 (0.37−1.76)	1.01 (0.64−1.59)	1.15 (0.67−1.96)
Family history	0.82 (0.35−1.93)	0.71 (0.25−2.01)	1.05 (0.62−1.79)	0.96 (0.49−1.86)
Age	1.08 (1.04−1.11)*	1.08 (1.05−1.12)*	1.01 (0.99−1.02)	0.99 (0.98−1.01)
Gender	1.11 (0.47−2.62)	0.87 (0.31−2.46)	2.12 (1.24−3.62)*	2.21 (1.19−4.14)*
BMI	0.93 (0.91−1.04)	0.97 (0.89−1.04)	1.01 (0.97−1.05)	0.99 (0.95−1.05)

* $p<0.05$.

employed models (see Table 3). Importantly, the leukocyte count was included in these multivariate analyses to rule out the possibility that the association between genotype and clinical outcome was not driven by the observed difference in leukocyte counts. For the end-point of hospitalization no association was observed in univariate analysis. For cardiovascular hospitalization an association was observed after univariate analysis; however, the association did not remain significant after univariate adjustment for creatinine and after multivariate adjustment according to Model 1. Table 3 shows the results of the Cox proportional hazard analyses for all four end-points. Univariate adjustments for covariates, which also proved to be independent predictors, are also displayed in Table 3. The sensitivity and specificity of the GG genotype to diagnose the indicated end-points within the observation interval were 76.0 and 48.4% for death, 80.0 and 47.9% for cardiovascular death, 63.0 and 57.1% for hospitalization, and 62.7 and 57.6% for the combined end-point, respectively.

Table 3
Univariate and multivariate Cox proportional hazard analyses for genotypic model

Variable	Hazard ratio (95% CI)	p
All-cause mortality		
GG genotype	2.99 (1.19−7.50)	0.020
Adjusted for age	3.02 (1.20−7.61)	0.019
Adjusted for LV function	3.21 (1.28−8.10)	0.013
Multivariable model		
Model 1	3.16 (1.17−8.53)	0.024
Model 2	2.88 (1.13−7.33)	0.026
Cardiovascular mortality		
GG genotype	3.74 (1.25−11.21)	0.019
Adjusted for age	3.80 (1.26−11.44)	0.018
Adjusted for LV function	4.00 (1.33−12.05)	0.014
Multivariable model		
Model 1	4.05 (1.25−13.06)	0.019
Model 2	3.49 (1.15−10.61)	0.027
Hospitalization		
GG genotype[a]	1.43 (0.84−2.43)	0.194
Cardiovascular hospitalization		
GG genotype	1.65 (1.03−2.64)	0.038
Adjusted for creatinine	1.49 (0.92−2.41)	0.106
Adjusted for hyperlipoproteinemia	1.72 (1.07−2.77)	0.025
Adjusted for BMI	1.67 (1.04−2.69)	0.035
Multivariable model		
Model 1	1.40 (0.85−2.29)	0.182
Model 2	1.97 (1.18−3.25)	0.009

CI, confidence interval. Model 1, NT-proBNP, creatinine, hsCRP, MPO, leukocyte count, LV function. Model 2, hypertension, hyperlipoproteinemia, diabetes, current smoking, family history, age, gender, body mass index.
[a] No adjustments were carried out because there was no significance on univariate analysis.

Plasma MPO levels were, neither as continuous nor as categorical variable, not associated with any of the investigated end-points (χ^2 for the lowest vs the two highest MPO tertiles 1.69, $p=0.194$ for death; χ^2 2.634, $p=0.105$ for cardiovascular death; χ^2 0.160, $p=0.689$ for hospitalization; and χ^2 0.083, $p=0.773$ for cardiovascular hospitalization). Moreover, no significant difference in plasma MPO levels was observed between patients who reached end-points and those who did not (death, 30.0 (IR 17.9–56.2) vs 23.7 (13.0–48.3) ng/ml, $p=0.219$; cardiovascular death, 33.1 (18.4–97.5) vs 23.6 (13.1–45.4) ng/ml, $p=0.052$; hospitalization, 23.9 (14.0–47.4) vs 23.7 (12.3–47.2) ng/ml, $p=0.372$; combined end-point, 24.6 (15.8–49.6) vs 23.7 (12.3–47.2) ng/ml, $p=0.259$). However, in the subgroup of patients with severely impaired LV function (EF <30%), MPO plasma levels were significantly higher in those participants who reached the end-points death and cardiovascular death (33.6 (IR 15.5–68.9) vs 21.8 (14.2–28.5) ng/ml, $p=0.04$; and 38.4 (20.0–278.2) vs 22.6 (14.0–30.5) ng/ml, $p=0.018$, respectively). Importantly, leukocyte levels in this subgroup between patients reaching the end-points did not differ from those who were event-free (death, 7.8 ± 1.3 vs 7.8 ± 1.7, $p=0.97$; cardiovascular death, 7.8 ± 1.5 vs 7.8 ± 1.6, $p=0.99$).

Neither MPO plasma levels nor MPO activity showed an association with the −463 G/A promoter polymorphism (data not shown).

Allelic analyses

At baseline the G allele was associated with higher leukocyte counts ($8.28\pm1.97\times10^6$/ml vs $7.61\pm1.88\times10^6$/ml, $p=0.02$) and a higher frequency of acetylsalicylic acid intake (38% vs 56%, $p=0.02$). All remaining baseline parameters revealed no difference between the two alleles.

The G allele was associated with all-cause mortality (χ^2 5.49, $p=0.019$) and cardiovascular mortality (χ^2 5.33, $p=0.021$). Associations with hospitalization (χ^2 2.69, $p=0.101$) and cardiovascular hospitalizations (χ^2 3.56, $p=0.059$) failed to reach statistical significance. The Kaplan–Meier graphs for the MPO alleles are shown in Fig. 2.

The G allele was independently associated with all-cause mortality according to both models of multivariate Cox regression analysis. For cardiovascular death an independent association was observable only according to Model 1 (Table 4).

Discussion

Herein we report the GG genotype of the −463 G/A MPO promoter polymorphism to be associated with an increased long-

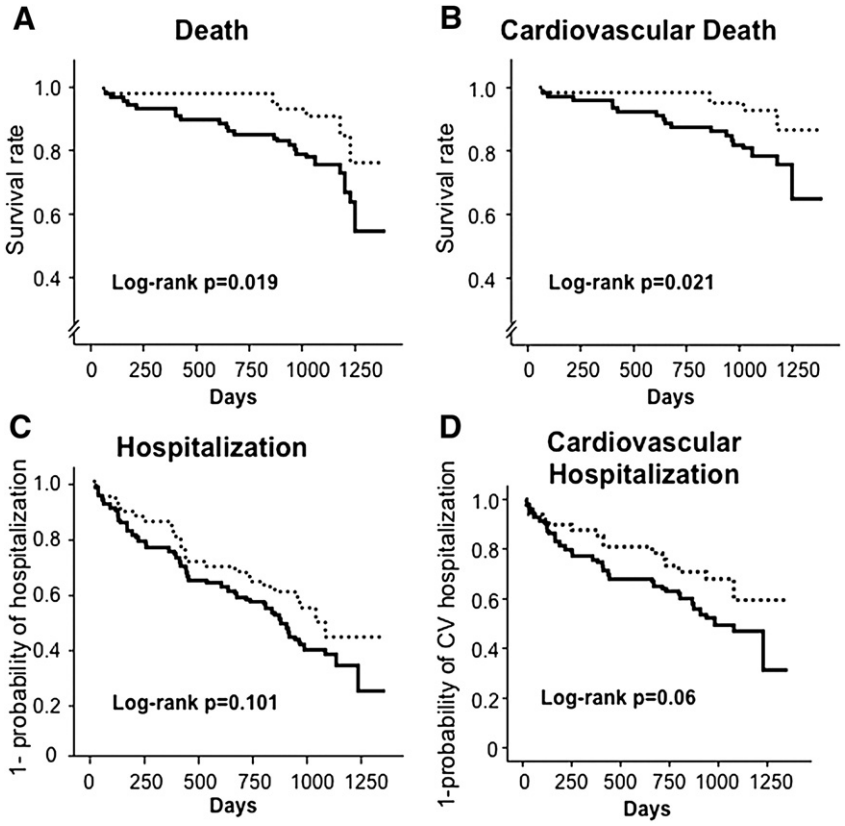

Fig. 2. Allelic model. Kaplan–Meier analyses for death, cardiovascular death, hospitalization, and cardiovascular hospitalization. Solid line, G allele; dotted line, A allele.

term all-cause mortality of patients with impaired LV function. This association remained present even after multivariate adjustment for plasma levels of NT-proBNP, creatinine, hsCRP, and MPO; the leukocyte count; and LV function as well as after adjustment for classical cardiovascular risk factors, age, gender, and BMI. Similarly, in an allelic model the G allele was shown to be independently associated with all-cause mortality. In contrast, plasma MPO levels were not associated with the clinical outcome in this group of patients characterized by a broad range of LV dysfunction.

Table 4
Univariate and multivariate Cox proportional hazard analyses for allelic model

Variable	Hazard ratio (95% CI)	*p*
All-cause mortality		
G allele	2.50 (1.13−5.57)	0.023
Adjusted for age	2.39 (1.01−5.32)	0.033
Adjusted for diabetes	2.48 (1.11−5.51)	0.026
Adjusted for NT-proBNP	2.76 (1.24−6.17)	0.013
Adjusted for LV function	2.58 (1.16−5.74)	0.020
Multivariable model		
Model 1	2.45 (1.07−5.61)	0.035
Model 2	2.27 (1.01−5.08)	0.047
Cardiovascular mortality		
G allele	2.86 (1.12−7.30)	0.028
Adjusted for age	2.39 (1.08−5.32)	0.033
Adjusted for diabetes	2.79 (1.09−7.13)	0.032
Adjusted for NT-proBNP	3.27 (1.27−8.40)	0.014
Adjusted for LV function	2.95 (1.15−7.54)	0.024
Multivariable model		
Model 1	1.41 (0.96−2.07)	0.081
Model 2	2.48 (0.96−6.40)	0.060
Hospitalization		
G allele	1.43 (0.92−2.19)	0.105
Cardiovascular hospitalization		
G allele	1.71 (0.97−3.02)	0.064

CI, confidence interval. Model 1, NT-proBNP, creatinine, hsCRP, MPO, leukocyte count, LV function. Model 2, hypertension, hyperlipoproteinemia, diabetes, current smoking, family history, age, gender, body mass index, acetylsalicylic acid intake. Multivariate analyses were not carried out for hospitalization and cardiovascular hospitalization because associations were not significant in univariate analysis.

The GG-genotype of the −463 G/A MPO promoter polymorphism has been linked to progression and adverse outcome of various diseases such as hepatic fibrosis [19], multiple sclerosis [20], and cystic fibrosis [21]. Furthermore, this genotype has been connected to vascular inflammatory pathology, in which leukocyte activation and concomitant release of MPO are now considered critical pathophysiological events: patients exhibiting the GG-genotype tended to have progressive arteriosclerosis [22], were revealed to have a 1.6-fold increased risk of coronary artery disease [16], and displayed reduced coronary flow reserve [23]. Moreover, in patients with stable coronary artery disease, the GG genotype was associated with decreased survival [24]. So far, however, the role of this polymorphism in the outcome of heart failure patients remains elusive.

Heart failure is no longer regarded solely as a mechanicostructural disorder but appreciated as a systemic disease involving many other organ systems. In addition to neurohumoral imbalance, the activation of proinflammatory pathways, free radical generation, and monocyte and lymphocyte activation are viewed as important pathophysiological phenomena [25]. Despite the established role for inflammatory pathways in this disease, a direct involvement of PMN in the pathology of heart failure was not anticipated until recently: circulating MPO plasma levels and a readout of systemic leukocyte and in particular PMN activation were particularly associated with advanced LV dysfunction [6,12,13].

Consistent with these findings we herein report the GG genotype to be associated with all-cause and cardiovascular mortality in patients with LV dysfunction, even after adjustment for established risk factors of cardiovascular mortality. In contrast, no association of

the genotype with hospitalization was observed and an association with cardiovascular hospitalization was observed only in univariate analysis, whereas it was lost after adjustment for creatinine and after multivariate adjustment according to Model 1. In the allelic model the G allele was also demonstrated to be independently associated with an increase in all-cause mortality, whereas for cardiovascular morality this association could not be verified only in multivariate analysis. No association between the G allele and hospitalization or cardiovascular hospitalization was observed in the allelic model.

It is intriguing to speculate whether our findings could be explained by increased expression of MPO and thus an increased overall burden of MPO, which has been ascribed to the G allele of this polymorphism [14]. However, although this tenet is strongly supported by mechanistic in vitro studies [26], recent studies have challenged the association of the −463 G/A polymorphism with increased MPO expression [27,28]. In all of these studies MPO content or activity has been measured in neutrophils. However, intracellular MPO levels and activity of PMN are dependent on the activation state of the PMN, with activation leading to a reduction in MPO after its secretion [29]. None of the mentioned studies have included other markers indicating the activation state of neutrophils, which can potentially confound the correlation between MPO expression and levels of MPO measured in neutrophils. Interestingly, in the two recently published studies equivocal findings were also reported for the −129 G/A MPO polymorphism, with one study reporting and one study not reporting an association between polymorphism and intracellular MPO activity. This further supports the concept that neutrophil activation plays a role as a potentially confounding factor. In our study there was no difference in plasma MPO activity in terms of $^{\bullet}$NO consumption between the three genotypes.

On the other hand, circulating MPO protein levels in plasma might not directly correlate with PMN activation because MPO is immobilized in the vascular wall because of electrostatic interactions with the endothelial glycocalyx. These considerations may explain why a family-based genetic study did not show a correlation between MPO plasma levels and the genotype of the −436 G/A polymorphism [30] and ultimately also the disparity between MPO polymorphism and MPO plasma levels in our study. Thus, the reliable assessment of the overall MPO burden, which indeed reflects the rate of expression of MPO, is challenging and the question whether MPO polymorphisms are associated with levels of MPO expression has not been convincingly clarified in a clinical setting.

At first glance the results of this study seem to contradict previous findings of our group reporting a lack of *diagnostic* value of the −463 G/A MPO promoter polymorphism in identifying patients with LV dysfunction [5]. In this cross-sectional study it was reported that MPO plasma levels are elevated in patients with impaired LV function, whereas no such association was observed for the −463 G/A MPO polymorphism. It is important to discern that the current study in contrast is a longitudinal study, which aims to assess the *prognostic* value of the −463 G/A MPO promoter polymorphism on the outcome of patients with impaired LV function and that thus it seems reasonable to regard both observations as compatible.

Interestingly, the present study did not demonstrate an association between plasma MPO levels and clinical outcome. Only if the analysis was restricted to patients with severely impaired LV function (EF <30%) were the plasma MPO levels higher in non-event-free patients. This is in accordance with the findings of Tang et al. [6], who showed increased plasma MPO levels to be predictive of an adverse long-term clinical outcome in patients with *severely* impaired LV function (EF <25%). The fact that MPO plasma levels predominantly predict mortality in patients with advanced stages of heart failure resembles the observation that the prognostic value of circulating MPO levels in coronary artery disease is most profound in patients with acute coronary syndromes [14,31–34]. As discussed above, plasma and serum levels of MPO seem to be of particular prognostic information when MPO levels are acutely and highly elevated. In this context it might also be important to note that in a previous study, which demonstrated elevated MPO plasma levels in patients with impaired LV function, a cutoff for the EF of <50% instead of <60% was chosen. This difference in general makes it difficult to compare both studies and might account for some of the observed differences.

Death and cardiovascular death were independently associated with the −463 G/A polymorphism in this study, whereas no independent association was observed for hospitalization or cardiovascular hospitalization. Considering the limited sample size and given a study design with overall mortality being the primary endpoint it can only be speculated whether patients with the GG genotype might also be at a higher risk for hospitalization. Future studies should be performed to address this question.

The results of this study are certainly limited by its small size, in particular with respect to the subgroup of patients with severely impaired LV function. However, the careful selection of patients, the long-term and complete follow-up, and the combined analysis of established biomarkers in heart failure in addition to circulating MPO and its genotype as performed in this study further underscore the involvement of leukocytes in the pathophysiology of this disease. These results advocate further investigations aiming to elucidate the precise molecular mechanisms by which MPO aggravates this disease and to explore this enzyme as a potential target for treatment.

Acknowledgments

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Myeloperoxidase attracts neutrophils by physical forces

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Recruitment of polymorphonuclear neutrophils (PMNs) remains a paramount prerequisite in innate immune defense and a critical cofounder in inflammatory vascular disease. Neutrophil recruitment comprises a cascade of concerted events allowing for capture, adhesion and extravasation of the leukocyte. Whereas PMN rolling, binding, and diapedesis are well characterized, receptor-mediated processes, mechanisms attenuating the electrostatic repulsion between the negatively charged glycocalyx of leukocyte and endothelium remain poorly understood. We provide evidence for myeloperoxidase (MPO), an abundant PMN-derived heme protein, facilitating PMN recruitment by its positive surface charge. In vitro, MPO evoked highly directed PMN motility, which was solely dependent on electrostatic interactions with the leukocyte's surface. In vivo, PMN recruitment was shown to be MPO-dependent in a model of hepatic ischemia and reperfusion, upon intraportal delivery of MPO and in the cremaster muscle exposed to local inflammation or to intraarterial MPO application. Given MPO's affinity to both the endothelial and the leukocyte's surface, MPO evolves as a mediator of PMN recruitment because of its positive surface charge. This electrostatic MPO effect not only displays a so far unrecognized, catalysis-independent function of the enzyme, but also highlights a principal mechanism of PMN attraction driven by physical forces. (*Blood*. 2011; 117(4):1350-1358)

Introduction

Recruitment of polymorphonuclear neutrophils (PMNs) is considered a hallmark in host defense.[1] With vessel- and tissue-infiltrating PMNs also being mechanistically linked to a broad range of vascular inflammatory diseases including coronary artery disease, heart failure and ischemia/reperfusion injury, the pathophysiologic significance of PMN motility reaches far beyond innate immunity.[2-4] So far, PMN migration is primarily viewed to be energy-dependent and cytoskeleton-dependent with G-protein coupled receptors and integrins initiating and orchestrating signaling pathways obligatory for neutrophil adhesion, spreading, diapedesis and chemotactic agitation.[5-7] On activation, PMN releases myeloperoxidase (MPO), an abundant heme protein in PMN with potent bactericidal and vascular-inflammatory properties. The enzyme accumulates along the endothelium and in the subendothelial space,[8] where it binds to anionic glycocalyx residues such as heparan sulfate glycosaminoglycans. Given the affinity of MPO to both PMN and endothelial cells, we evaluated whether MPO affects PMN locomotion and recruitment.

Methods

Isolation of PMN

Peripheral blood was drawn from healthy human volunteers and heparinized, and isolation of PMN was performed as previously described.[9] In brief, after sedimentation in dextran solution (45 mg/mL), the supernatant was placed over Histopaque 1077 (Sigma-Aldrich) for density gradient centrifugation. Remaining red blood cells were eliminated by hypotonic lysis and the pellet was resuspended in Hanks balanced salt solution (HBSS; Invitrogen) containing 0.25% bovine serum albumin (BSA) (*cell buffer*) and stored on ice until use.

Microslide motility experiments

Microslides (Ibidi μ-slide I, ibitreat; IBIDI) were coated with fibrinogen (250 μg/mL). PMN or red blood cells (RBCs; 1×10^6/mL in cell buffer or plasma, where indicated) were incubated with 4-amino-benzoic acid hydrazide (ABAH; 50μM, Calbiochem) and H_2O_2 (50μM, 30 minutes, Merck), (-)-Blebbistatin (100μM, 30 minutes, Sigma-Aldrich), Cytochalasin D (1μM, 30 minutes, Sigma-Aldrich), LY 294002 (200μM, 2 hours, Calbiochem), or with sodium azide and 2-deoyxglucose (50mM, 1 hour, Sigma-Aldrich). 100 μg of poly-L-arginine (PLA, Sigma-Aldrich), protamine (Protamin Valeant, MEDA Pharma), or histone H2A (Millipore) was added directly before application to microslides, where indicated. For experiments in pH 9.2, PMN were resuspended and lyophilized MPO was reconstituted in cell buffer of pH 9.2. 100 μL of PMN suspension was applied into the microslide channel; cells were allowed to attach for 5 minutes. Subsequently, 30 μL of MPO (120nM unless otherwise indicated, Planta Natural Products), recombinant, mutant MPO Q91T or M243T (120nM), IL-8 (25nM, PeproTech), or human serum albumin (120nM, Sigma-Aldrich) in cell buffer or plasma, optionally supplemented with inhibitors, PLA, protamine, or histone, respectively, was added from one side into the channel. Where indicated, Sepharose beads with anionic

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(HiTrap SP HP, GE Healthcare/Amersham) or cationic (HiTrap Q HP, GE Healthcare) coating were used instead of PMN. The area of the steepest gradient was calculated as before (www.ibidi.com) and captured for 20 minutes with a light microscope (CK 2, Olympus)–mounted charge-coupled device (CCD) camera (Retiga 1300, QImaging).

Image acquisition and processing of in vitro experiments

During leukocyte or Sepharose-bead motility experiments, pictures were captured at 0 minutes and 20 minutes, and time-lapse imaging was used to create a stack of images (iVision version 4.0, BioVision). The percentage of cells oriented in an angle of less than 180° toward the segment of upward gradient was evaluated (PMN in up-gradient segment). Twenty PMNs per stack were tracked and transformed to 2-dimensional plots, with the x-direction indicating the upward gradient orientation (manual tracking and chemotaxis-tool, created for ImageJ by IBIDI). The mean accumulated distance and the x-forward-migration index (x-displacement × accumulated distance^{-1}) were calculated.

Quantification of f-actin with flow cytometry

PMN (1×10^6/mL cell buffer) were incubated with MPO (120nM) or IL-8 (25nM). After indicated times, cells were fixed, permeabilized, and incubated with Alexa Fluor 488–Phalloidin (20 minutes, 5 U/mL in phosphate buffer solution [PBS] with 1% BSA, Molecular Probes). Flow cytometric analysis was performed using a FACSCalibur flow cytometer and CellQuest Pro 5.1 software (BD Biosciences).

Mouse liver experimental protocols

Male C57BL/6J (wild-type; WT) and MPO_tm1lus (*Mpo*$^{-/-}$) mice aged 12-15 weeks were treated either to occlude the blood supply to the median and left hepatic lobes with an atraumatic vascular clamp for 90 minutes, and reperfusion then initiated; or, their inferior mesenteric veins were injected with recombinant human serum albumin (Prospec); recombinant active MPO (R&D Systems); inactive MPO M243T, Q91T MPO, or recombinant MPO preincubated with 50μM ABAH and H_2O_2 (4 μg in 200 μL of physiologic saline), respectively. Two hours after intraportal injection or 20 hours after initiation of reperfusion, mice were again anaesthetized. The liver was flushed by intraportal injection of 1 mL of PBS and excised. All animal experiments were approved by the local ethics committees (Hamburg, Germany, G100/06) and in accordance with the US National Research Council's "Guide for the Care and Use of Laboratory Animals."

Quantitative assessment of hepatic PMN infiltration by immunohistochemistry

Frozen liver specimens were fixed with acetone. Sections were incubated with anti-mouse Ly6G primary antibody (ratio of 1:40; Hycult Biodiagnostics), and endogenous peroxidase activity was blocked. The secondary antibody was horseradish peroxidase (hrp)–labeled rabbit IgG to rat IgG (1:100, Dako), and the tertiary antibody was hrp-labeled goat anti–rabbit IgG (1:500, Vector Laboratories) in 3% normal mouse serum. PMNs were stained with 3-amino-9-ethylcarbazol (AEC) solution and tissue was counterstained with hematoxyline. The number of neutrophils attached to sinusoids or extravasated into hepatic parenchyma was counted in 30 high-power fields per animal (original magnification ×600) under a light microscope (Olympus).

Mouse cremaster muscle experimental protocols

Recombinant murine tumor necrosis factor (TNF)-α (500 ng per mouse, R&D) in sterile saline was injected into the scrotum of male C57BL/6J (WT) mice and MPO_tm1lus (*Mpo*$^{-/-}$) mice aged 12-15 weeks. Two hours after injection, the carotid artery was cannulated and the cremaster muscle surgically prepared for intravital microscopy as previously described.[10] During intravital microscopy, the cremaster muscle preparation was continuously superfused with a 37°C warm buffer solution. In separate experiments, the cremaster muscle was surgically prepared without prior treatment (trauma induced inflammation). Twenty minutes after surgical preparation of the cremaster muscle, the animals received a bolus injection of active MPO (20 μg per mouse) or recombinant, inactive MPO Q91T (20 μg per mouse) in a volume of 0.2 mL of sterile saline, or an injection of saline alone, into the carotid artery. During all experiments systemic blood samples were obtained (10 μL in 90 μL of Tuerk solution; Merck) for quantification of systemic white blood cell count. All animal experiments were approved by the local ethics committees (Regierung von Oberbayern, Az. 55.2-1-54-2531-80-07) and in accordance with the "Guide for the Care and Use of Laboratory Animals."

Intravital microscopy and data analysis

Intravital microscopy was performed on an upright microscope (Olympus BX51) with a saline immersion objective (original magnification 40×, 0.8 numerical aperture) as described.[10] Experiments were recorded on an S-VHS recorder using a CCD camera (model CF8/1, Kappa) for later offline analysis. Vessel diameter, segment length of postcapillary cremaster muscle venules, and PMN diameter were measured with a digital image processing system.[11] Centerline red blood cell velocity was determined by a dual photodiode and a digital online cross-correlation program (Circusoft Instrumentation) and used for offline calculation of mean blood flow velocity and wall shear rates as previously reported.[12] Supplemental Tables 1 and 2 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) give an overview of microvascular parameters observed during intravital microscopy experiments. PMN adherence was quantified by counting the number of leukocytes per mm^2 of vessel surface that remained stationary for more than 30 seconds.

Whole-mount histology

To assess leukocyte extravasation on intrascrotal injection of TNF-α, whole mounts of cremaster muscles were prepared as previously described.[13] Leukocyte extravasation was quantified by counting the number of extravascular cells per mm^2 of cremaster muscle tissue using a Zeiss upright microscope with an oil immersion objective (original magnification 100×, 1.3 numerical aperture).

MPO and Alcian blue staining

For hepatic MPO staining, human MPO (4 μg, Planta Natural Products) or human serum albumin (4 μg, Sigma-Aldrich) was injected to the mesenteric vein of C57Bl/6J mice as described. Hepatic sections were incubated with the primary antibody to human MPO (rabbit, Calbiochem). For cremasteric MPO staining, human MPO (20 μg) was injected into the carotid artery of WT mice, and after 10 minutes an antibody to human MPO (40 μg, rabbit, Calbiochem) was injected. After another 10 minutes, the vena cava inferior was incised and 3 mL of PBS were flushed via the carotid catheter. Where indicated, Alcian blue 8 GX (1 mL, 0.1% solution, Sigma-Aldrich) was injected into the artery and flushed with 1 mL of PBS and 1 mL of 4% formaldehyde. The cremaster muscle was excised as described, placed on a microslide, and fixed with 4% formaldehyde. All samples were treated with secondary antibody Alexa 488 to rabbit IgG (Molecular Probes) and tissue was counterstained with DAPI. Images were acquired with a Leica microscope (DMLB) and IVision software.

Statistical analysis

Data were tested for normal distribution using the Kolmogorov-Smirnov test. When normality was shown, Student unpaired *t* test was used for pairwise comparison; otherwise, the Mann-Whitney rank sum test was used. A before-after comparison was performed with the Student paired *t* test. For multiple comparison, one-way ANOVA followed by a Bonferroni post hoc test, or Kruskal-Wallis ANOVA on ranks test followed by Dunn post hoc test, was used as appropriate. A P value $< .05$ was considered statistically significant. All statistical analyses were carried out with SPSS Version 13 (SPSS). Data are presented as mean ± SEM.

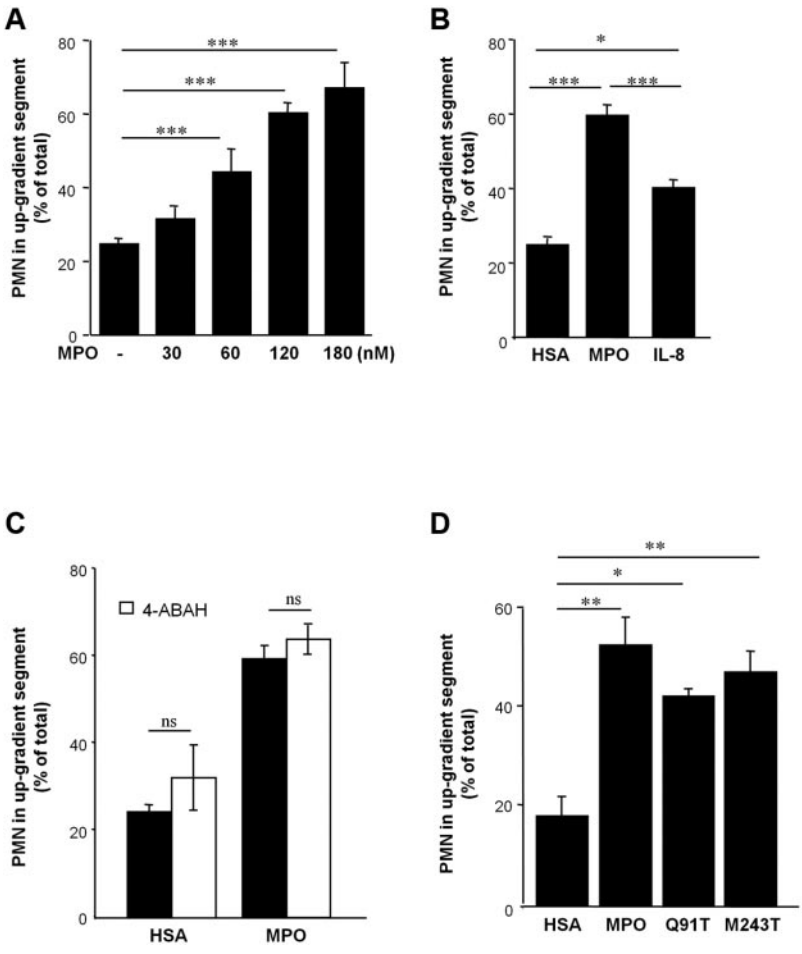

Figure 1. MPO-dependent PMN motility in vitro. PMN motility was evaluated at the steepest gradient of a chemotactic factor or control in Ibidi microslides. (A) MPO provoked directed locomotion of PMN in a concentration-dependent manner (n = 4, one-way ANOVA; $P < .0001$). (B) Directed PMN motility after application of human serum albumin (HSA, n = 21), MPO (n = 34), and interleukin-8 (IL-8, n = 9) was investigated (one-way ANOVA; $P < .0001$). (C) Directed motility toward HSA or MPO was evaluated on preincubation with the MPO-inhibitor 4-amino-benzoic acid hydrazide (ABAH; white bars, n = 3-4). (D) MPO-variants Q91T (n = 4) and M243T (n = 9) devoid of catalytic activity also yielded increased directed PMN motility (one-way ANOVA $P < .003$). Number of independent experiments denoted by n; number of donors of PMN ≥ 3. Bars represent means; error bars, standard error of the mean (SEM). *$P < .05$, **$P < .01$, ***$P < .001$.

Results

To test PMN motility in response to MPO, isolated human PMN added to microslides were challenged with MPO administered to one pole of the chamber. The resulting gradient of MPO provoked PMN motility in a concentration-dependent manner (Figure 1A). The presence of human plasma did not affect MPO-directed motility (Suppl. Figure 1A). The extent of directed PMN locomotion toward MPO was significantly elevated compared with human serum albumin (HSA) and the chemokine interleukin-8 (IL-8, Figure 1B), with eosinophil peroxidase (EPO) attracting PMN to a similar extent as MPO (supplemental Figure 1B). Interestingly, inactivation of MPO by the inhibitor 4-amino-benzoic acid hydrazide (ABHA) did not attenuate MPO-dependent PMN motility. Furthermore, 2 MPO variants, which lack either total enzymatic activity (Q91T) or the chlorinating, brominating, NO-oxidizing and NO-nitrating activity of the enzyme (M243T[14]), provoked PMN attraction to a similar extent as active MPO, thus indicating a mechanism that operates irrespective of the enzyme's catalytic activity (Figure 1C, D).

Remarkably, although MPO-exposed PMN traveled a shorter distance compared with IL-8, MPO-dependent motility was strikingly more vectored (as expressed by the x-forward-migration index[15]; Figure 2A-C, supplemental Figure 2). These differences between MPO-evoked compared with IL-8–evoked motility were accompanied by a discrepancy in cell morphology: PMN exposed to MPO lacked the morphologic characteristics observed on migration in response to IL-8, that is, polarization and formation of lamellipodia[16] (Figure 2D). Given these directional and morphologic peculiarities in PMN motility toward MPO, characterization of central intracellular signaling cascades involved in mediating leukocyte migration were explored. Whereas inhibition of nonmuscle myosin II by blebbistatin, phosphatidylinositol 3-kinase activity by LY 294002, and actin filament polymerization using cytochalasin D impaired IL-8-induced migration,[17-19] MPO-induced PMN motility remained unaffected. The rise in filamentous actin content (f-actin), which is a prerequisite of active PMN migration and observed in IL-8 treated cells, was blunted in MPO-exposed cells. This indicates that PMN motility on MPO exposure is independent of cytoskeletal rearrangements (Figure 2E, F). Instead, MPO provoked passive PMN locomotion irrespective of energy consumption, as evidenced by preserved cell motility in the presence of respiratory chain and glycolysis-inhibiting sodium azide and 2-deoxyglucose, respectively (Figure 2G). Moreover, PMN movement toward MPO was retained after fixation of the cells using methanol (Figure 3A).

Given that MPO-dependent PMN motility was revealed to be independent of energy-consuming cytoskeletal modifications, electrostatic interactions between the cationic MPO and the negatively charged PMN-surface were explored. In support of electrostatic interactions as a prerequisite for MPO-evoked PMN motility, alkalization of the assay buffer to the isoelectric point of MPO (p*I* 9.2) entirely prevented motility of methanol-fixed PMN (Figure

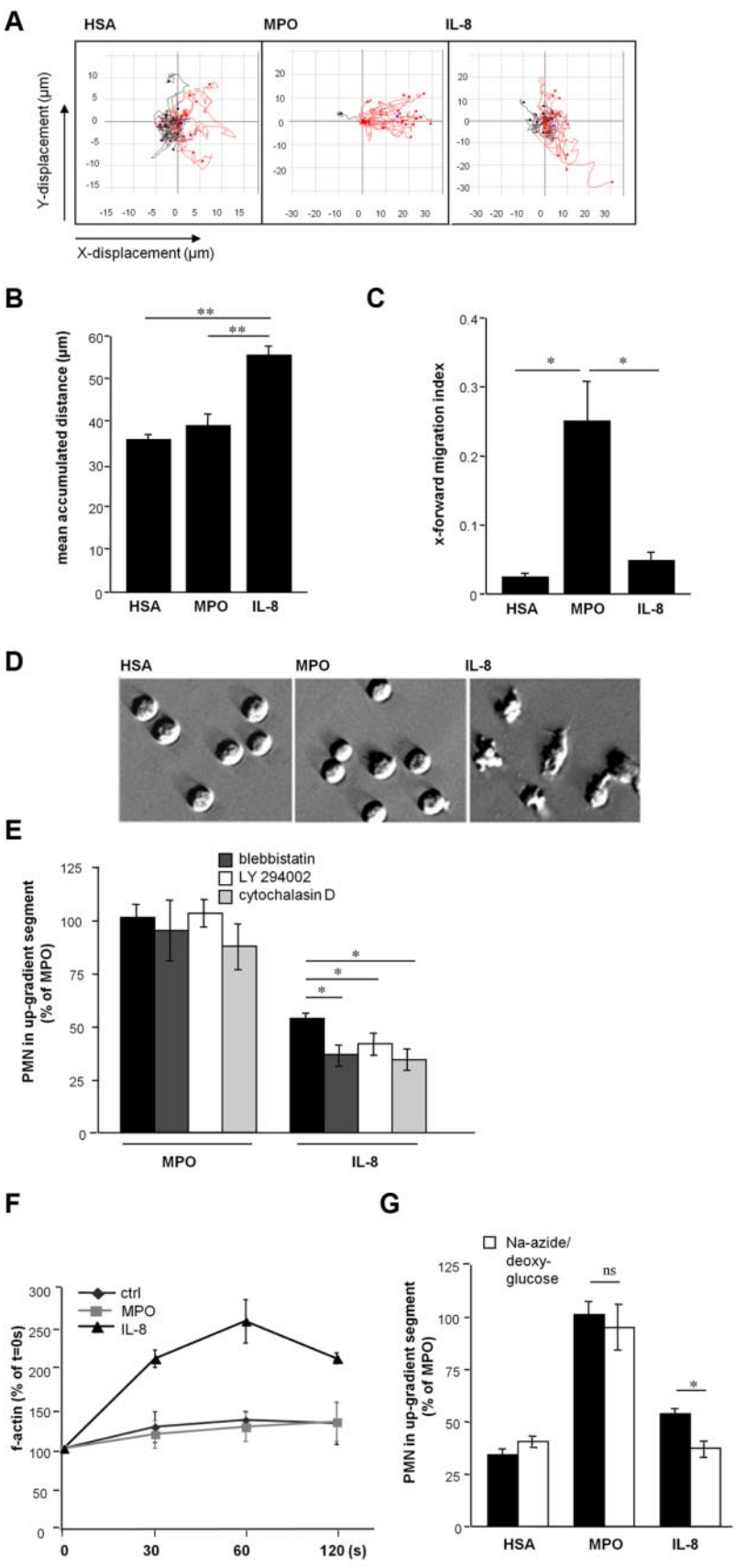

Figure 2. MPO-mediated PMN motility is highly directed and independent of cytoskeletal rearrangements. (A) Plots of PMN locomotion in microslides are depicted, red lines represent tracks of cells moving toward the up-gradient segment. Cell motility was followed under an Olympus CK2 inverted microscope with a mounted CCD camera (Retiga 1300, QImaging). Time-lapse microscopy was performed with iVision version 4.0 (Biovision) software and tracks created with ImageJ. (B) The mean accumulated distance of plotted tracks was calculated (n = 3-4 plots including 20 tracks, respectively, one-way ANOVA $P < .002$). (C) The x-forward–migration index (x-displacement × accumulated distance^{-1}) of plotted tracks (n = 3-4 plots including 20 tracks) represents the extent of vectored PMN movement (one-way ANOVA $P < .01$). (D) PMN administered to microslides in HSA-, MPO-, and IL-8–gradients were visualized with relief contrast microscopy (magnification ×400, digital interference contrast (DIC) microscopy, Olympus CKX31). (E) Motility toward MPO or IL-8 after preincubation of PMN with blebbistatin (dark gray), LY294002 (white), or cytochalasin D (light gray) was tested (n = 3-4, one-way ANOVA $P < .0001$). (F) Intracellular f-actin content of PMN after exposure to HSA, MPO, or IL-8 for indicated times was assessed by flow cytometry (n = 3-4). (G) Chemotaxis experiments in microslides were performed after preincubation of PMN with sodium azide and 2-deoxyglucose (white bars) to deplete energy metabolism. (n = 3-6, one-way ANOVA $P < .0001$). In this case, n denotes number of independent experiments, number of donors of PMN ≥ 3. Bars represent means; error bars, SEM. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$.

3A). The profound effect of the monomeric MPO mutants on PMN motility (Figure 1D) suggests that cleavage of MPO to hemimyeloperoxidase, which occurs on alkalization, is not responsible for attenuation of PMN motility under alkaline conditions.[20] The significance of electrostatic interactions for MPO-directed cell motility was further stressed by the observation, that addition of

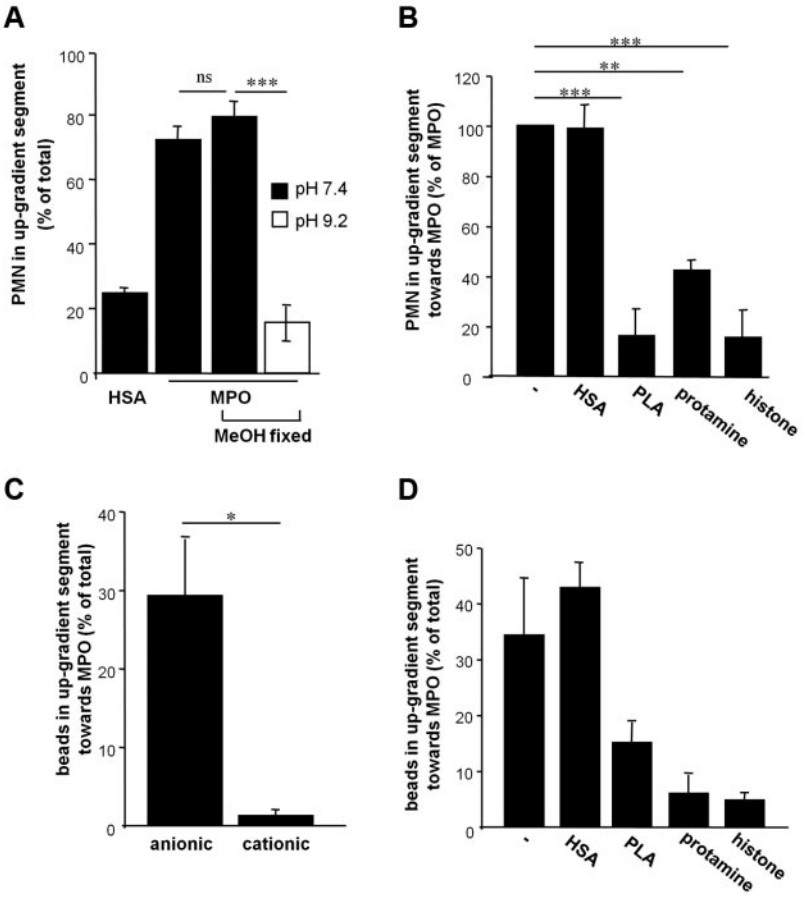

Figure 3. MPO-mediated PMN-motility is dependent on electrostatic interactions. (A) Methanol (MeOH) treatment of PMN did not impair MPO-induced motility, while directed movement of MeOH-fixed PMN toward MPO was blunted in buffer with the pH raised to the isoelectric point of MPO (p*I* 9.2, white bar, n = 3–4, one-way ANOVA $P < .0001$). (B) Poly-L-arginine (PLA), protamine and histone H2A were coadministered to PMN-suspension and MPO to equalize the cationic gradient. HSA was coadministered as a control protein (n = 3, one-way ANOVA $P < .0001$). (C) MPO-directed locomotion of sepharose beads with anionic or cationic coating was tested (n = 3-6). (D) Coadministration of PLA, protamine, and histone H2A impaired the motility of anionic beads toward MPO. In this case, n denotes number of independent experiments, number of donors of PMN ≥ 3. Bars represent means; error bars, SEM. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$.

highly polycationic molecules such as protamine, poly-L-arginine (PLA), and histone H2A coadministered to blunt the cationic gradient indeed abrogated the MPO-effect (Figure 3B). Along these lines, sepharose particles carrying a negative surface charge displayed a highly directed motility toward MPO, whereas cationic particles hardly did. In accordance with experiments performed with PMN, PLA, protamine, and histone H2A diminished MPO-directed locomotion of negatively charged particles (Figure 3C-D), underscoring the necessity of a cationic gradient as a prerequisite for cell motility. Moreover, and in support of MPO-mediated cell attraction, coating of microslides with MPO caused increased leukocyte adhesion under flow conditions compared with BSA (supplemental Figure 3).

To test whether these observations translate into MPO-dependent changes in PMN accumulation in vivo, we applied different mouse models using WT and MPO-deficient ($Mpo^{-/-}$) animals. In a first set of experiments, WT and $Mpo^{-/-}$ mice were subjected to 90 minutes of hepatic ischemia, followed by 20 hours of reperfusion. This resulted in systemic and hepatic accumulation of endogenous MPO in WT mice (supplemental Figure 4A-C), accompanied by a profound increase in PMN-infiltration compared with sham-operated animals. In $Mpo^{-/-}$ mice, the extent of PMN infiltration was markedly reduced (Figure 4A-B), reconfirming observations previously made in myocardial ischemia and renal ischemia and reperfusion.[3,21] Likewise, TNF-α–treated $Mpo^{-/-}$ mice revealed significantly reduced endothelial PMN adhesion and extravasation compared with WT mice (Figure 4C-D), as monitored by intravital microscopy and histologic evaluation of cremaster muscle postcapillary venules. Endothelial deposition of MPO on intrascrotal TNF-α injection in WT mice was confirmed immunohistochemically (supplemental Figure 5). Importantly, basal systemic leukocyte numbers and PMN surface expression of Mac-1 integrins did not differ between the mouse strains (supplemental Figure 6A-C).

To assess the direct contribution of MPO for PMN recruitment in vivo, MPO was injected into the superior mesenteric vein of WT mice. Accumulation of MPO in hepatic tissue was confirmed by immunofluorescence staining (Figure 4E). Administration of active MPO and the catalytically inactive MPO variants M243T and Q91T, as well as ABAH-inactivated MPO, resulted in significantly increased hepatic PMN accumulation compared with HSA, implying that the effect of MPO on PMN recruitment remains independent of the enzyme′s catalytic activity (Figure 4F). In line with these observations, injection of active MPO and inactive Q91T MPO into the carotid artery of WT and $Mpo^{-/-}$ mice provoked rapid PMN adhesion in cremaster muscle postcapillary venules (Figure 4G, supplemental Figure 7A, supplemental Video 1A-B). Microcirculatory parameters remained similar between the experimental groups (supplemental Tables 1-2). Of note, determination of cell diameters perpendicular to the endothelium[22] revealed that vessel-adherent PMN in MPO-treated mice was characterized by less spreading compared with PMN in animals challenged with TNF-α (Figure 4H). This observation recalls the in vitro findings and demonstrates distinctions between mechanisms evoked by established inflammatory cytokines. Importantly, MPO application did not affect PMN recruitment in arterioles of the cremaster muscle (supplemental Figure 7B-C, supplemental Video 1C). This underscores that MPO does not replace but rather supports the multiple mechanisms responsible for PMN recruitment, which hardly occurs in arterioles. In support of this MPO-dependent, charge-mediated PMN recruitment in vivo, MPO indeed profoundly

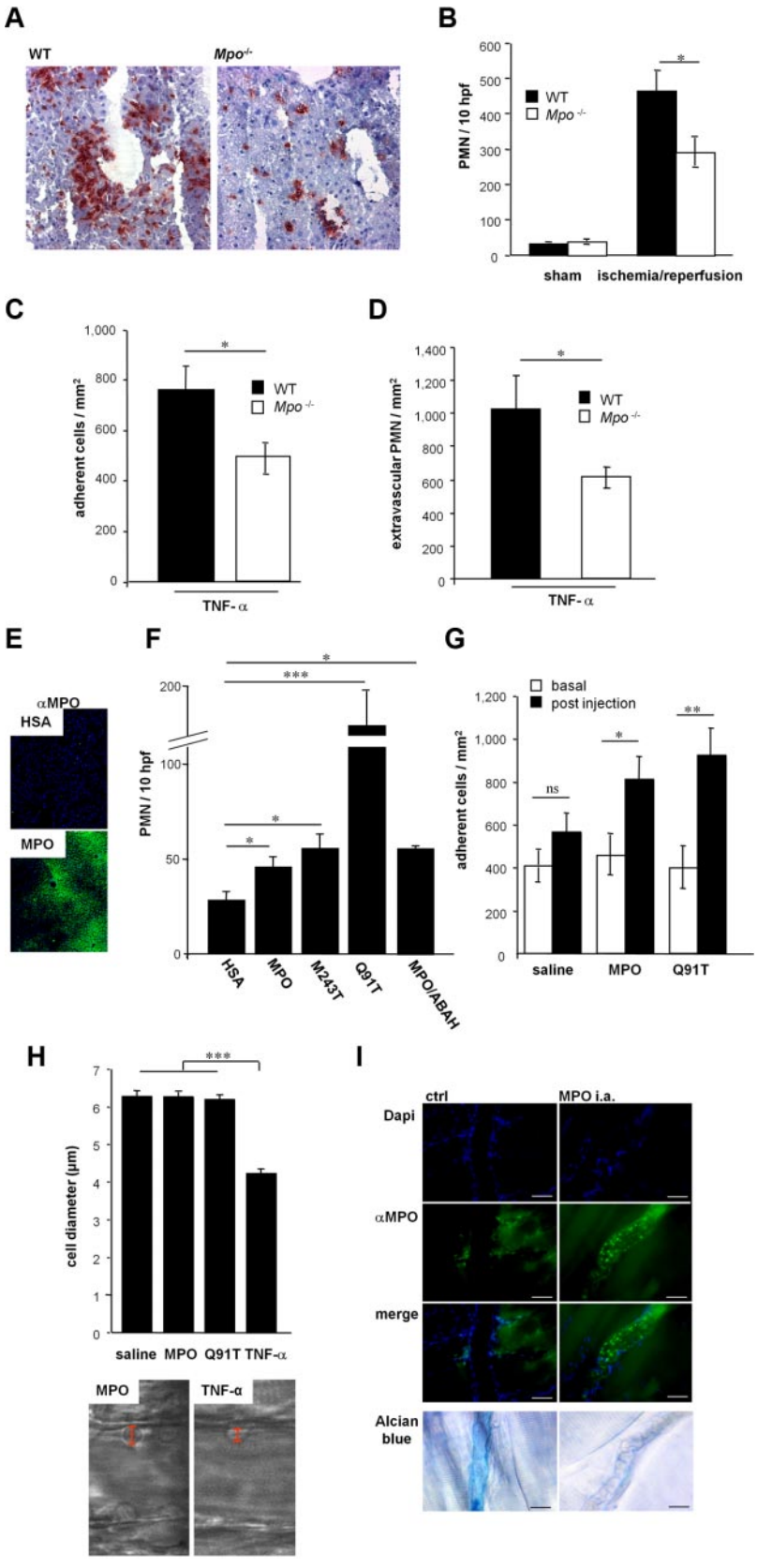

Figure 4. MPO provokes neutrophil recruitment in vivo. (A) Representative liver sections of WT and *Mpo*$^{-/-}$ mice after 90 minutes of hepatic ischemia with subsequent 20 hours of reperfusion are shown (PMN = red-brown; ×200, captured with Zeiss AxioCam HRc mounted on a Zeiss Axioskop with AxioVision 3.1. Tonal value correction, brightness, and contrast were adjusted with Adobe Photoshop CS3 extended 10.0). (B) PMN were quantified in liver sections of WT or *Mpo*$^{-/-}$ mice on hepatic ischemia and reperfusion (hpf = high-power field; magnification ×600; sham n = 5-8, treated n = 9-10, one-way ANOVA $P < .0001$). (C) Number of adherent leukocytes in postcapillary venules of the cremaster muscle of WT (19 vessels in 4 mice) and *Mpo*$^{-/-}$ mice (18 vessels in 4 mice) stimulated with TNF-α was assessed by intravital microscopy. (D) Number of perivascular PMN in TNF-α stimulated cremaster muscle of WT (n = 4) and *Mpo*$^{-/-}$ mice (n = 4) was evaluated in Giemsa-stained whole mounts. (E) MPO-immunoreactivity (αMPO, green, Alexa Fluor 488) on intraportal injection of HSA or human MPO is shown in hepatic sections of WT mice (blue = Dapi, original magnification ×100, captured with Retiga 1300 CCD camera mounted on Leica DMLB fluorescence microscope by iVision version 4.0, processed as described in panel A). (F) Hepatic PMN-infiltration was quantified on intraportal injection of HSA, active MPO (MPO), inactive MPO M243T and Q91T, or ABAH-inactivated MPO (n = 3-8, hpf = high-power field, magnification ×600, one-way ANOVA $P < .01$). (G) Number of adherent leukocytes in cremaster muscle postcapillary venules was assessed before (basal, white bars) and after (post injection, black bars) injection of saline solution (16 vessels in 5 mice), active (15 vessels in 3 mice) or inactive MPO Q91T (12 vessels in 3 mice) into the carotid artery of WT mice. (H) Mean diameter of PMN adherent to the endothelium of cremaster muscle venules in mice on injection of saline, MPO, or Q91T MPO to the carotid artery or intrascrotal TNF-α application (500 ng) and a representative image of vessel-adherent PMN in MPO and TNF-α–treated mice are shown (magnification ×400; intravital microscopy was performed with an Olympus BX51 microscope with a CF8/1 CCD-camera [Kappa]), recorded on S-VHS recorder and digitalized with MetaMorph software [Molecular Devices]). (I) Intraluminal staining of MPO (green) and negatively charged glycocalyx residues (Alcian blue) in WT mice on MPO injection of the carotid artery (MPO i.a.) or control (ctrl). Scale bars, 30 μm; fluorescence images captured and processed as described in panel E and bright-field images as described in panel A. Bars represent means; error bars, SEM. *$P < .05$, **$P < .01$, ***$P < .001$.

attenuated the anionic endothelial surface charge in cremaster muscle venules, illustrated by diminished deposition of the cationic dye Alcian blue, which binds to the glycocalyx in a charge-dependent fashion[23-25] (Figure 4I).

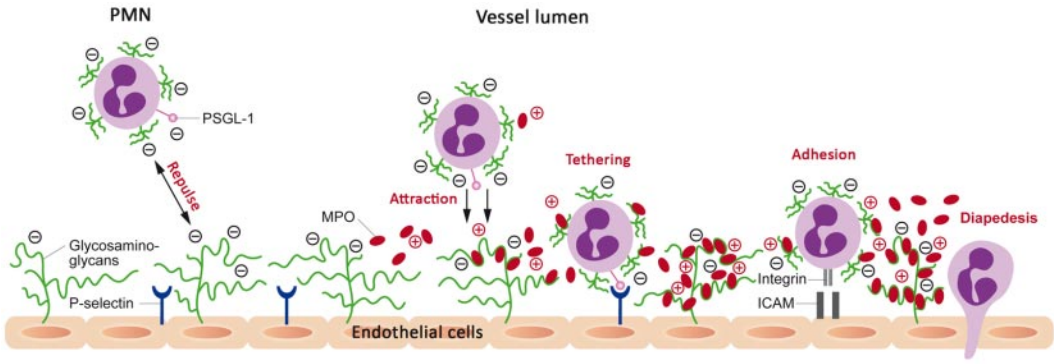

Figure 5. Scheme of the suggested mechanism of MPO-mediated PMN recruitment. Electrostatic repulsion between the negatively charged glycocalyx of PMN and the vessel wall prevents the interactions of PMN with the endothelium. Given the difference in height, the glycocalyx (~ 500 nm) shields selectins (~ 40 nm), which communicate the definite contact of PMN to the vessel wall (for instance, binding of constitutively expressed P-selectin glycoprotein ligand-1 [PSGL-1] on the PMN membrane with P-selectins on the endothelial surface). Thus binding of MPO to glycosaminoglycans reduces the negative surface charge and allows for electrostatic attraction of PMN to the endothelium, which then mediates receptor-ligand interactions, resulting in PMN tethering and rolling, adhesion, and diapedesis.

Discussion

Recruitment of PMN during inflammation implies a complex multistep process, which is initiated by capture, tethering, and rolling of leukocytes followed by leukocyte adhesion, spreading, and crawling on the inflamed vessel wall, and finally diapedesis into tissue. We herein propose a central role of MPO during the early steps of PMN interaction with the endothelium, this role being mediated by electrostatic forces between the cationic MPO surface and the anionic PMN surface.

Whereas the initial steps of the migration cascade are primarily considered to be selectin-dependent, nonselective physical forces have been suggested as alternative and potentially preceding mechanisms allowing PMN interaction with the vessel wall. Previous reports speculated on cationic proteins being responsible for the initiation of leukocyte emigration by alteration of the surface charge.[26] The attenuation of electrostatic repulsive forces has been hypothesized to permit short-range forces like Van der Waals and hydrophobic forces between leukocytes and the endothelium to act, and thereby facilitate leukocyte adhesion.[27] In fact, reports in the 1970s and early 1980s suggested that PMN-derived proteins enhance PMN margination and aggregation by decreasing negative surface charge, but these reports failed to identify the underlying effector molecules.[28-30] In accordance with these observations, sulfated glycosaminoglycans became established epitopes mediating the repulsion of blood cells and attenuating adhesion of circulating leukocytes, in part because of their negative charge, and by shielding endothelial adhesion molecules. Depletion of heparan sulfate chains has been shown to increase leukocyte adhesion in postcapillary venules,[31] whereas the negative charge of the leukocyte surface protein leukosialin (CD 43) appears to exert antiadhesive properties.[32]

The current data now expand on these findings and introduce MPO as a powerful inducer of PMN recruitment. In fact, as the enzyme is capable of attracting PMN in vitro independently of cytoskeletal-dependent and energy-dependent rearrangements of the leukocyte, electrostatic forces may indeed account for essential steps of PMN migration in vivo. Given its high affinity to glycosaminoglycans and matrix proteins such as collagen, fibronectin,[8,33] and fibrinogen (data not shown), MPO, released at and covering sites of inflammation, attenuates the antiadhesive properties of the vessel wall. This cationic "coat" may ease the interplay of PMN with the endothelium and allow PMN to interact with adhesion molecules harbored within the glycocalyx.[34] Furthermore, MPO deposition by adherent and crawling leukocytes on the endothelial surface layer may even help to guide upcoming PMN to the exit point into tissue (Figure 5). This concept of MPO supporting established mechanisms of PMN recruitment rather than unselectively promoting firm PMN adhesion is underscored by the fact that the enzyme did not provoke PMN recruitment in arterioles, where leukocyte interaction with the endothelium remains restricted because of high shear forces and a limited expression of adhesion molecules.

Alternative mechanisms apart from electrostatic interactions may be responsible for the current observations. For example, one could speculate that MPO increases vascular permeability as shown for other cationic molecules.[35,36] Vascular permeability is mainly regulated by the adhesion molecule vascular endothelial (VE)–cadherin, with its blockade resulting in increased permeability and enhanced PMN emigration.[37] However, research does not confirm that MPO affects vascular permeability.[8] Moreover, MPO binding to the neutrophil integrin Mac-1[38,39] may facilitate PMN adhesion and thus support electrostatically driven cell recruitment, as previously described for elastase and proteinase 3.[40,41]

It is possible to deduce from the current study that other cationic granular proteins should exhibit similar effects as MPO. However, electrostatically driven PMN recruitment does not only depend on the p*I* of the affecting protein: For example, lactoferrin, α-chymotrypsinogen A, and histone, despite a similar p*I*, promote endothelial leukocyte adhesion in varying degrees.[42] In fact, the different binding affinities of the proteins to the endothelium appear to be of critical importance as well.[43] MPO is characterized by its high affinity to heparan sulfate glycosaminoglycans,[8,44] the major components of the endothelial surface layer. In conjunction with its abundant granular expression, this results in profound deposition on the endothelial surface and might account for the enzyme's profound effects on PMN recruitment.

Of note, the proposed mechanism might also affect the affinity of other blood corpuscles toward the vessel wall as shown, for example, for red blood cells in vitro (supplemental Figure 8). However, MPO-mediated affinity of leukocytes to the vessel wall might be of particular significance, given that leukocytes subsequently adhere to endothelial cells and extravasate in an MPO-independent fashion.

In conclusion, these findings describe a novel mechanism underlying neutrophil motility and highlight a so-far unrecognized role for MPO in the recruitment of PMN, which is independent of

the enzyme's catalytic activity. Instead, PMN attraction is communicated by MPO's positive charge, an observation that calls for revisiting the role of MPO in innate immunity and the impact of physical forces on the recruitment of neutrophils.

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Authorship

Contribution: A.K. designed the project, performed experiments, and wrote the manuscript; C.N. designed and performed the intravital microscopy experiments and wrote the manuscript; L.K., K.F., D.B., D.L., and K. Szocs provided suggestions on the project and performed experiments; H.-J.P. performed flow cytometric experiments; C.S. and U.S. were responsible for flow chamber experiments; P.H. designed animal experiments; P.G.F. synthesized MPO variants and made suggestions on the in vitro experiments; T.K.R., V.R., K. Sydow, H.-J.D., H.E., and T.M. provided suggestions on the project and revised the manuscript; and M.S. and S.B. supervised the project and wrote the manuscript.

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Original Contributions

Myeloperoxidase induces the priming of platelets

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ABSTRACT

The release of myeloperoxidase (MPO) from polymorphonuclear neutrophils is a hallmark of vascular inflammation and contributes to the pathogenesis of vascular inflammatory processes. However, the effects of MPO on platelets as a contributory mechanism in vascular inflammatory diseases remain unknown. Thus, MPO interaction with platelets and its effects on platelet function were examined. First, dose-dependent binding of MPO (between 1.7 and 13.8 nM) to both human and mouse platelets was observed. This was in direct contrast to the absence of MPO in megakaryocytes. MPO was localized both on the surface of and inside platelets. Cytoskeleton inhibition did not prevent MPO localization inside the three-dimensional platelet structure. MPO peroxidase activity was preserved upon the MPO binding to platelets. MPO sequestered in platelets catabolized NO, documented by the decreased production of NO (on average, an approximately 2-fold decrease). MPO treatment did not affect the viability of platelets during short incubations; however, it decreased platelet viability after long-term storage for 7 days (an approximately 2-fold decrease). The activation of platelets by MPO was documented by an MPO-mediated increase in the expression of surface platelet receptors P-selectin and PECAM-1 (of about 5 to 20%) and the increased formation of reactive oxygen species (of about 15 to 200%). However, the activation was only partial, as MPO did not induce the aggregation of platelets nor potentiate platelet response to classical activators. Nor did MPO induce a significant release of the content of granules. The activation of platelets by MPO was connected with increased MPO-treated platelet interaction with polymorphonuclear leukocytes (an approximately 1.2-fold increase) in vitro. In conclusion, it can be suggested that MPO can interact with and activate platelets, which can induce priming of platelets, rather than the classical robust activation of platelets. This can contribute to the development of chronic inflammatory processes in vessels.

Cardiovascular diseases remain the leading cause of death and morbidity in the industrialized world, with a rapidly rising prevalence in developing countries [1]. Cardiovascular diseases are connected with multifactorial pathological processes involving various players including endothelial cells, leukocytes, and platelets [1,2].

Myeloperoxidase (MPO) is a hemoprotein comprising up to 5% of the total proteins of polymorphonuclear neutrophilic leukocytes (neutrophils), typically perceived to primarily mediate host defense reactions [3,4]. Recent evidence from both our research and other studies suggests the importance of MPO in the regulation of cellular and tissue physiology not directly related to host defense [3,4]. Reactive intermediates formed by MPO-catalyzed reactions may interfere with, and modulate, cellular signaling pathways. This can be by means of interference with signaling mediators owing to their catabolism (e.g., nitric oxide (NO), epoxides of arachidonic acid, and linoleic acid mediators) or through the modulation of their structures (e.g., posttranslational modifications of proteins, the oxidative modification of a wide range of biologically active lipid mediators) [2–6].

Recently, the release of MPO by activated neutrophils has also emerged as a hallmark of vascular inflammation [7–9]. Released MPO can bind to endothelial cells (ECs) through electrostatic interaction with the negatively charged endothelial cell glycocalyx, particularly heparin glycosaminoglycans (GAGs), with the consequent subendothelial deposition of MPO in the vessel wall [8,9]. MPO bound to the endothelium was shown to contribute to vascular dysfunction by suppressing the bioavailability of NO through NO catalytic consumption altering NO-dependent signaling pathways [8,10]. Further, MPO-mediated activation of neutrophils, resulting in increased interaction with the endothelium and the

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extravasation of neutrophils, was also described [2,11]. MPO was suggested to bind to neutrophils both through a nonspecific electrostatic interaction with the neutrophil surface and through specific interaction with CD11b receptors [2,11]. All current data suggest that MPO has significant effects on the vascular endothelium and circulating neutrophils, disturbing vascular homeostasis.

Blood platelets are crucial in homeostasis regulation and also important participants in inflammatory processes [12,13]. The activation of platelets and the release of a wide range of biologically active mediators significantly contribute to chronic and acute inflammation and consequently to pathological processes in vasculature [13]. Under normal conditions, nonactivated platelets circulate in blood without significant interaction with either the vascular endothelium or other blood cells. Platelets produce NO, which acts as a negative feedback mechanism to inhibit platelet aggregation and platelet recruitment and, in general, to reduce inflammatory response [14,15]. Under inflammatory conditions, the importance of an association between neutrophil interactions with platelets and vascular disorders is suggested in a wide range of studies [16–18]. The impaired production of NO by platelets was suggested as contributing to the pathophysiology of acute coronary syndrome. Activated platelets increase the expression of surface receptors, which mediate increased interaction not only with the endothelium and subendothelial matrix but also with circulating neutrophils [19–21]. Further, activated platelets produce a wide range of inflammatory mediators during partial or full degranulation [22,23]. Stimulated platelets also produce reactive oxygen species (ROS) through the enzymatic activity of NADPH oxidase [14,24–26]. ROS were shown to further potentiate platelet activity, adhesion, the release of proinflammatory mediators, and aggregation [14,24–26].

All current data indicate the important role of MPO in the regulation of vascular and blood homeostasis. However, the effects of MPO on the physiological functions of other blood elements such as platelets have not been evaluated. Interestingly, pathological events connected with high platelet activation such as acute myocardial infarction are more severe in patients with elevated MPO levels [27]. It is our contention that neutrophil-derived MPO can play an important role in the regulation of the physiological functions of platelets under inflammatory conditions. On the basis of the observed data, it can be speculated that MPO can mediate changes in the physiological function of platelets through its interaction with the negatively charged glycocalyx on the platelet surface. Further, the catalytic consumption of NO catalyzed by MPO bound to platelets could be proposed.

Materials and methods

The isolation of platelets

Blood was collected either from healthy volunteers by antecubital venipuncture using a 21-gauge butterfly needle (Braun, Germany) or from C57BL/6J mice by heart puncture into anticoagulant 3.8% (v/w) trisodium citrate dihydrate (Lachema, Czech Republic) 1:9 (v/v). The blood donors had given their informed consent. All animal experiments were approved by the local ethics committees (IBP, Brno, Czech Republic and UKE, Hamburg, Germany) and in harmony with the U.S. National Research Council's *Guide for the Care and Use of Laboratory Animals.* Platelets were obtained by the centrifugation of blood mixed 1:1 with Tyrode buffer with 0.2% EDTA (0.1% glucose, 136.9 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, and 1 mM $MgCl_2$ $6H_2O$, pH 7.4) at 250 *g* for 10 min at room temperature (RT). The platelet-rich plasma (PRP) was repeatedly centrifuged (250 *g,* 10 min, RT). Next, the PRP was spun (900 *g,* 15 min, RT), and the platelet pellet was resuspended in Tyrode buffer without EDTA to a concentration of 4×10^7/ml. The platelets were counted by a BC-2800 Auto Hematology Analyzer (Mindray, China). Isolated mouse platelets were prepared in the same manner with slight modifications. The first and second centrifugations were at 130 *g* for 10 min, the third centrifugation was at 1000 *g* for 10 min.

Isolation of mouse megakaryocytes

Femurs and tibias obtained from C57BL/6J mice were cleaned and flushed with modified PBS (13.69 mM sodium citrate, 0.1% glucose, 1% bovine serum albumin (BSA) and 2 μM prostaglandin E_1). A total of 4 ml of a single-cell suspension was mixed with 3 ml Percoll in PBS (1.02 g/ml; GE Healthcare, Sweden). This mixture was gently layered on top of 4 ml Percoll in PBS (1.05 g/ml) and centrifuged for 20 min at 400 *g.* The interface was collected and washed with modified PBS. A portion of the megakaryocyte suspension was incubated with MPO as described later. The cells were stained with anti-mouse CD42d (phycoerythrin, PE; eBioscience, USA) and anti-mouse CD41 (fluorescein isothiocyanate, FITC; eBioscience) antibodies at 4 °C in the dark for 20 min. An aliquot of the cell suspension was incubated under identical conditions with matching isotype antibodies to set up the gates. Positive cells based on the high expression of both markers (less than 0.1% of the stained cells), which were recognized as megakaryocytes, were sorted by FACSAria II (BD Biosciences, USA).

Preparation of the MPO-rich supernatant from activated polymorphonuclear leukocytes (PMNLs)

Human PMNLs were isolated from blood, which was layered over a separation mixture consisting of 4% dextran (Sigma–Aldrich, Germany) and 60% Telebrix (Guerbet, France) (3.7:1 v/v, final density 1.08 g/cm^3). The obtained leukocyte- and platelet-rich supernatant was placed over Lymphoprep (Fresenius Kabi Norge, Norway) for density gradient centrifugation (400 *g*/45 min/RT). The pellet was resuspended in Hanks' buffered salt solution (HBSS) without Ca^{2+} and Mg^{2+} after being washed in PBS (250 *g,* 5 min, RT). Next, PMNLs were diluted to a concentration of 5×10^6/ml and activated with opsonized zymosan (prepared as described previously [28]) for 40 min at 37 °C. Supernatant containing MPO was obtained by centrifugation (1000 *g,* 5 min, RT). The concentration of MPO in the supernatant was 347 nM as determined by ELISA according to the manufacturer's instructions (CardioMPO kit; PrognostiX, USA).

Treatment of isolated platelets, whole blood, and isolated megakaryocytes with MPO

The isolated platelets were incubated with various concentrations (1.7–69 nM) of purified human MPO (Planta Natural Products, Austria) for various durations (2–60 min or 7 days) at RT. Then, the platelets were extensively washed, typically three times, in Tyrode buffer with EDTA (900 *g,* 10 min, RT) and either analyzed or used for further experiments, as described under the particular methodological section. In some cases, human anticoagulated whole blood was incubated directly with the purified human MPO (34.5 nM; Planta Natural Products) or with the MPO-rich supernatant obtained from degranulated PMNLs activated by opsonized zymosan (as described above) for 30 min at RT. Then, platelets were isolated as described above and analyzed for the presence of MPO, as described subsequently. In the case of megakaryocytes, their suspension was incubated with MPO (34.5 nM) for 15 min and washed. Next, megakaryocyte surface receptors were labeled by specific antibodies and the cells were sorted as described above.

Detection of MPO in platelet lysates

Platelets were incubated with MPO (1.7–13.8 nM, 30 min, RT) and washed as described above. They were then lysed in PBS with 0.1% Triton and the MPO concentration in the lysates was determined by ELISA according to the manufacturer's instructions (human; CardioMPO kit). To assess the effects of glycosaminoglycans on cell–MPO binding, platelets were incubated simultaneously with MPO (13.8 or 34.5 nM) and heparin (2 or 5 μg/ml; Sigma–Aldrich) for 30 min. In other experiments, platelets were exposed to MPO (13.8 or 34.5 nM) for 30 min. Then the platelets were treated with heparin (2 or 5 μg/ml) for 30 min. After several washes with Tyrode buffer with 0.2% EDTA, the pellet was lysed in PBS with 0.1% Triton and the MPO concentration in the lysates was determined by ELISA.

Detection of proteins in platelet lysates

The lysates from control and MPO-treated platelets were prepared as described above and the protein concentration was determined using commercial BCA protein assay (Pierce, USA).

Presence of MPO on the platelet surface

The presence of MPO on the surface of platelets was determined with isolated platelets incubated with purified MPO (MPO 3.4–13.8 nM, 30 min, RT) and platelets isolated from whole blood after being incubated with purified MPO (MPO 34.5 nM, 30 min, RT) or the MPO-rich supernatant (40 times diluted from the stock supernatant of 347 nM MPO, e.g., final 8.7 nM MPO, 30 min, RT). Washed platelets were subsequently fixed with 1% formaldehyde in PBS and incubated first with rabbit anti-MPO antibody (Calbiochem, USA) and then with anti-rabbit IgG conjugated with Alexa Fluor 488 (Thermo Scientific, USA). Platelets were analyzed using a FACScalibur flow cytometer (BD Biosciences, USA). In all cases of flow cytometric determination, platelets were defined by forward- and side-scatter characteristics, and 10,000 individual events were collected within the platelet gate. The cells stained only with secondary antibody were used to determine the nonspecific background signal.

Immunocytochemistry

The cellular localization of MPO was determined by confocal microscopy. Isolated platelets and megakaryocytes incubated with MPO (34.5 nM) were fixed with 0.5% formaldehyde in PBS and allowed to adhere to the surface of glass slides (90 min, RT). In some experiments, the effect of cytochalasin D on MPO internalization by platelets was investigated. Human platelets were preincubated with cytochalasin D (10 μM; Santa Cruz Biotechnology, USA). After being washed, the platelets were incubated with MPO (34.5 nM), fixed with 0.5% formaldehyde in PBS, and allowed to adhere to the surface of glass slides (90 min, RT).

After fixation, the cells were permeabilized using 0.5% Triton X-100 in PBS (5 min, RT) and blocked with 1% (w/v) BSA (Sigma–Aldrich) and 5% goat serum (Sigma–Aldrich) in PBS (1 h, RT). Primary Ab labeling was performed with rabbit anti-MPO pAb (Thermo Scientific or Calbiochem, diluted 1:200) in PBS with 1% BSA (Sigma–Aldrich) and 5% goat serum (Sigma–Aldrich) (1 h, RT). After being washed three times in PBS, the cells were incubated with secondary antibody (goat anti-rabbit IgG–Alexa Fluor/DyLight 568 or 694, diluted 1:100) (Thermo Scientific) for 1 h. Glass slides were washed and mounted in Mowiol (Calbiochem) solution (10% Mowiol 4-88 was prepared in 25% glycerol, 100 mM Tris–HCl, and 0.6% 1,4-diazabicyclo-[2.2.2]-octane, pH 8.5). Images were acquired on a confocal microscope (TCS SP5; Leica, Germany) using a 63 × 1.4 oil immersion objective. Negative controls obtained by omitting primary antibodies revealed no autofluorescence or nonspecific fluorescence. A three-dimensional projection was reconstructed from a two-dimensional *Z*-stack series of platelets using LAS AF Lite software (Leica).

MPO activity in platelet lysates

Platelets were incubated with MPO (6.9–41.3 nM, 30 min, RT) and washed as described above. They were subsequently lysed in PBS with 0.1% Triton X-100. The MPO activity of platelet lysates was measured as the oxidation of tetramethylbenzidine (TMB; 1 mM; Sigma–Aldrich) in 300 mM sodium acetate buffer (pH 5.4) in the presence of 0.3, 0.075, or 0.032 mM hydrogen peroxide (H_2O_2) within 2.5 min. In some experiments, 50 μM 4-aminobenzoic hydrazide (4-ABAH; Sigma–Aldrich) to detect the effect of MPO inhibitor or 20% plasma was added to the TMB reaction mixture in the presence of 0.075 mM H_2O_2. In some experiments, MPO activity was determined in variously diluted lysates (obtained from platelets incubated with 17.2 nM MPO, 30 min RT) in the presence of 0.075 mM H_2O_2. The formation of the reaction product was determined spectrophotometrically as the increase in absorption at 350 nm using an Infinite M200 microplate spectrofluorimeter (Tecan, Switzerland).

Platelet viability

Platelet viability was assessed by measuring the accumulation of the vital probe calcein-AM by viable platelets and the lipophilic plasma membrane probe FM4-64 by nonviable platelets according to Albanyan et al. [29] with slight modification. Briefly, platelets were incubated with various concentrations of MPO (3.4–34.5 nM) or the calcium ionophore A23187 (CaI; 2.8 μM) as a positive control for 30 min at RT or 7 days at 37 °C. Platelets were then incubated with calcein-AM (50 nM; Invitrogen) and FM4-64 (2 μM; Invitrogen) for 10 min at 37 °C followed by the addition of Tyrode buffer with 0.2% EDTA (1:1) and immediately analyzed by flow cytometer (FACScalibur). Unstained samples were used to adjust for a nonspecific background signal and autofluorescence.

Surface expression of P-selectin (CD62P) and the platelet endothelial cell adhesion molecule (PECAM-1, CD31)

The surface expression of P-selectin and PECAM-1 receptors was measured after the incubation of isolated platelets with various concentrations of MPO (3.5–34.5 nM) or CaI (2.8 μM, positive control) for 30 min at RT. Platelets were washed as described above, stained with PE-conjugated anti-human CD62P (P-selectin) (Invitrogen) or anti-human CD31 (PECAM-1) (Invitrogen) antibodies at 4 °C in the dark for 20 min, fixed subsequently with 1% formaldehyde in PBS, and analyzed by a FACScalibur flow cytometer. Platelets stained with appropriate isotype controls were used to determine the nonspecific background signal. Data present the geometric mean fluorescence intensity (MFI).

ROS production by platelets

Isolated platelets were preincubated with MPO (34.5 nM) with subsequent activation with or without CaI (2.8 μM). After a 1-h incubation with hydroethidine (HE; 20 μM, Invitrogen) or dihydrorhodamine-123 (DHR-123; 10 μM; Invitrogen) probes, the intensity of the fluorescence-represented production of ROS was measured by an Infinite M200 microplate spectrofluorimeter at excitation and emission wavelengths of 520 nm—HE, 484 nm—DHR-123 and 600 nm—HE, 590 nm—DHR-123, respectively.

F-actin in platelets

The intracellular F-actin content of isolated platelets was assessed according to Klinke et al. with slight modification [11]. Briefly, platelets were incubated with various concentrations of MPO (3.5–34.5 nM) or CaI (2.8 μM) as a positive control for 2 min. After the indicated times, cells were immediately fixed with 0.5% formaldehyde in PBS (15 min, RT). This step was followed by permeabilization with 0.1% Triton X-100, incubation with Alexa Fluor 488–phalloidin (30 min, 0.1 U/ml in PBS; Invitrogen), and flow cytometric analysis using FACScalibur.

NO production by platelets

Isolated platelets were incubated with MPO (34.5 nM) for 30 min and washed as described above. Subsequently, cells were placed into glass vials and transferred to the NO-measuring chamber (World Precision Instruments, USA) preheated to a constant temperature of 37 °C. After response stabilization, CaI (2.8 μM) was injected into the cell suspension. NO production was measured continuously with an NO-specific electrode (AmiNO 700; Innovative Instruments, USA) connected to an ISO-NO MARK II potentiostat (World Precision Instruments). The calibration was performed with distilled water saturated with NO gas (concentration after saturation, 1.9 mM at RT). The integral area under the resulting curve measured for 2.5 min corresponded to the total amount of NO present in the glass vial with platelet suspension after CaI activation.

Catabolism of NO by platelet lysates

Platelets were incubated with MPO (6.9–68.9 nM, 60 min, RT) and washed three times as described above. An NO-specific electrode (AmiNO 700) connected to the ISO-NO MARK II potentiostat was placed into glass vials containing 850 μl of PBS and kept at RT. The solution was stirred vigorously. The injection of H_2O_2 (15 μM) was performed, followed by the injection of 5 μl of the NO donor, which resulted in a rapid peak that slowly decayed until it reached the background current. Then, the platelet–lysate suspension or control buffer (50 μl) was added and the consumption of NO in the various reaction mixtures was evaluated. The electrochemical signal was followed for 2.5 min to obtain kinetic curves. The calibration was performed with distilled water saturated with NO gas (concentration after saturation, 1.9 mM at RT). Values were calculated from the initial rate of NO consumption after subtraction of background NO consumption before H_2O_2 addition and are expressed as nM/s.

Platelet aggregation

Platelet aggregation was studied in isolated platelet suspensions using a dual-channel aggregometer (Chrono-log, USA). After the incubation of platelets with MPO (34.5 nM) for 5 or 30 min and a subsequent 1-min stabilization period at 37 °C in the aggregometer, the reaction was recorded for 8 min. Aggregation was initiated by the addition of adenosine diphosphate (ADP; 5 μM; Sigma–Aldrich), collagen (16.8 nM; BD Biosciences), and thrombin (0.025 and 0.5 U/ml; Sigma–Aldrich). Changes in aggregation were evaluated from the amplitudes of aggregation curves in millimeters at 2, 4, and 8 min.

Assay of platelet adhesion to endothelial cells

Human umbilical vein endothelial cells (HUVECs) from Lonza (Switzerland) were cultivated in EGM Bullet Kit medium (Lonza) (37 °C in a humidified atmosphere with 5% CO_2). For the adhesion experiments, HUVECs were cultured in gelatinized 96-well tissue culture plates (Genetix, USA) until confluent. The platelets were fluorescently loaded with calcein-AM (1 μM) for 20 min at 37 °C in the dark and washed as described above. Platelets were subsequently incubated with human MPO (34.5 nM) for 30 min at RT and washed.

The adhesion of platelets to EC cultures was quantified under static conditions. Before the adhesion assay was performed, cultures were left either unstimulated or stimulated for 3 h with TNF-α (1 μg/ml; PeproTech, USA). ECs were washed and serum-free medium was applied to cells. Preincubated and labeled platelets were applied at a density of 4×10^6 cells/well to the EC surface and allowed to adhere for 1 h at 37 °C with gentle rocking. Nonadherent cells were removed by washing two times with HBSS. The relative fluorescence intensity of adherent platelets was analyzed by an Infinite M200 microplate spectrofluorimeter with excitation and emission wavelengths of 490 and 520 nm.

Platelet–granulocyte aggregates (PGAs)

Anticoagulated human whole blood was incubated with or without MPO (34.5 nM) or CaI (2.8 μM) for 30 min at RT. PGAs were labeled with monoclonal antibodies for 30 min in the dark. Anti-human CD41–PE (eBioscience) is a platelet-specific monoclonal antibody that recognizes the glycoprotein complex on resting and activated platelets. Anti-human CD15–FITC (eBioscience) is a marker of neutrophil granulocytes. The formation of PGAs was detected by a FACScalibur flow cytometer and PGAs were quantified as a percentage of the total granulocyte population. A minimum of 200,000 cells were collected per each sample.

Bleeding time

For these experiments male wild-type C57BL/6J (WT) and MPO-deficient mice ($MPO^{-/-}$; The Jackson Laboratory) on a C57BL/6 background, age 12–15 weeks and matched for weight, were employed. Bleeding time was tested in control WT and $MPO^{-/-}$ untreated mice, WT and $MPO^{-/-}$ mice treated with endotoxin for 24 h, and WT mice infused with MPO. WT and $MPO^{-/-}$ mice were given a single intraperitoneal injection of *Escherichia coli* lipopolysaccharide (10 μg/g) 24 h before bleeding determination. Another cohort of WT was infused with human MPO (6.9 pmol/g) or human serum albumin (15.5 pmol/g) (Sigma–Aldrich) as control via the jugular vein with a 1-F microtip catheter, and the bleeding time was determined after 30 min. For bleeding time determination, mice were anesthetized by a continuous isoflurane gas flow and placed on a heating pad 10 min before the experiment. Tails were transected 2 mm from the tip with a razor blade and immediately immersed into a tube filled with 50 ml PBS (37 °C). The time until the cessation of bleeding was recorded.

Statistical analysis

For multiple comparisons, one-way ANOVA followed by Tukey post hoc test or Dunnett post hoc test was used as appropriate. Before–after comparisons of two data sets were analyzed with the Student *t* test for pairwise-dependent samples. A *p* value equal to or lower than 0.05 was considered statistically significant. Data are presented as the means ± standard error of the mean (SEM). All statistical analyses were carried out with Statistica 10 (StatSoft, USA).

Results

MPO binds dose dependently to platelets

To test MPO binding to human and mouse platelets, purified MPO was incubated with isolated platelets. The analysis of MPO presence in platelet lysates revealed dose-dependent binding to both human and mouse platelets (Fig. 1). The number of platelets and the amount of proteins in platelet lysates did not differ significantly among controls and MPO-treated samples, thus the correction of MPO levels in lysates for these values revealed completely analogous results (data not shown). Given the significant sequestration of MPO by platelets, characterization of the localization of MPO bound to platelets was explored. The analysis of surface-bound MPO indicated that at least a portion of MPO sequestered on platelets was localized on the surface of both human and mouse platelets, as was shown by flow cytometric determination (Fig. 2). Further, confocal microscopy revealed the presence of exogenously added MPO inside the three-dimensional structure of human platelets primarily localized in granule-like structures (Fig. 3A–D). To determine whether cytoskeletal organization representing active transport is required for the internalization of MPO, we utilized cytochalasin D, a cytoskeleton inhibitor inducing actin depolymerization. This inhibition should have prevented any active transport of MPO through the platelet membrane. As is shown in Fig. 4, the incubation of platelets with cytochalasin D did not influence MPO sequestration. Thus, it can be proposed that the sequestration of exogenously added MPO by platelets is not related to platelet cytoskeletal reorganization and that MPO was not transported through the platelet membrane. Therefore, these data suggest that the observed MPO inside the three-dimensional structure of MPO can be a result of the passive diffusion of MPO into the platelet canalicular system, which is open to extracellular space [30].

Any significant presence or staining of MPO compared to background was not observed in platelets isolated from healthy volunteers without MPO incubation (Figs. 1 and 3A). Similarly, the determination of the presence of mouse MPO in platelets isolated from mice did not reveal any significant MPO staining, and the observed background signal was comparable to the signal obtained with platelets isolated from MPO-deficient mice (data not shown). These data suggest that MPO is not significantly detectable in platelets isolated from healthy human volunteers or control mice.

The absence of MPO in platelets was further supported by the determination of MPO presence in megakaryocytes isolated from bone marrow, which revealed that megakaryocytes are negative for MPO staining (Fig. 5A). However, the incubation of isolated megakaryocytes with exogenous MPO showed the ability of MPO to bind to megakaryocytes as a precursor of platelets (Fig. 5B).

Finally, it was proven that MPO can be sequestered on the surface of platelets after addition of purified MPO (34.5 nM,

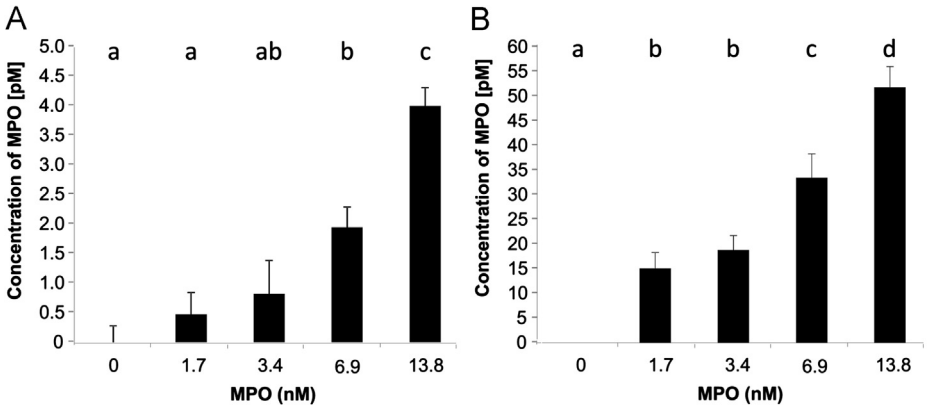

Fig. 1. Overall binding of purified MPO to both (A) human and (B) mouse platelets. Isolated platelets were incubated with various concentrations of MPO. After extensive washes, the platelets were lysed and the concentration of MPO in the lysates was detected by MPO ELISA. Data are presented as means ± SEM ($n=4$ and $n=6$, respectively). Letters above bars indicate statistical differences between MPO-untreated and MPO-treated platelets according to the Tukey test ($p\leq 0.05$). Values with the same letter do not differ significantly.

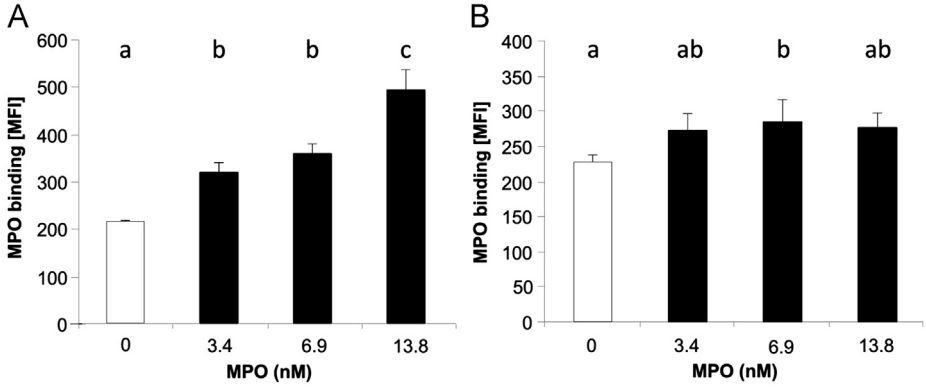

Fig. 2. Binding of purified MPO to the surface of isolated (A) human and (B) mouse platelets. Isolated platelets were incubated with various MPO concentrations. After extensive washes, the platelets were fixed and stained with rabbit anti-MPO antibody followed by incubation with Alexa Fluor 488-labeled anti-rabbit IgG and analyzed by flow cytometry. Data present geometric mean fluorescence intensity (MFI) and are expressed as means ± SEM ($n=4$ and $n=5$, respectively). Letters above bars indicate statistical differences between MPO-untreated and MPO-treated platelets according to the Tukey test ($p\leq 0.05$). Values with the same letter do not differ significantly.

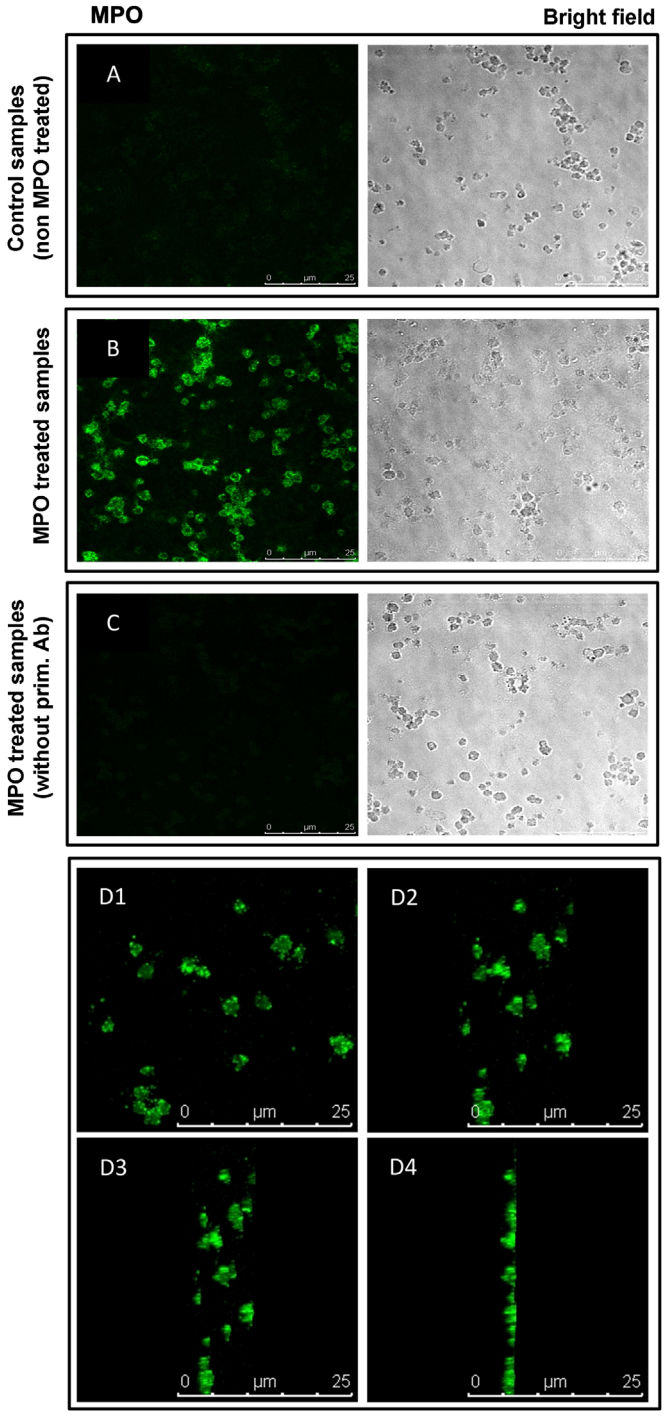

Fig. 3. Localization of bound MPO on human platelets. Isolated human platelets were incubated (A) without MPO or (B, C, D) with MPO (34.5 nM), fixed, and deposited on glass slides. After permeabilization with 0.5% Triton X-100, the platelets were incubated with rabbit anti-MPO antibody and then with anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 568. Samples without primary antibody served as a control (C). Samples were analyzed by scanning confocal microscopy. Images of cells were also taken in bright light (BL). Bar, 25 µm. (D) 3D projection of MPO localization on human platelets with rotation. 3D image reconstruction was generated from 59 confocal sections. Rotations of D1, 0°; D2, 33°; D3, 69°; D4, 90°.

30 min, RT) to the whole blood (control blood samples 56.3 ± 8.3 MFI; MPO-treated blood samples 129.9 ± 49.2 MFI, $n=4$). To further exclude the possibility that the observed phenomenon is not dependent on the application of purified MPO, we performed the binding assay with the MPO-rich buffer obtained from degranulated PMNLs activated by opsonized zymosan (the final concentration of MPO in platelet suspension 8.7 nM, 30 min, RT) (control blood samples 56.3 ± 8.3 MFI; blood samples with addition of the MPO-rich buffer 257.0 ± 91.6 MFI, $n=4$). Altogether, it can be suggested that platelets can bind MPO after they are released into systemic circulation from bone marrow.

MPO binding to platelets can be modulated by GAGs

To characterize the mechanism of platelet–MPO interaction and to evaluate the role of negatively charged GAGs, human platelets were incubated with heparin and MPO simultaneously. This excess of heparin led to a significant reduction in the detectable amount of MPO in platelet lysates (Fig. 6A). Correspondingly, heparin exposure significantly liberated MPO bound to the platelets after the preincubation of platelets with MPO (Fig. 6B). The presence of albumin up to 1.5 µM during the incubation did not have any significant effect on this process (data not shown).

MPO sequestered within platelets remains catalytically active

Because the interaction of MPO with binding partners such as ceruloplasmin can inhibit MPO enzymatic activity [31], the activity of MPO sequestered by platelets was evaluated. MPO peroxidase activity in the presence of TMB as substrate was preserved in platelet lysates (Fig. 7). The specificity of this TMB reaction can be suggested from the application of MPO inhibitor, which completely prevented the TMB oxidation (Fig. 7A). Further, the TMB reaction revealed dose dependency on the hydrogen peroxide concentration (Fig. 7C) and also correlated with the dilution of platelet lysate in a dose-dependent manner (data not shown). The TMB reaction was also observed in the presence of plasma (Fig. 7B), suggesting the potential relevance of this MPO-mediated oxidation under physiological conditions.

On the basis of the previously described MPO-mediated decrease in NO bioavailability from endothelial cells [8,10], the MPO-dependent modulation of platelet NO production was evaluated. Interestingly, platelets incubated with MPO produced a significantly lower level of detectable NO in response to CaI (Fig. 8A). This was similar for platelets from wild-type and MPO-deficient mice. Correspondingly, the rate of NO decomposition in the presence of platelet lysate and hydrogen peroxide was significantly higher in the case of platelets incubated with MPO compared to the lysate of control untreated platelets (Fig. 8B).

MPO does not induce platelet aggregation, only partial platelet activation

Given that previously described interactions of MPO with other cell types manifested the modulation of the physiological function of these cells, the effect of MPO on platelet functions was evaluated. MPO did not induce any detectable platelet aggregation measured by classical turbidimetric methods (data not shown). Similarly, the preincubation of platelets with MPO did not increase the sensitivity nor the grade nor the speed of response to classical agonists such as collagen, ADP, or thrombin used for the induction of platelet aggregation (data not shown). Similarly, MPO did not induce any significant release of RANTES or PDGF-AB by platelets into supernatants (data not shown). Thus, these data do not suggest any massive degranulation of platelets mediated by MPO.

MPO did not induce any significant decrease in platelet viability over the evaluated period of 30 min (Fig. 9A). This was in contrast to treatment by CaI as positive control, which already at this time point revealed a tendency to decrease platelet viability (Fig. 9A). Similarly, MPO did not induce a decline in platelet number in suspension under any conditions up to 2 h of incubation (data not shown). In contrast, the long-term incubation for 7 days showed

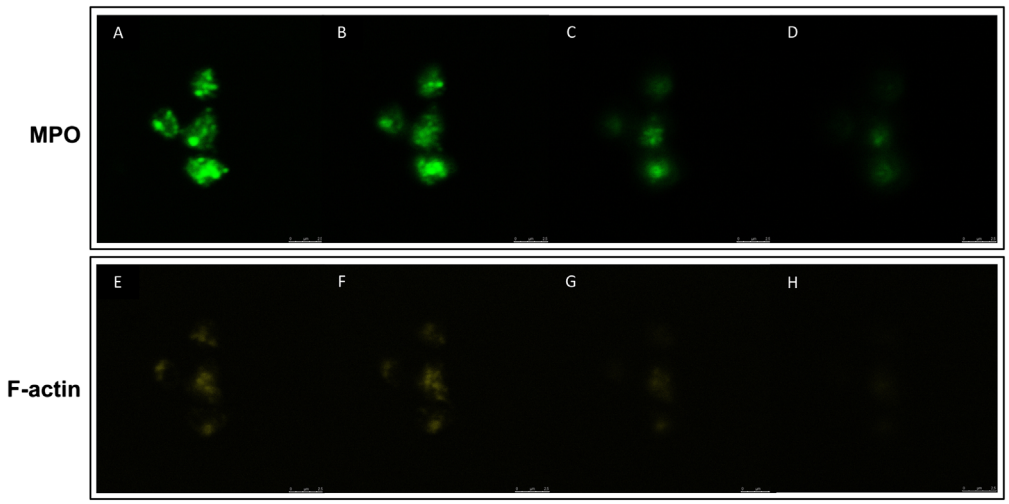

Fig. 4. Effect of cytochalasin D on MPO internalization by platelets. Isolated human platelets were preincubated with cytochalasin D (10 μM). After being washed, the platelets were incubated with MPO (34.5 nM), fixed, and deposited on glass slides. After permeabilization with 0.5% Triton X-100, the platelets were incubated with rabbit anti-MPO antibody and then with anti-rabbit IgG secondary antibody conjugated with DyLight 649 (green; A, B, C, D) and Phalloidin Alexa 488 (yellow; E, F, G, H). Samples were analyzed by scanning confocal microscopy. Images are shown in *x*–*y* orientation and represent a series of individual *Z*-slices selected from *Z*-dimension scanning (each step 0.13 μm) (A, E) 3.4 μm, (B, F) 2.8 μm, (C, G) 2.2 μm, (D, H) 1.5 μm. Bars, 2.5 μm.

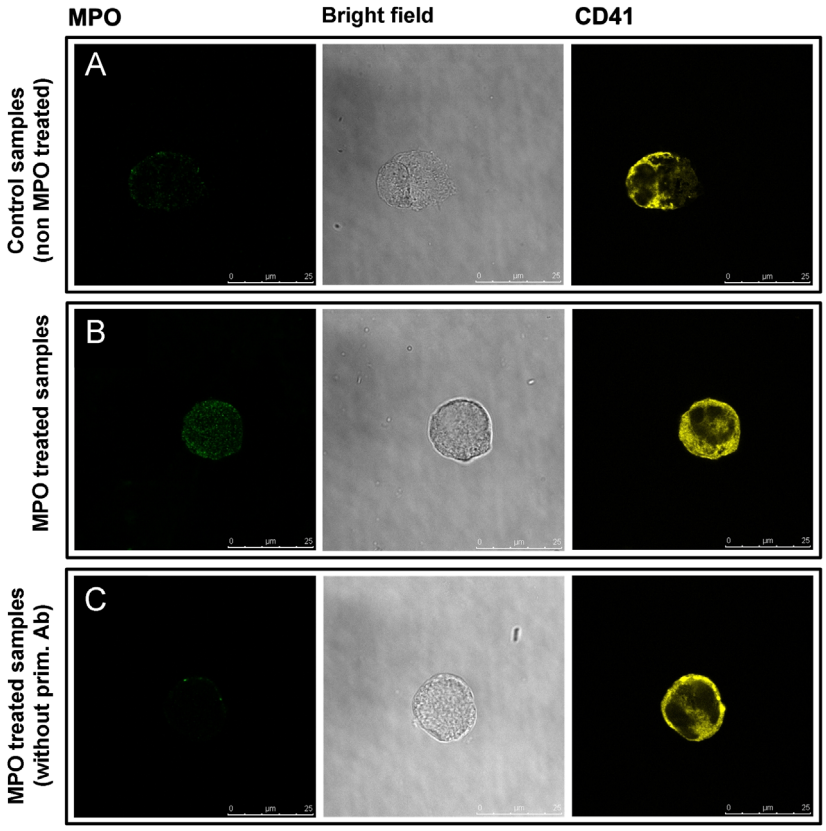

Fig. 5. Determination of MPO presence and the localization of bound MPO on megakaryocytes. Megakaryocytes isolated from mouse bone marrow, (A) MPO-untreated and (B, C) incubated with MPO (34.5 nM), were deposited on glass slides and fixed. After permeabilization with 0.5% Triton X-100, the megakaryocytes were incubated with rabbit anti-MPO antibody and then with anti-rabbit IgG secondary antibody conjugated with DyLight 649 (green). FITC-labeled anti-CD41 antibody was used for megakaryocyte identification (yellow). Samples without primary antibody served as a control (C). Samples were analyzed by scanning confocal microscopy. Images of cells were also taken in bright light (BL). Bars, 25 μm.

a dose-dependent decrease in platelet viability compared to control (Fig. 9B). The functionality of this test was further confirmed by the observation that no viable platelets could be observed in positive control CaI-treated samples (Fig. 9B). Overall, these data suggest the partial activation of platelets by MPO, which led to the potentiation of platelet death after long storage.

Further, the incubation of platelets with MPO induced a mild, yet significant, increase in the surface expression of the receptors

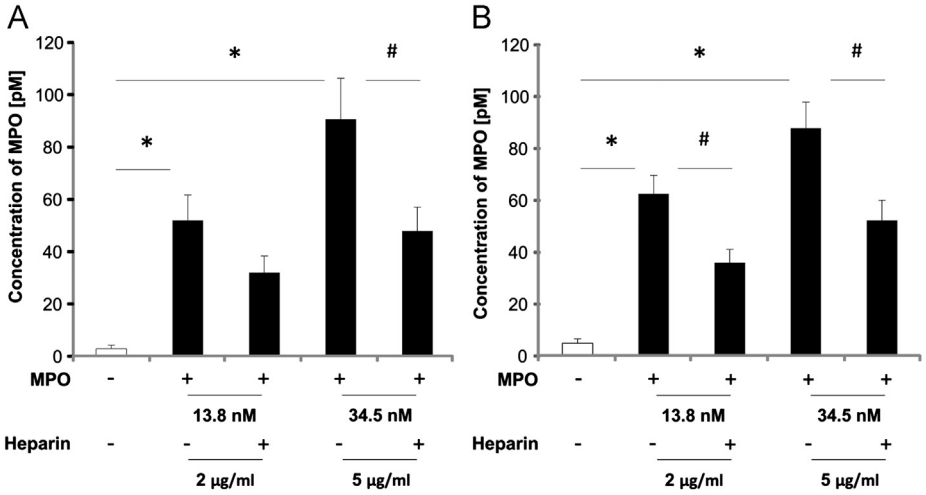

Fig. 6. Effect of heparin on overall binding of MPO to human platelets. (A) Isolated platelets were incubated with heparin (2 μg/ml) and MPO (13.8 nM) simultaneously for 30 min. (B) Isolated platelets were preincubated with MPO (13.8 or 34.5 nM), washed, and then exposed to heparin (2 or 5 μg/ml). In both cases, after extensive washes, the platelets were lysed, and cell-associated MPO in platelet lysates was assessed by ELISA. Data are expressed as means ± SEM ($n=6$, $n=5$). $^{*}p\leq0.05$, statistically significant difference between MPO-untreated and MPO-treated platelets without heparin, according to the Dunnett test, and $^{\#}p\leq0.05$, statistically significant difference between MPO-treated platelets with and without heparin, according to pairwise t test.

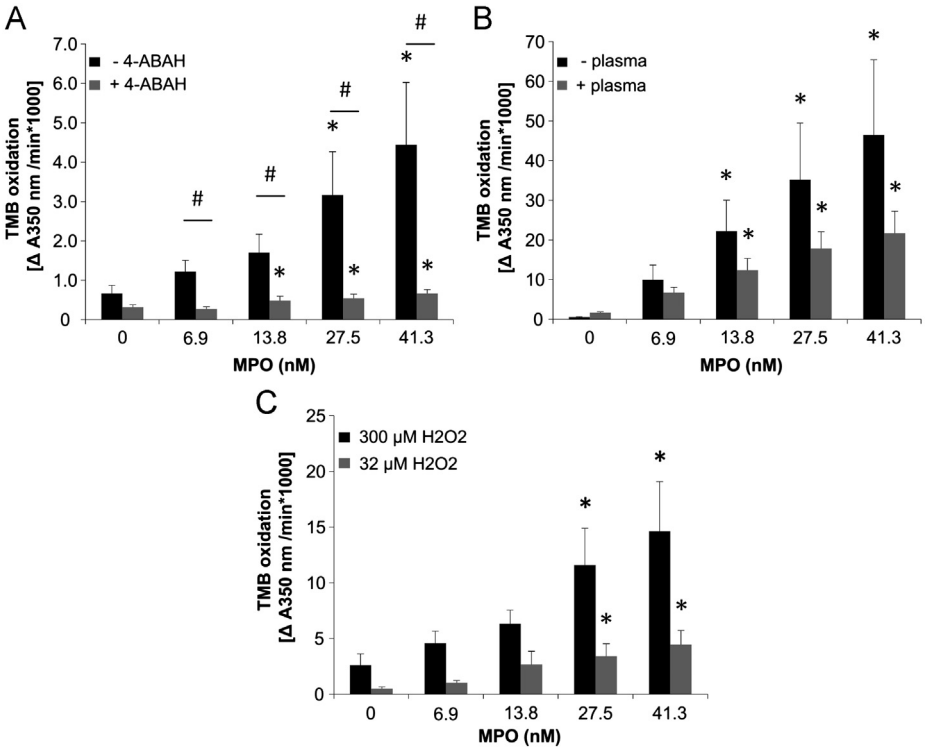

Fig. 7. Peroxidase enzymatic activity of MPO deposited on human platelets (A) with and without MPO inhibitor 4-ABAH, (B) with and without 20% plasma, and (C) in the presence of various concentrations of H_2O_2. Isolated platelets incubated with various concentrations of MPO were extensively washed and lysed, and enzymatic activity was detected by spectrophotometry employing TMB. Data are presented as the increase in absorption at 350 nm per minute and are expressed as means ± SEM ($n=5$). $^{*}p\leq0.05$, statistically significant difference between MPO-untreated and MPO-treated platelets according to the Dunnett test, and $^{\#}p\leq0.05$, statistically significant difference between results with and without inhibitor according to pairwise t test.

on platelet P-selectin (Fig. 10A) and PECAM-1 (Fig. 10B). In agreement with this, the increased manifestation of F-actin, a marker of actin filament activation, was observed in platelets after incubation with MPO (Fig. 10C). Another marker of platelet activation was the significantly increased formation of ROS determined by the HE (Fig. 10D) and DHR-123 fluorescent probes (Fig. 10E). All these data together further support the theory of the partial activation, or so-called "priming", of platelets by MPO.

MPO potentiates platelet interaction with neutrophil granulocytes and adhesion to ECs in vitro

In general, partially activated "primed" platelets adhere and interact more avidly with other cell types they come into contact with within the blood circulation. Thus, the effects of MPO on the formation of platelet–neutrophil granulocyte aggregates and the adherence of platelets to endothelial cells were determined.

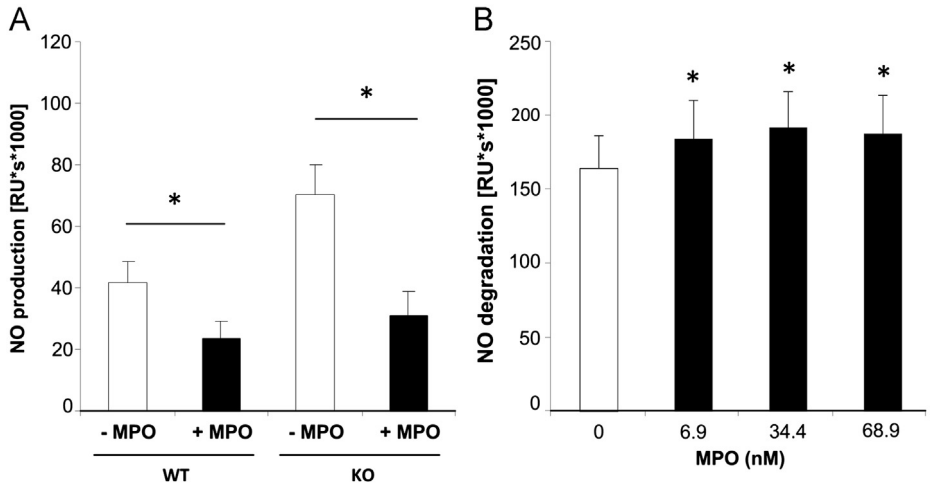

Fig. 8. Metabolism of NO mediated by MPO bound to platelets. (A) NO production was determined after preincubation of isolated mouse wild-type (WT) and MPO-deficient (KO) platelets with MPO (34.5 nM), followed by extensive washing and activation with Cal employing an NO electrode. Data are expressed as means ± SEM ($n=3$). *$p \leq 0.05$, statistically significant difference between MPO-untreated and MPO-treated platelets according to the pairwise t test. (B) NO catabolism was determined in lysates from human platelets incubated with various concentrations of MPO. Data are expressed as means ± SEM ($n=12$). *$p \leq 0.05$, statistically significant difference between MPO-untreated and MPO-treated platelets according to the Dunnett test.

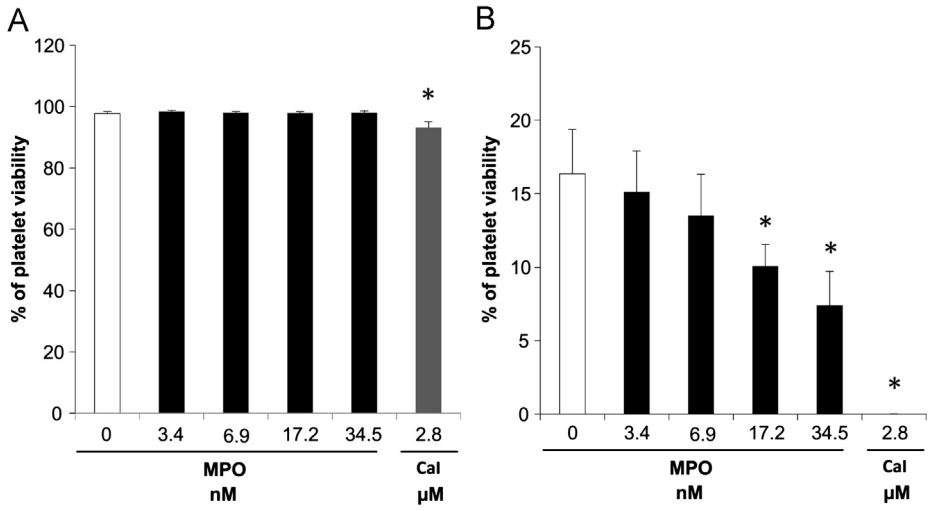

Fig. 9. The effect of MPO on platelet viability. Platelets were incubated with various concentrations of MPO or Cal (positive control) for (A) 30 min at RT and (B) 7 days at 37 °C. Platelet viability was assessed by the ability of platelets to accumulate calcein-AM but not FM4-64 and was quantified as a percentage of the number of viable platelets in control untreated samples. Data are expressed as means ± SEM ($n=4$). *$p \leq 0.05$, statistically significant difference between MPO-untreated and MPO/Cal-treated platelets according to the Dunnett test.

As expected, platelets preincubated with MPO formed aggregates with neutrophil granulocytes with greater frequency compared to control untreated platelets (Fig. 11A).

Further, a higher amount of MPO-preincubated platelets adhered to the untreated and TNF-α-treated HUVEC monolayer compared to untreated control platelets (Fig. 11); however, these data were not statistically significant because of higher variability.

MPO modulates bleeding time in mice in vivo

To test whether these observations translate into MPO-dependent changes in platelet functions in vivo, purified MPO was infused into mouse systemic circulation and bleeding time from the tail was evaluated. This resulted in significantly shorter bleeding times compared to mice infused with human serum albumin (HSA; Fig. 12).

Further, the importance of endogenously produced MPO on bleeding time was evaluated employing the model of MPO-deficient mice. In contrast to exogenously added MPO, a comparison of bleeding time in untreated MPO-deficient mice and their wild-type counterparts did not reveal any significant differences (data not shown). This could be due to the minimal release of MPO in healthy mice. Thus, systemic inflammation was induced by the intraperitoneal application of endotoxin to induce the release of MPO into the systemic circulation. However, the bleeding time in this model was very short — up to 5 s in both wild-type mice and MPO-deficient mice (data not shown).

Discussion

Alterations to the endothelium and circulating leukocyte functions caused by MPO released from activated neutrophils have emerged as hallmarks of vascular inflammation [2]. In this study, the current concept was extended with respect to the importance of MPO in altering platelet functions after being sequestered within platelets. This phenomenon can be proposed as another important

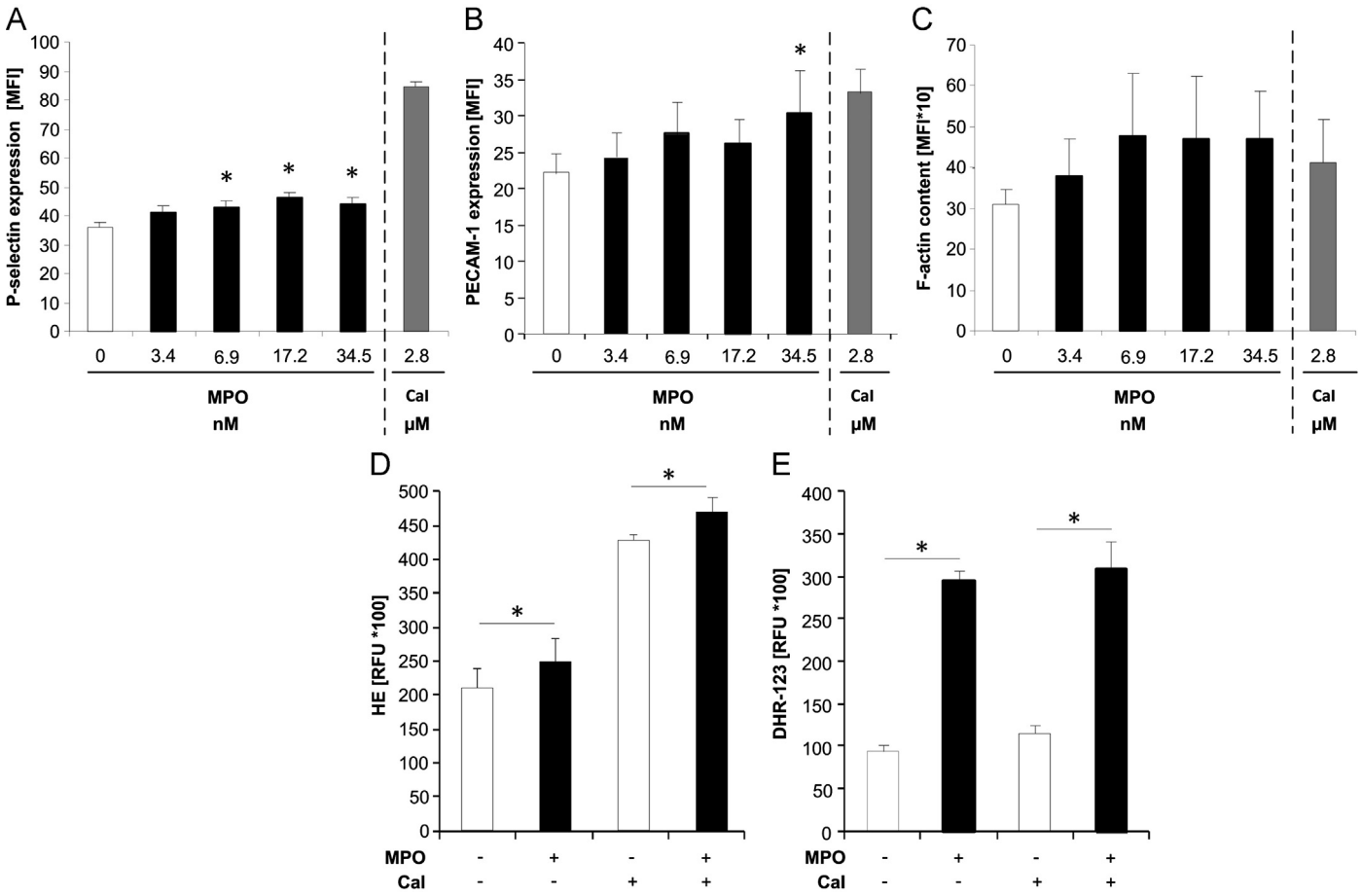

Fig. 10. The activation of human platelets was determined based on the increase in (A) surface P-selectin, (B) surface PECAM-1, and (C) F-actin expression and increased ROS production determined by (D) HE and (E) DHR-123. (A, B) The surface expression of P-selectin and PECAM-1 was measured after the incubation of isolated platelets with various concentrations of MPO or CaI (positive control). After extensive washes, platelets were stained with PE-conjugated anti-P-selectin or anti-PECAM-1 antibodies and analyzed by flow cytometry. Data are presented as the geometric mean fluorescence intensity (MFI) and are expressed as means ± SEM ($n=5$). *$p\leq 0.05$, statistically significant difference between MPO-untreated and MPO-treated platelets according to the Dunnett test. (C) F-actin content was measured after the incubation of isolated platelets with various concentrations of MPO or CaI for 2 min followed by fixation. Platelets were stained with Alexa Fluor 488-phalloidin and analyzed by flow cytometry. Data are presented as the geometric mean fluorescence intensity (MFI) and are expressed as means ± SEM ($n=5$). (D, E) ROS production was determined after preincubation of isolated human platelets with MPO (34.5 nM), followed by activation with or without CaI by fluorimetry using hydroethidine (HE) and dihydrorhodamine-123 (DHR-123) probes. Data are presented as relative fluorescence units (RFU) and expressed as means ± SEM ($n=6$, $n=3$). *$p\leq 0.05$, statistically significant difference between MPO-untreated and MPO-treated platelets according to pairwise *t* test.

mechanism through which MPO affects blood hemostasis and can contribute to the development of cardiovascular diseases.

The binding of MPO to platelets was depicted using several techniques showing MPO presence both on the surface of platelets and inside their three-dimensional structure. The MPO sequestered by platelets remained catalytically active as was shown by the TMB reaction employing the inhibitor 4-ABAH. Experiments with cytoskeleton inhibitor to break the active internalization of molecules indicate that the presence of MPO inside platelets is probably due to passive MPO binding on the membrane surface of the canalicular system, which is open to extracellular space and penetrates the platelet body. In this case, any active transport of MPO through the platelet membrane would not be required. The idea of platelet sequestration of MPO is supported by Zabucchi et al. [30], who observed MPO staining both on the cell surface and in the canalicular system using electron microscopy. In general, the ability of platelets to incorporate proteins such as horseradish peroxidase was suggested by Handagama et al. [32]. However, in neither of these studies did the authors provide information about the importance of the active transport of heterogeneous protein across the platelet membrane.

In general, most of the previously reported interaction between MPO and the cell surface was thought to be due to the highly cationic MPO (an isoelectric point higher than 10) interaction with polyanionic GAGs on the cell surface [8,9,11,33]. In this study, the importance of the electrostatic interaction of MPO with platelets was documented by the ability of an excess of negatively charged heparin to prevent the binding and the ability of heparin to liberate MPO sequestered on platelets. Similarly, previous studies showed the prevention and reversion of MPO binding to endothelial cells by the presence of heparin [8,9,33,34]. Some authors suggest the interaction of MPO with albumin as a prerequisite of MPO binding to endothelial cells [35]. In our study, in contrast to heparin, the presence of albumin during incubation did not have any significant effect on this process.

MPO was not detectable in platelets isolated from healthy volunteers or control mice using immunohistochemical and ELISA methods. Further, in control wild-type mice, the observed background signal from immunohistochemical determinations was comparable to the signal obtained with platelets isolated from MPO-deficient mice. Similarly, determinations of MPO presence in platelet lysates determined using enzymatic assays did not reveal any difference between platelets from wild-type and MPO-deficient mice (data not shown). These data suggest that MPO is not significantly present in platelets isolated from healthy human volunteers or control mice. However, all these methodological

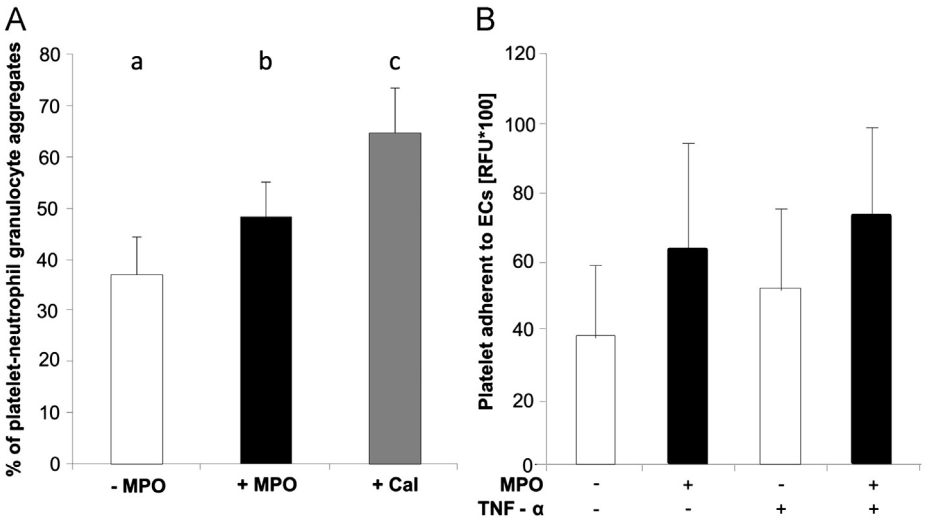

Fig. 11. Effects of MPO (A) on platelet–granulocyte aggregate (PGA) formation and (B) on the interaction of platelets with endothelial cells. (A) Whole blood was preincubated with MPO (34.5 nM) or Cal. PGAs were labeled with FITC–anti-CD15 antibody (a marker of neutrophil granulocytes) and PE–anti-CD41a antibody (a marker of platelets). PGAs were detected by flow cytometry and were quantified as a percentage of the total granulocyte population (means ± SEM; $n=5$). Letters above bars indicate statistical differences between MPO-untreated and MPO/Cal-treated platelets according to the Tukey test ($p \leq 0.05$). (B) Calcein-AM-labeled platelets were incubated with MPO (34.5 nM). Washed platelets were co-incubated with adherent endothelial cells under static conditions. The fluorescence signal of bound platelets was determined by fluorimetry in relative fluorescence units (RFU). Data are presented as means ± SEM ($n=4$).

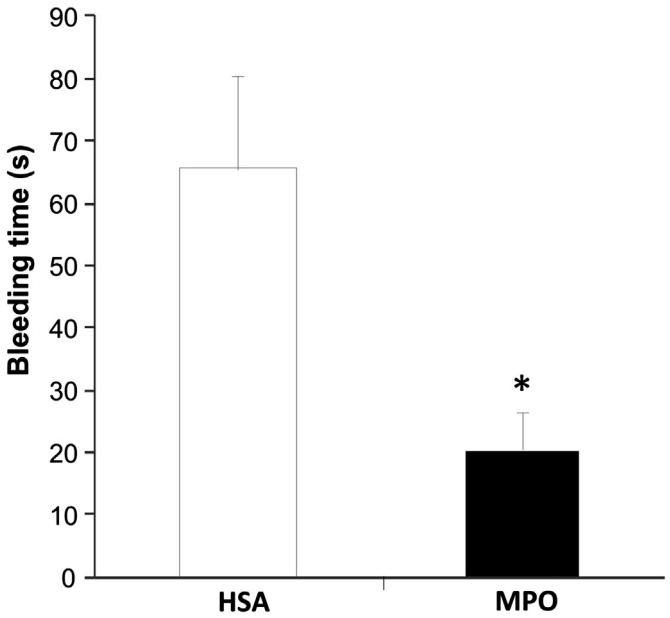

Fig. 12. The effect of MPO on bleeding time in vivo. After the infusion of MPO (69 pM per mouse) or HSA (151 pM per mouse) as a control through the jugular vein, the time of bleeding from the tail vein was determined. Data are presented as means ± SEM ($n=5$). *$p \leq 0.05$, statistically significant difference between HSA- and MPO-infused groups.

approaches retain a certain level of nonspecific background signal. Thus, in general, a theoretical residual presence of MPO in the platelets of healthy human individuals and mice cannot be excluded. Interestingly, the immunohistochemical analysis of the presence of MPO in platelet precursor megakaryocytes did not reveal any positive signal in these cells. The absence of MPO in megakaryocytes was further highlighted by the detectable presence of MPO after the incubation of megakaryocytes with MPO. The absence of MPO in megakaryocytes is in agreement with the generally accepted assumptions that are applied for the cytochemical differentiation of bone marrow cells in hematology. The hematological differentiation of hematopoietic precursors recognizes megakaryocytes and precursors from this lineage as MPO-negative cells [36]. Thus, our data suggest that platelets are produced as MPO-free cells after their release from bone marrow. MPO could be sequestered on their surface membranes during an acute increase in MPO concentration in the blood circulation.

In general, the activation of platelets is associated with a shape change connected with the reorganization of the cytoskeleton and the translocation of some adhesion molecules such as P-selectin and PECAM-1 from the α-granule membrane to the outer surface, which is considered to be one of the indicators of platelet activation [37]. Our data show a mild, yet significant, MPO-mediated increase in the surface expression of these receptors, accompanied by an increase in the asSEMbly of actin monomers into F-actin. This suggests the mild MPO-mediated activation of platelets. In agreement with this, MPO mediated an increase in ROS production by platelets, which is considered to be another typical marker of platelet activation [14,38]. However, these data should be taken as qualitative and not as quantitative indicators of ROS production, because the fluorescent probes used have significant limitations such as the formation of 2-hydroxyethidium by the oxidation of HE and the nonselective oxidation, autoxidation, and redox cycling of 2′,7′-dichlorofluorescin (DCFH) [39,40]. Despite this, however, because increased ROS production by platelets treated by MPO was observed with both of these probes, an MPO-mediated increase in ROS production can be suggested. The significantly higher signal obtained with DCFH could be due to DCFH sensitivity to peroxidase-catalyzed oxidation, which could further confirm the enzymatic activity of bound MPO. The MPO-mediated priming of platelets can also be suggested from the determination of platelet viability. The MPO did not affect platelet viability during short-term incubation, in contrast to strong activators such as Cal. However, MPO did mediate a decrease in viability compared to untreated control after long-term storage up to 7 days.

Interestingly, in contrast, no significant MPO-mediated increase in granule content in cultivation medium was detected, suggesting that the observed increase in the surface expression of receptors was not accompanied by any massive degranulation of platelets. Similarly, MPO did not induce any detectable platelet aggregation.

Nor did it potentiate or increase the sensitivity of platelets to model inducers of platelet activation, including collagen, ADP, or thrombin. Thus, this suggests that MPO induces only a partial activation of platelets, which is not characterized by extensive platelet degranulation or aggregation. This low level of platelet activation can be recognized as platelet "priming," and various molecules or compounds were found to induce this partial platelet activation that can amplify the response of platelets to activating stimuli [12]. For example, Begonja et al. [14] demonstrated that platelets activated by various agonists produced intracellular ROS, which significantly affected the activation of αIIbβ3 integrin, but not α/dense granule secretion or platelet shape change. The platelet is suggested to increase multicellular events that involve interactions among platelets and other cellular components of blood and connective tissue [12]. In agreement with this, platelets preincubated with MPO adhered more avidly to the activated endothelium, and MPO potentiated the increased formation of platelet leukocyte aggregates. One of the factors contributing to this effect could be the observed increase in P-selectin surface expression, which is suggested as being required for the formation of platelet–leukocyte conjugates [20,21,41–43]. However, the experiment was performed with endothelial cells isolated from umbilical vein under static conditions; thus, confirmation of this phenomenon with endothelial cells isolated from various arteries and veins under conditions of shear stress is required.

One of the key mechanisms by which MPO contributes to vascular inflammation processes is the disturbance of vessel function through the catabolism of NO leading to the decreased bioavailability of NO for vascular and blood cells [10,34]. In this study, the catalytic degradation of NO by lysates of platelets preincubated with MPO was demonstrated, further confirming the catalytic activity of MPO sequestered within platelets. On the basis of the significant production of ROS by MPO-preincubated platelets, it was possible to assume that MPO sequestered within platelets can catalytically degrade NO. Accordingly, the decreased production of NO by platelets preincubated with MPO was revealed for both unstimulated platelets and CaI-stimulated platelets. Given the generally accepted role of NO as a negative feedback molecule that inhibits platelet activation [14,15,38,44], we can speculate that the catalytic consumption of NO can contribute to platelet activation. Freedman et al. [45] indicated that the impairment of platelet-derived NO may be linked to the pathogenesis of acute coronary syndromes. Because platelet recruitment is a primary means of thrombus propagation, it is reasonable to speculate that the loss of platelet NO could increase the risk of developing diseases such as unstable angina, in which coronary thrombosis is a primary event [45].

Finally, bleeding time was determined as a functional evaluation of observed effects on blood hemostasis. The infusion of MPO into the mouse systemic circulation significantly shortened bleeding time from the tail vein. However, comparing MPO-deficient mice to their matching wild-type controls did not reveal any differences among bleeding times between MPO-deficient mice and their WT counterparts, either control untreated or treated with endotoxin. Inflammatory models were employed to induce an increase in MPO in mouse blood circulation; however, any increase in MPO plasma levels was not detected in these mice. This is in contrast to our previous studies and could be explained by the limited degranulation of MPO in mice compared to humans and the low reproducibility of inducing increased MPO blood plasma levels in these experimental mouse inflammatory models. Interestingly, there are no data reporting any significant differences in thrombus formation under acute or chronic vascular inflammation when comparing wild-type and MPO-deficient mice, e.g., in pulmonary vasculature.

Overall, our data together with current literature suggest that MPO activates platelets; however, MPO is not a strong activator of platelet activation. Interestingly, Morrell and Maggirwar [46] have reported newly discovered platelet activators that are not strong agonists but, rather, important modifiers of platelet activation, and, in the context of vascular inflammation, their platelet activation effects may contribute significantly to accelerated vascular inflammation without overt thrombosis. Thus, MPO could be suggested to be considered among these activators. The importance of the MPO–platelet interaction in the vascular inflammation pathology is supported by the facts that platelet and neutrophil granulocyte activation is an early event during local inflammation and the expression of P-selectin, the formation of platelet–leukocyte aggregates, and elevated levels of serum MPO are reported as early predictors of coronary artery complications [47,48]. Moreover, the contribution of decreased platelet NO production to an increase in circulating platelet–monocyte aggregates during hypertension was demonstrated [49]. Thus, MPO binding to platelets can contribute to the currently well-described negative effects of MPO on the development of chronic and acute vascular inflammatory pathological processes.

Acknowledgments

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Original article

Red blood cells serve as intravascular carriers of myeloperoxidase ☆

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ABSTRACT

Myeloperoxidase (MPO) is a heme enzyme abundantly expressed in polymorphonuclear neutrophils. MPO is enzymatically capable of catalyzing the generation of reactive oxygen species (ROS) and the consumption of nitric oxide (NO). Thus MPO has both potent microbicidal and, upon binding to the vessel wall, pro-inflammatory properties. Interestingly, MPO – a highly cationic protein – has been shown to bind to both endothelial cells and leukocyte membranes. Given the anionic surface charge of red blood cells, we investigated binding of MPO to erythrocytes. Red blood cells (RBCs) derived from patients with elevated MPO plasma levels showed significantly higher amounts of MPO by flow cytometry and ELISA than healthy controls. Heparin-induced MPO-release from patient-derived RBCs was significantly increased compared to controls. *Ex vivo* experiments revealed dose and time dependency for MPO-RBC binding, and immunofluorescence staining as well as confocal microscopy localized MPO–RBC interaction to the erythrocyte plasma membrane. NO-consumption by RBC-membrane fragments (erythrocyte "ghosts") increased with incrementally greater concentrations of MPO during incubation, indicating preserved catalytic MPO activity. *In vivo* infusion of MPO-loaded RBCs into C57BL/6J mice increased local MPO tissue concentrations in liver, spleen, lung, and heart tissue as well as within the cardiac vasculature. Further, NO-dependent relaxation of aortic rings was altered by RBC bound-MPO and systemic vascular resistance significantly increased after infusion of MPO-loaded RBCs into mice. In summary, we find that MPO binds to RBC membranes *in vitro* and *in vivo*, is transported by RBCs to remote sites in mice, and affects endothelial function as well as systemic vascular resistance. RBCs may avidly bind circulating MPO, and act as carriers of this leukocyte-derived enzyme.

1. Introduction

Myeloperoxidase (MPO) is a heme-containing enzyme abundantly expressed in immune cells, including polymorphonuclear neutrophils (PMN), monocytes and macrophages. MPO is stored in large amounts in the azurophilic granules of neutrophils and is secreted into the phagosome and the extracellular space upon activation by inflammatory stimuli [1]. Enzymatically capable of generating reactive oxygen species (ROS), in particular hypochlorous acid (HOCl), it is considered one of the major bactericidal proteins, taking part in the first line of host defense [2].

Besides its bactericidal properties, MPO has been found to potently affect vascular homeostasis. MPO catalytically consumes nitric oxide (NO), thereby directly impairing endothelial function. Also, MPO catalyzes the oxidation of thiols by HOCl, such as thioester and tyrosyl residues, thereby structurally impairing vascular integrity [3]. Moreover, MPO exhibits leukocyte-attracting properties irrespective of its catalytic function. Electrostatic interactions between the highly-cationic MPO and the anionic glycocalyx residues of PMNs and endothelial cells lead to attraction of PMNs to the endothelial surface [4]. Given the potent pro-inflammatory properties of MPO, its distribution in the microcirculation is crucial for its pathobiological significance.

Red blood cells (RBCs) are the most common cells in peripheral blood, with a concentration of 4–6 million cells per microliter. High amounts of sialic acid, bound to glycophorins in the erythrocyte membrane, are responsible for a halo of negative charge that surrounds RBCs [5].

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We postulated that cationic MPO would be attracted to erythrocyte membrane. Due to the large number of RBCs and their close interaction with endothelial cells and other blood cells or even with RBCs themselves, depending on the region of vasculature, we investigated RBC capability to bind to, transport, and deliver MPO, with potential relevance for changing endothelial function and vascular tone.

2. Methods

Human studies were performed after informed consent, in accordance with the Declaration of Helsinki and approved by the Board of Physicians Ethics Committee Hamburg. All animal experiments were approved by the local ethics committee an in accordance with the US National Research Council's Guide for the Care and Use of Laboratory Animals.

2.1. Isolation of RBCs

Blood for erythrocyte isolation was collected either from healthy volunteers or from patients in intermediate or intensive care unit. Patients had either been diagnosed with acute coronary syndrome within the last week or had proof of severe heart failure, and were excluded for fever (body temperature >38 °C) or for c-reactive protein (CRP) level >50 mg/dl. Investigators were blinded to anticoagulation regime or additional clinical data.

Venous blood was mixed with EDTA (0.5 M pH 8.0) 100:1 and centrifuged for 10 min, supernatant and buffy-coat with leukocytes were removed, and 2 subsequent washing steps with phosphate buffered saline (PBS-Invitrogen) were performed. The pellet was diluted to 5 million RBC/μl stock solution and further diluted with PBS as needed. Isolation of RBCs from C57BL/6J mice (Jackson Laboratory) was performed using the same protocol.

2.2. Isolation of RBC-ghosts

RBCs were lysed in a 10-fold volume of 5 mM Tris–HCl, pH 7.0, 1 mM EDTA and mixed for 15 min at room temperature. Afterwards, samples were centrifuged at 21,000 ×*g* for 30 min at 4 °C. The pellet was resuspended in 5 mM Tris–HCl, pH 7.0, 1 mM EDTA and washed again. High salt wash was performed with 0.05 M Tris–HCl, pH 7.0, 1 mM EDTA, 0.5 M NaCl, and then twice resuspended and washed with 5 mM Tris–HCl, pH 7.0, 1 mM EDTA, while centrifuging at 21,000 ×*g* for 12 min at 4 °C. RBC ghosts were washed until they were white in color. Storage of RBC ghosts was performed in a small volume of 0.05 M Tris–HCl, pH 7.0 (500 μl per pellet) for a maximum of 1 day. Before use, membrane fragments were transferred into PBS and diluted to a stock solution using spectrometric OD 280 nm absorbance to create arbitrary units.

2.3. Flow cytometry

The presence of MPO on the surface of RBC was determined using isolated RBCs from healthy volunteers and patients (described above). After washing steps, RBCs were incubated with R-Phycoerythrin (PE)-conjugated anti-MPO antibody or isotype control (Acris, USA) for 10 min at RT. RBCs were analyzed using flowcytometer FACSCanto (BD Biosciences, USA). RBCs were defined by forward- and side-scatter characteristics, and 10,000 individual events were collected within the RBC gate. Cells stained with appropriate isotype controls were used to determine non-specific background signal.

2.4. Fluorescent staining of RBCs and confocal microscopy

RBC suspension was streaked on microscope slides and dried overnight. In the first set of experiments, RBC suspension was fixed in 100% ethanol for 30 min. Subsequently, samples were washed with PBS, blocked and permeabilized with 10% goat serum and 0.1% Triton X-100 in PBS for 30 min at RT. Non-permeabilized samples were blocked with 10% goat serum in PBS at RT for 1 h. Samples were incubated with antibodies against MPO (rabbit, Calbiochem EMD Millipore 1:250; rabbit polyclonal, Thermo Scientific, USA 1:125) and hemoglobin (goat, Thermo Scientific, 1:250) at 4 °C overnight, and finally labeled with secondary antibodies (Alexa 488 IgG anti-rabbit, DyLight 488 Thermo Scientific, Alexa 594 IgG anti-goat). Images were acquired with a Leica microscope (DMLB) and IVision software or a confocal microscope (TCS SP5, Leica, Germany) using a 63 × 1.4 oil immersion objective. A three-dimensional projection was reconstructed from a two-dimensional z-stack series using LAS AF Lite software (Leica).

2.5. Immunofluorescence staining of 3-chlorotyrosine in murine tissue

Harvested organs (as described in 2.6) were embedded and frozen in optimal cutting temperature compound (OCT) and cut to 4 μm sections. Sections were thawed, fixed in 3.7% formaldehyde solution, incubated with 0.1% Triton X-100 and blocked with 10% goat serum. Sections were treated with first antibody rabbit IgG to 3-chlorotyrosine (1:100, Hycult Biotech) and secondary antibody goat IgG to rabbit labeled with Alexa Fluor 488 (Molecular Probes). Nuclei were counterstained with DAPI. Images were acquired with a Keyence BZII Analyzer Software (Keyence).

2.6. NO-consumption by RBC-ghosts

RBC-ghosts of different concentrations (0, 10, 100 arbitrary units) after incubation with different MPO concentrations (0, 10, 50 μg/ml) were placed into glass vials. Using an NO electrode (AmiNO 700, Innovative Instruments, USA), connected to the ISO-NO MARK II potentiostat (WPI, USA) while stirring at 37 °C, NO concentration was measured as previously described [6] after administration of 15 μM H_2O_2 with 10 μM PAPANO as a NO-donor. Values were calculated from the maximal rate of NO consumption per second and are expressed as pA/s.

2.7. Animal experiments

Male C57BL/6J (Jackson Laboratory) aged 10–15 weeks were treated with an infusion of MPO-loaded RBCs. Briefly, murine blood was harvested in EDTA 1:100. RBCs were isolated as described above, and diluted in standard fashion with NaCl (Fresenius) for incubation with MPO or NaCl. Two subsequent washing steps followed. Treated murine RBCs were then reinfused into animals of the same gender and age via the right carotid artery under anesthesia with isoflurane and buprenorphine. After circulation for 30 min, animals were euthanized and organs were harvested in standard fashion after perfusing the animal with 5 ml of PBS via the carotid catheter.

2.8. Langendorff-perfusion

Freshly isolated mouse hearts were mounted on a Langendorff-perfusion system and rinsed with 500 μl of isotonic NaCl-solution at 37 °C. Subsequently, hearts were perfused with 2 ml of PBS-heparin (50 IU/ml) at 37 °C and the rinse was collected. The resulting solution was concentrated to 200 μl using speedvac-centrifugation and MPO-concentration was quantified by ELISA. Unless otherwise stated, all ELISA analysis was performed using a CardioMPO kit (Cleveland Heart Lab) according to manufacturer's instructions.

2.9. Organ bath

Aortic segments of euthanasized mice were carefully dissected, and endothelium-dependent and -independent relaxation were determined in response to increasing doses of acetylcholine.

(Ach, 10^{-9}–5×10^{-6} mol/l) and nitroglycerine (NTG) as previously described [6,7].

2.10. Sialidase treatment

Isolated human RBCs were diluted and incubated with or without 200 mU/ml sialidase (Sigma Aldrich) at 37 °C for 1 h. RBC were washed twice with PBS, resuspended in PBS and incubated with 0.3 μg/ml MPO at 37 °C for 30 min. Next, RBCs were washed 3 times with PBS and lysed in 1% hexadecyltrimethylammonium hydroxide/PBS. MPO-concentration was determined with a MPO ELISA kit (Enzo Life Sciences).

2.11. Systemic vascular resistance

Mice (littermates (+/+) of $Mpo^{-/-}$ colony) were anesthetized with isoflurane (Abbott, Wiesbaden, Germany), received low dose buprenorphine (Essex-Pharma, Munich, Germany; 0.05 mg/kg body-weight subcutaneously) for analgesia and were placed on a heating pad to maintain body temperature. Following endotracheal intubation, animals were ventilated with 150 strokes/min and stroke volume of 7 μl/g bw. The left jugular vein was cannulated with PE-10 tubing and a solution of 12.5% bovine serum albumin (Sigma-Aldrich Corp., St. Louis, MO, USA, 2 μl/g bw) was infused. A microtip conductance pressure–volume catheter (1.4F, SPR-839 NR, Millar Instruments, Houston, TX, USA) was inserted into the carotid artery. Heart rate was maintained between 400 and 500 bpm by adjusting the concentration of isoflurane accordingly. The thorax was opened, the apex was punctured with a 26 G cannula and another 1.4 F microtip conductance catheter was inserted into the left ventricle as described [8]. Left ventricular (LV) pressure and volume and carotid pressure were recorded continuously with an ADInstruments PowerLab 8/30 system (ADInstruments, Spechbach, Germany). After baseline measurements, 200 μl of RBCs (isolated from $Mpo^{-/-}$ mice and incubated with MPO or NaCl, as described above), was injected into the left ventricle and measurements were continued for 10 min. Volume calibration was performed using ADInstruments volume calibration cuvette.

Cardiac output (CO) was calculated from LV pressure volume loops. Mean arterial pressure (MAP) was calculated from carotid pressure, and systemic vascular resistance (SVR) was estimated as follows: $SVR = 80 \times MAP \times CO^{-1}$.

2.12. Statistical analysis

All calculations were made with SPSS 17.0 (Statistical Package for Social Science, Chicago).

Normal distribution led to pairwise testing with Student's unpaired *t* test; otherwise the Mann–Whitney rank sum test was performed. One-way ANOVA and mixed-model regression were used for multiple comparisons, and as post-hoc tests the Fisher's least significant difference test was performed. A p value of <0.05 was considered statistically significant. Data are presented as mean ± SEM.

3. Results

3.1. Measurement of endogenous MPO on human RBCs

Isolated RBCs from healthy human subjects were diluted to different concentrations. After cell lysis, MPO was measured via ELISA to reveal *in vivo* MPO-RBC-binding in healthy subjects. As seen in Fig. 1, MPO concentrations increased with increasing RBC-concentrations (64.0 ± 2.5 pmol/l, 376.0 ± 37.4 pmol/l, 822.0 ± 45.4 pmol/l, and 1840.0 ± 189.7 pmol/l for 1, 10, 20, 5 and 0 Mio RBCs per ml, $p < 0.01$).

To test the binding of MPO to RBCs in patients *in vivo*, blood was taken from healthy volunteers, and patients who had been admitted to cardiac intensive care unit for acute coronary syndrome or heart failure. To verify that MPO plasma levels were higher in these ill patients, as seen in former studies, we measured MPO-plasma levels via ELISA (Fig. 2a). There were significantly higher concentrations of MPO in patients' plasma compared to healthy controls (1200.1 ± 288.4 pmol/l vs. 273.8 ± 20.4 pmol/l, $p < 0.01$). Subsequently, isolated RBCs from the same samples were measured via ELISA and flow cytometry for MPO concentration on their surfaces. Mean MPO concentration on RBCs (413.4 ± 103.2 pmol/l vs. 2169.9 ± 540.6 pmol/l, $p < 0.01$; Fig. 2b) and mean PE-fluorescence (683.1 ± 73.2 vs. 486.9 ± 55.9, $p < 0.05$; Fig. 2c) of RBC derived from patients were significantly higher than in controls.

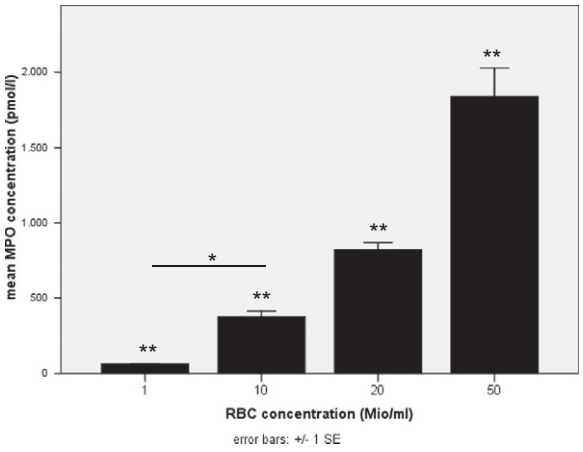


Fig. 1. MPO concentration on isolated RBCs from healthy human subjects. Concentration of MPO in pmol/l measured via ELISA in different concentrations of isolated RBCs from healthy subjects. $N = 5$ per group. * = $p \leq 0.05$, ** = $p \leq 0.01$ versus all other groups. Level of significance was determined using one-way ANOVA with posthoc LSD test.

To prove the specificity of these results and to elucidate reversibility of MPO-binding to RBCs, we performed a heparin-washing step of isolated RBCs. As previous studies indicate, binding to heparan sulfate-based glycosaminoglycans mobilizes MPO from extracellular matrix proteins, cultured endothelial cells and saphenous vein grafts [9]. As seen in Fig. 2d, MPO concentrations in supernatants of heparin-washed RBCs were significantly higher than those from PBS-washed RBCs, indicating release of MPO from RBCs mediated by heparin. Additionally, heparin-induced release of MPO was significantly higher in patient samples than in control samples (913.3 ± 183.7 pmol/l vs. 513.1 ± 104.1 pmol/l, $p < 0.05$).

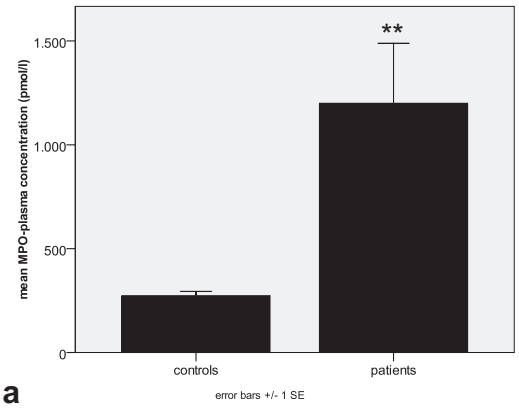
3.2. Ex vivo measurements of MPO on human RBCs

To test whether MPO binds to RBCs in a dose dependent fashion, isolated RBCs were incubated with MPO at varying concentrations. As shown in Figs. 3a and b, PE-fluorescence for MPO in flow cytometry (surface-bound MPO) and ELISA-measurements (total RBC-bound MPO) significantly increased with increasing concentrations of MPO-incubation. Additionally, MPO on RBCs increased over time when incubated with 5 μg/ml for different time points (Fig. 3c). To test whether erythrocyte sialic acid residues are involved in these MPO-binding-capacities of RBCs, we evaluated the amount of RBC-bound MPO in RBCs treated with sialidase prior to undergoing MPO-incubation. MPO-binding capacity of RBCs decreased in sialidase-treated RBCs to 74.2 ± 10.6% of the control group ($p < 0.05$, Fig. 3d).

After immunofluorescence staining of freshly isolated RBCs for MPO and hemoglobin, co-localization of hemoglobin and MPO further indicated a direct binding of MPO to RBCs. This was confirmed using confocal microscopy. We found that the distribution pattern of MPO was most likely RBC-membrane-associated and resembled the typical discoid shape of human RBCs (Fig. 4a). Interestingly, MPO co-localization with RBCs could be found both in permeabilized and non-permeabilized cells, indicating attraction of MPO to RBC surface membranes without apparent internalization of MPO (Fig. 4b).

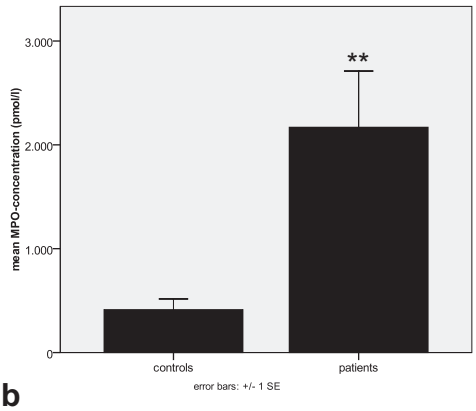
The impact of MPO on NO bioavailability and vascular function might be enhanced, if catalytically active MPO would be distributed over the vascular system by RBCs. Therefore, we investigated the

Elevated MPO-plasma levels and RBC-bound MPO in patients versus healthy controls



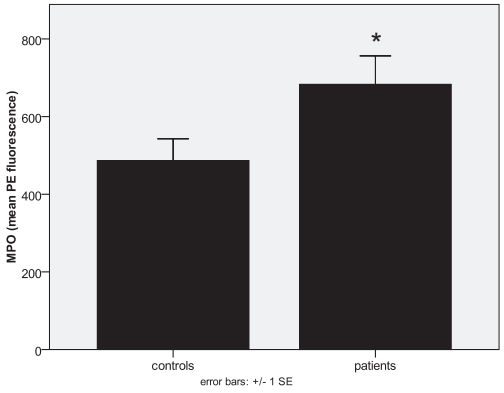
a

MPO plasma concentration in patients vs. controls



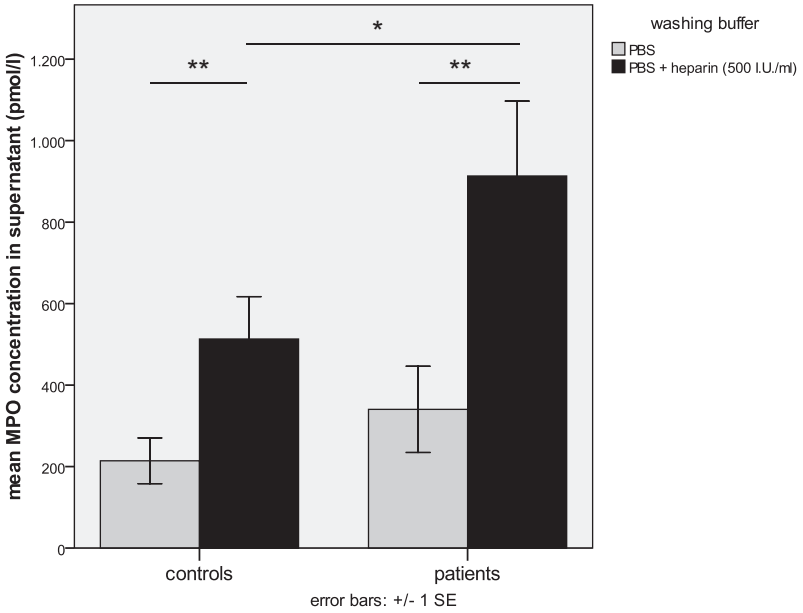
b

MPO-concentration on RBCs in patients vs. controls (ELISA)



c

MPO-binding on RBCs of patients vs. controls (flow cytometry)



d

Heparin-induced release of MPO from isolated RBCs in patients vs. controls

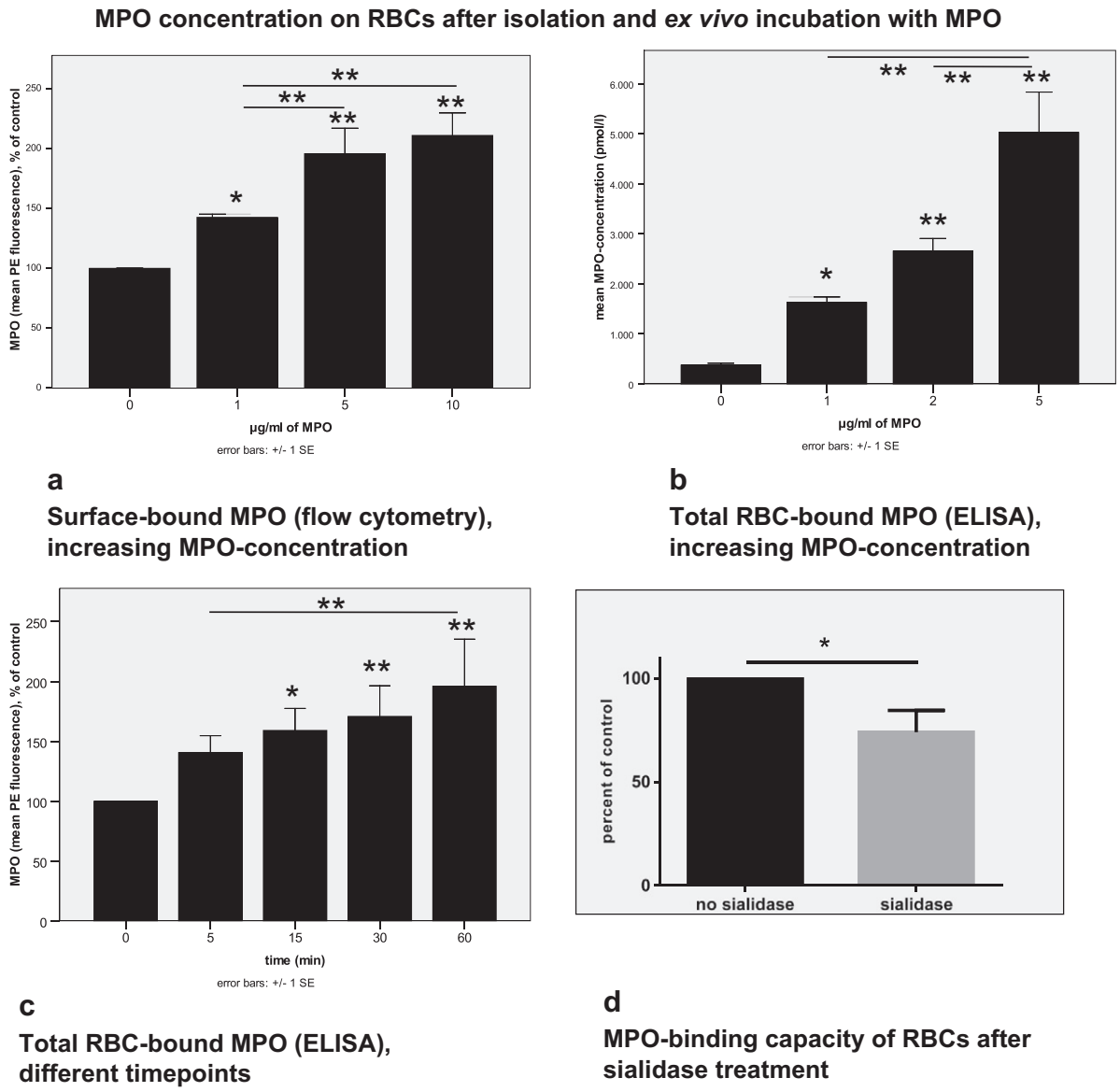


Fig. 3. MPO concentration on RBCs after isolation and ex vivo incubation with MPO. a: Surface-bound MPO (flow cytometry), increasing MPO-concentrations. After isolation, RBCs were incubated with MPO. After staining with PE-labelled MPO antibodies, fluorescence was measured using flow cytometry. $n = 5$ per each group $^{*} = p \leq 0.05$, $^{**} = p \leq 0.01$ versus 0 µg/ml unless otherwise indicated. Level of significance was determined using a one-way ANOVA with posthoc LSD test. b: Total RBC-bound MPO (ELISA), increasing MPO-concentrations. Isolated RBCs were diluted to 10 million cells per ml and incubated with MPO. Subsequently, RBCs were analyzed according to the manufacturer's instructions. $n = 5$ per group. $^{*} = p \leq 0.05$, $^{**} = p \leq 0.01$ versus 0 µg/ml unless otherwise indicated. Level of significance was determined using a one-way ANOVA with posthoc LSD test. c: Total RBC-bound MPO (ELISA), different timepoints. RBCs were incubated with 5 µg/ml MPO for 0, 5, 15, 30, 60 min at 37 °C in PBS. After staining with PE-labeled MPO antibodies, fluorescence was measured using flow cytometry. $n = 4$ per each group. $^{*} = p \leq 0.05$, $^{**} = p \leq 0.01$ versus 0 min unless otherwise indicated. Level of significance was determined using a one-way ANOVA with posthoc LSD test. d: MPO-binding capacity of RBCs after sialidase treatment. RBCs were treated with or without 200 mU/ml sialidase for 60 min and subsequently incubated with MPO (0.3 µg/ml). Concentration of MPO on RBCs in pmol/l was measured via ELISA after cell lysis. $n = 5$ per group. $^{*} = p \leq 0.05$ versus other group. Level of significance was determined using a two-sided unpaired *T*-test.

catalytic activity of membrane-bound MPO in RBC-membrane fragments (RBC-ghosts). These erythrocyte membrane remnants were created by utilizing cell lysis and subsequent washing steps and afterwards incubated with increasing MPO concentrations. MPO-binding to ghosts was verified by ELISA and Western blot (data not shown). As indicated in Fig. 5, NO consumption of RBC ghosts was augmented by increasing the concentrations of membrane fragments on the one hand. However, by applying different MPO-concentrations to the same amount of membrane remnants, catalytic peroxidase activity was also increased. Thus, NO consumption was dependent on both the availability of ghosts and

Fig. 2. Elevated MPO-plasma levels and RBC-bound MPO in patients versus healthy controls. a: MPO plasma concentration in patients vs. controls. Concentration of MPO in pmol/l measured via ELISA in human EDTA-plasma derived from healthy controls or patients on Intensive Care Unit with acute coronary syndrome or acute heart failure. $N = 19$ in controls, $N = 18$ in patients. $^{**} = p \leq 0.01$ versus other group. Level of significance was determined using a two-sided unpaired *T*-test. b: MPO-concentration on RBCs in patients vs. controls (ELISA). RBCs were isolated from whole EDTA-blood, lyzed, diluted and subsequently assayed according to manufacturer's instructions. Concentration of MPO in pmol/l measured via ELISA from healthy controls or patients on Intensive Care Unit with acute coronary syndrome or acute heart failure. $N = 8$ in controls, $N = 3$ in patients. $^{**} = p = 0.01$ versus other group. Level of significance was determined using a Mann–Whitney rank sum test. c: MPO-binding on RBCs of patients vs. controls (flow cytometry). RBCs from patients and controls were isolated using standardized protocols. RBCs were stained with a PE-conjugated MPO-antibody and mean PE-fluorescence was detected using flow cytometry. $N = 11$ in patients and controls. $^{*} = p \leq 0.05$ versus other group. Level of significance was determined using a two-sided unpaired *T*-test. d: Heparin-induced release of MPO from isolated RBCs in patients vs. controls. Isolated RBCs from patients and controls were incubated with PBS or PBS + heparin 500 IU/ml for 10 min, 37 °C. Supernatants were collected and heparin-induced detachment of MPO from RBCs was quantified via ELISA. $N = 14$ in controls, $N = 15$ in patients. $^{*} = p \leq 0.05$, $^{**} = p \leq 0.01$ versus groups indicated, level of significance was determined using a one-way ANOVA with posthoc LSD test.

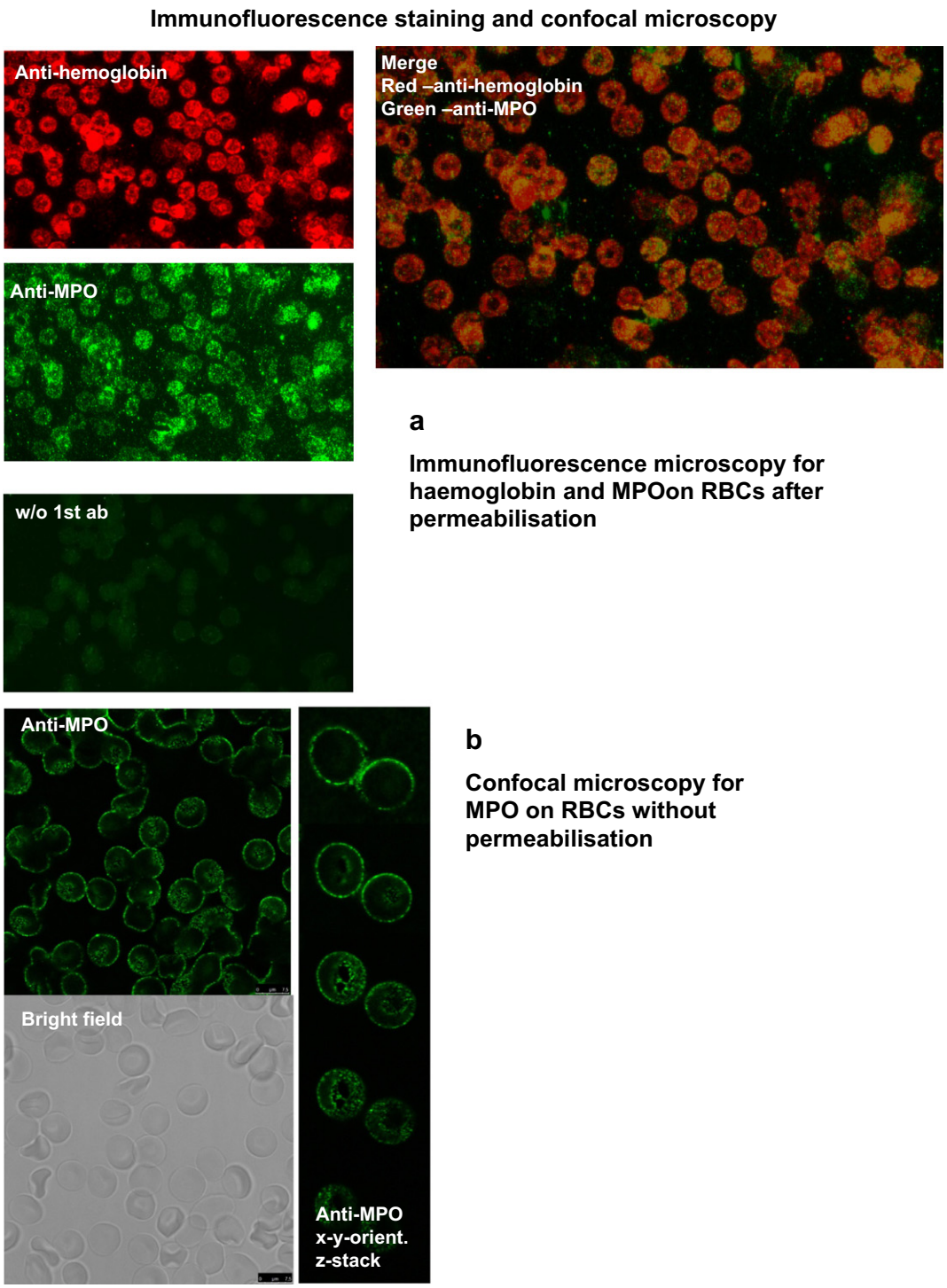


Fig. 4. Immunofluorescence staining and confocal microscopy. RBC smears were fixed and subsequently stained with primary antibodies against hemoglobin and MPO followed by secondary antibody labeling. a: Immunofluorescence microscopy for hemoglobin and MPO on RBCs after permeabilization. Immunofluorescence staining for MPO (green) and hemoglobin (red) on untreated human RBCs; merged picture calculated using corresponding fluorescence signals of the same picture. b: Confocal microscopy for MPO on RBCs without permeabilization. Isolated RCBs were incubated with MPO (5 μg/ml). MPO (green) was localized on RBCs after fixation and blocking without permeabilization. Samples were analyzed by scanning confocal microscopy.

the dosage of MPO-pre-incubation, indicating binding of catalytically active MPO to RBC-membrane ghosts.

3.3. Injection of MPO-loaded RBCs into C57BL/6J-mice

While our data suggested that binding occurs, more information was needed to elucidate if MPO-release from RBCs and distribution to vascular and extravascular tissues happens *in vivo*. Therefore, we loaded murine RBCs with MPO through incubation with MPO *ex vivo* and administered these RBCs to C57BL/6J-mice via carotid artery.

Thirty minutes after infusion of MPO-loaded RBCs, concentrations of MPO in hepatic tissue were significantly increased compared to control animals (4.2 ± 0.97 pmol/l vs. 1.0 ± 0.67 pmol/l, $p < 0.05$). As shown in Fig. 6a–d, similar results were seen in splenic tissue, lung tissue and cardiac tissue. To specifically evaluate the amount of vascular MPO, murine hearts were perfused with heparin-solution in a Langendorff-apparatus

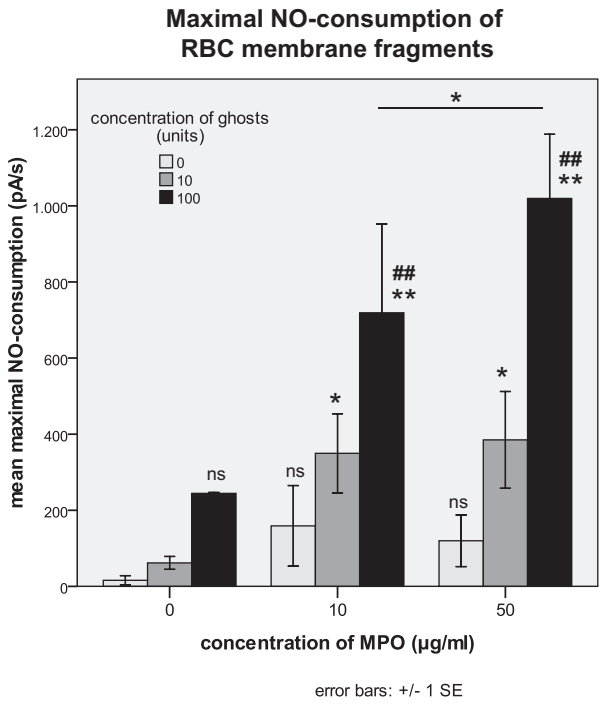


Fig. 5. Maximal NO-consumption of RBC membrane fragments. Arbitrary units of RBC-ghosts were incubated with different MPO concentrations (0, 10, 50 μg/ml). NO concentration was measured after application of H_2O_2 in the presence of a NO-donor. NO consumption per second was calculated. $n = 5$ for 0 and 10 units, $n = 6$ for 50 units of RBC-ghosts. * = $p \leq 0.05$, ** = $p \leq 0.01$ versus 0 μg/ml MPO. ## = $p \leq 0.01$ versus 0 units of ghosts in same MPO concentration; unless otherwise indicated. Level of significance was determined using a one-way ANOVA with posthoc LSD test.

immediately after explantation to elute endothelial-bound MPO. As seen in Fig. 6e, vascular-accessible cardiac MPO concentrations were significantly elevated upon infusion of MPO-loaded RBCs as compared to RBC-infusion alone (21.63 ± 3.5 pmol/l vs. 8.03 ± 1.2 pmol/l, $p < 0.01$).

Hemoglobin concentrations did not differ in hepatic, cardiac, lung or splenic tissues (Fig. 6f), indicating that the differentially measured MPO-concentrations in those tissues were not due to different amounts of residual RBCs.

Hypochlorous acid (HOCl) is generated in the presence of MPO and is considered a major agent in 3-chlorotyrosine (3-Cl-Tyr) formation. Chlorotyrosine formation can therefore be used as a marker of MPO activity [10]. We performed 3-Cl-Tyr staining in hepatic tissue of mice after infusion of control RBCs, free MPO and MPO-loaded RBCs. As Fig. 7 indicates, there was a slight increase of immunoreactivity for 3-Cl-Tyr in hepatic tissue upon infusion of mice with MPO. In mice infused with MPO-loaded RBCs, we could detect an even stronger increase in 3-Cl-Tyr immunoreactivity, indicating increased MPO-dependent post-translational protein modification.

3.4. Nitric oxide dependent vasorelaxation in explanted aortic rings

To prove physiological relevance of RBC-membrane-bound MPO, we evaluated its impact on relaxation of explanted aortic rings. After incubation of aortic rings from C57BL/6J WT mice with MPO-loaded vs untreated RBCs, we measured endothelium-dependent and -independent vasorelaxation. As seen in Fig. 8a, we found a significant decrease in NO-dependent vasorelaxation of aortic rings exposed to MPO-loaded RBCs indicating decreased endothelial function (−65.43 ± 2.77% vs. 73.5 ± 2.2% at maximal ACh concentration of 5×10^{-6} mmol/l), while NO-independent vasorelaxation was not altered.

3.5. In vivo hemodynamic changes after injection of MPO-loaded RBCs

After finding impaired NO-dependent relaxation of aortic rings *ex vivo*, we were specifically interested in *in vivo* hemodynamic effects after infusion of MPO-loaded RBCs into mice. Therefore, we measured systemic vascular resistance (SVR) in mice before and after infusion of MPO-loaded or control RBCs. As indicated in Fig. 8b, there was a significant increase in SVR in mice infused with MPO-loaded RBCs, whereas there was no change in the control group (117.6 ± 4.9 vs. 93 ± 8.0% compared to baseline measurements, $p < 0.05$).

4. Discussion

Here we show that RBC membranes avidly interact with MPO, acting as carriers of this leukocyte-derived enzyme, which lends important insight into MPO's impact on vascular function. MPO binds reversibly to RBCs in a dose-dependent fashion while MPO's catalytic activity is preserved. Aortic sections incubated with MPO-loaded RBCs show significantly decreased nitric oxide-dependent vasorelaxation. *In vivo* perfusion with MPO-loaded RBCs augments local MPO tissue levels and activity. Most importantly, MPO-RBC-infusion increases systemic vascular resistance in mice, implicating a role for RBC-bound MPO in hemodynamic regulation.

MPO as a central component of innate immunity binds effectively to microbial surfaces, due to its cationic charge, creating a locally increased level of oxidative stress [1]. However, during neutrophilic phagocytosis, relevant targets of MPO-driven oxidation are not microbial structures. The targets belong in large part to the host cells [11].

MPO and its catalytic products like hypohalous acids and resulting ROS have been shown to regulate the function of neutrophils, macrophages and endothelial cells [12–14]. MPO-derived oxidants cause enhanced leukocyte recruitment with induction of neutrophilic integrins [12] and upregulation of endothelial adhesion molecules [15]. Besides Mac-1 interactions, charge-driven MPO–membrane interactions increase crosstalk between neutrophils and endothelial cells, and lead to electrostatic-mediated neutrophil attraction [4] by binding of MPO to anionic heparan sulfate glucosaminoglycans in the luminal glycocalyx-layer of endothelial cells. Thus, MPO–membrane interactions are considered a key mechanism in MPO-driven neutrophil activation.

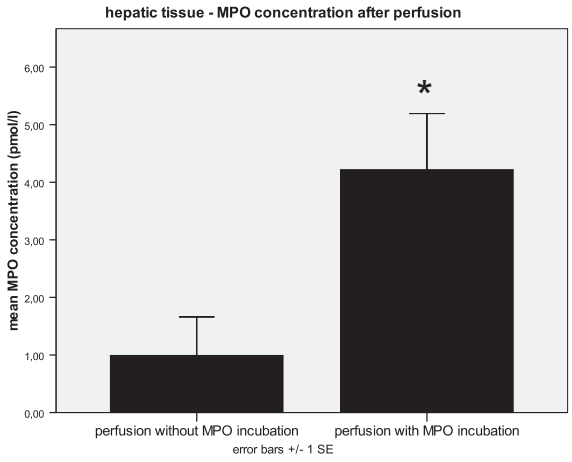
Our study shows that not only leukocyte and endothelial cell, but also RBC membranes are prone to MPO binding. While we did not demonstrate that RBC–MPO interactions are exclusively dependent on binding to the plasma membrane of erythrocytes, we have shown that RBC membranes contain MPO and that their fragments are able to provide catalytically active MPO. Additionally, we could provide some preliminary evidence that RBCs carry more MPO than other circulating cells like platelets, mononuclear cells or granulocytes.

Interestingly, RBC ghosts not treated with MPO had NO-consuming qualities themselves, apparently elicited by the remains of MPO collected *in vivo* before the isolation process. While we cannot rule out the presence of residual hemoglobin-peroxidase or other peroxidase activity of imperfectly cleared membrane fragments, these findings add significance to the concept of membrane-associated MPO-RBC interactions.

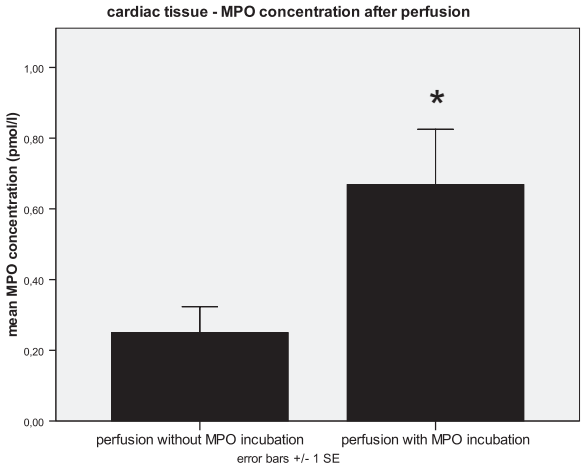
As suggested by the reversibility of MPO-binding to RBCs after heparin administration, MPO's binding site to the RBC is likely extracellular. This is further underlined by the fact that we could detect RBC-bound MPO with flow cytometry and confocal staining without cell permeabilization. Therefore, dynamic interactions between bound MPO (located on the extracellular side of the RBC membrane) and surrounding cells like neutrophils and endothelial cells may occur.

Glycophorin-bound sialic acids residues contribute to the negative halo surrounding RBCs. This so-called “zeta potential” is thought to be relevant to maintain distance between erythrocytes themselves [16] as well as between RBCs and the endothelium or other cellular blood components [17,18]. We demonstrated that MPO binding to the RBC

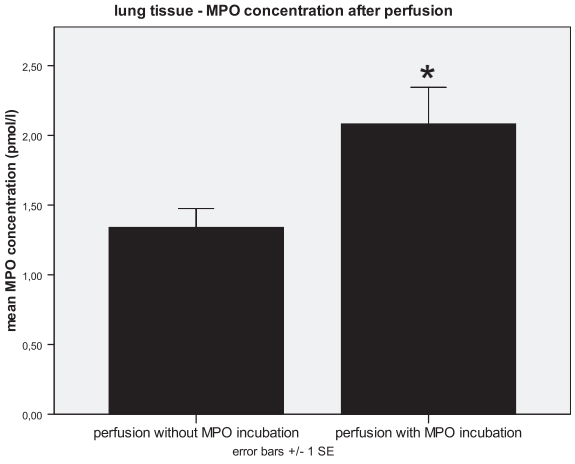
Local MPO-concentration in organs after administration of MPO-loaded RBCs *in vivo*



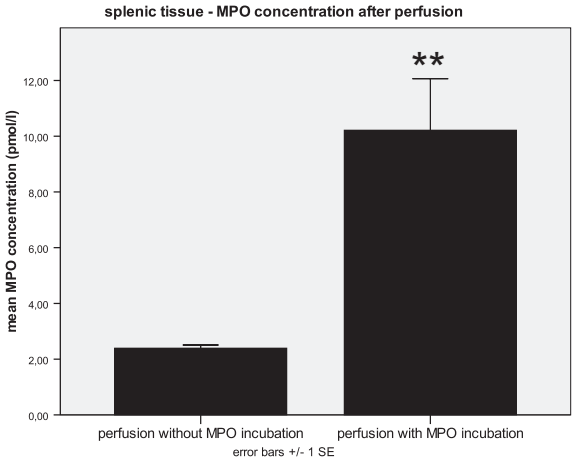
a) Hepatic tissue



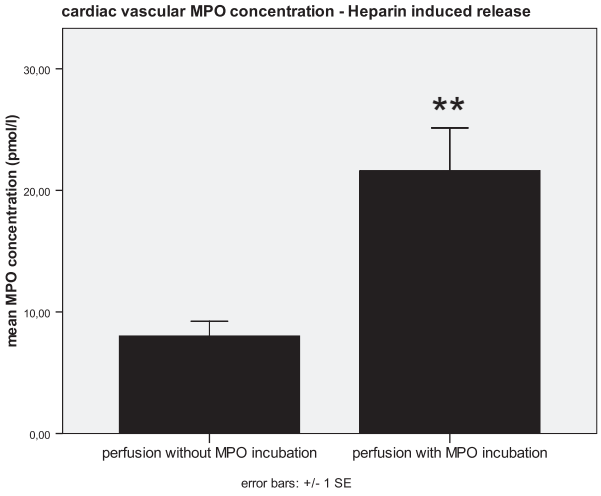
b) Cardiac tissue



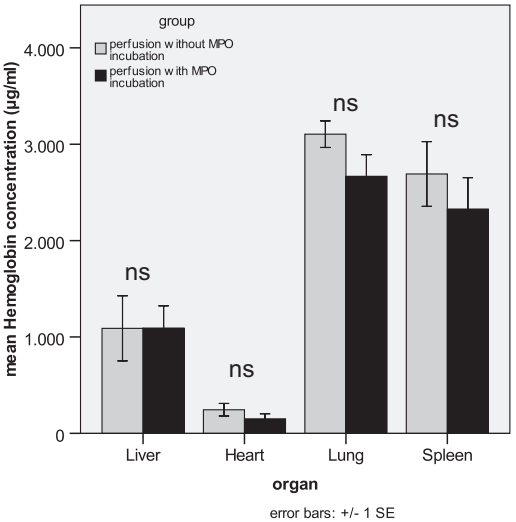
c) Lung tissue



d) Splenic tissue



e) Cardiac vascular MPO concentration



f) Local tissue concentration of hemoglobin

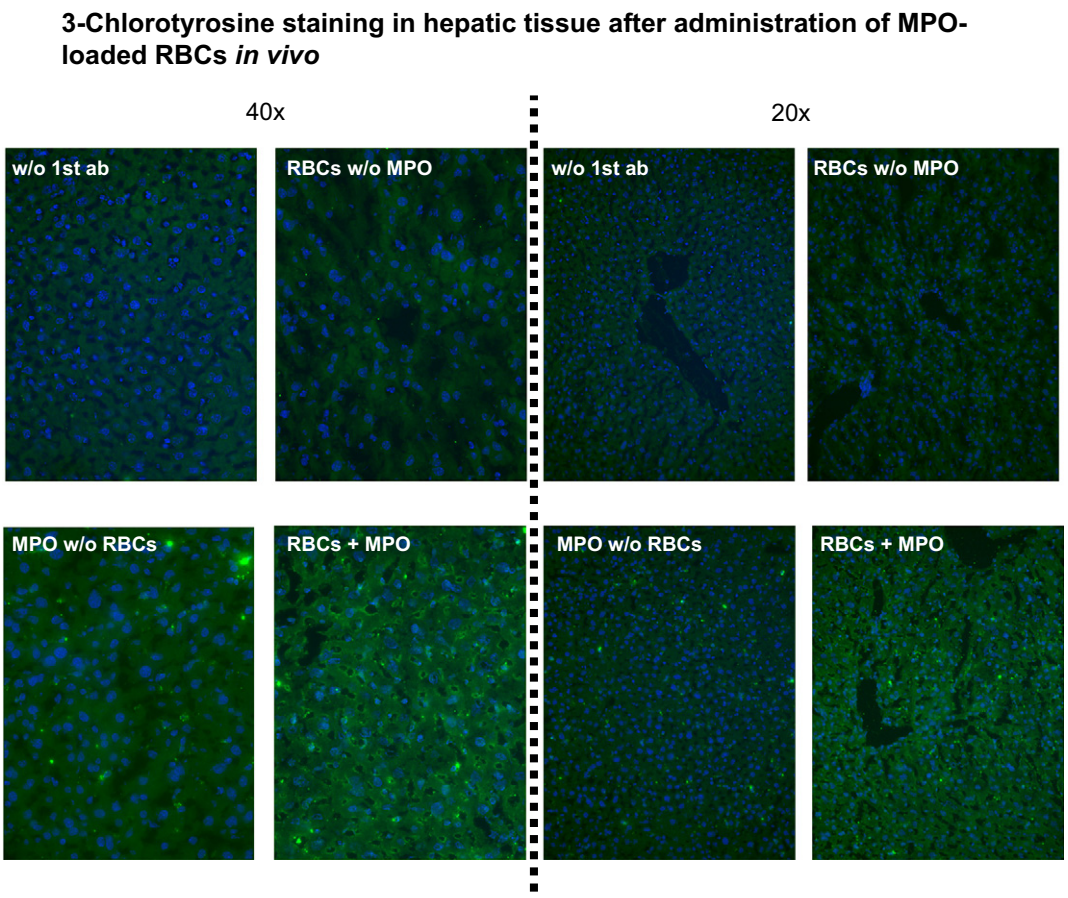


Fig. 7. 3-Chlorotyrosine staining in hepatic tissue after administration of MPO-loaded RBCs *in vivo*. Hepatic sections from mice upon 30 min infusion with RBCs, MPO or MPO-loaded RBCs were stained for 3-chlorotyrosine (green). Nuclei were stained with DAPI (blue). Representative images in 40× and 20× magnifications are shown.

membrane is at least in part dependent on sialic acid binding, as sialidase treatment ahead of MPO-incubation decreased the amount of RBC-bound MPO (Fig. 3d). Nevertheless, higher concentrations of MPO during incubation diminished this effect (data not shown), indicating that the binding capacity of erythrocytic sialic acid residues seem to be limited, and therefore other membrane structures are likely to participate in MPO-binding as well. In previous studies we showed that MPO attenuated the anionic endothelial surface charge in cremaster muscle venules [4]. Further studies are therefore needed to clarify the impact of MPO-binding to RBC membranes with regards to the changes in membrane zeta potential and the binding location of MPO on RBCs. This would have a relevant impact on the amount of possible vascular cell-cell interactions due to interference with the protective charge and function of certain membrane proteins.

We further provided evidence that *in vivo* perfusion with MPO-loaded RBCs increases local MPO tissue levels in heart, lung, spleen and liver. In general, increased local MPO levels are seen as a marker of local inflammation in different organs [19–21], and MPO could further attract neutrophils [4]. As has been well shown in previous studies looking at atrial fibrillation or myocardial infarction models, local levels of MPO do have a significant impact on disease formation. In this context, as in our hepatic sections after MPO-RBC-infusion, increased local atrial MPO concentrations were associated with a higher amount of 3-chlorotyrosine, a product of MPO-derived HOCl-activity. This was accompanied by MMP activation and increased susceptibility to atrial fibrillation [22]. In contrast, MPO knockout in LAD ligation was shown to be associated with decreased leukocyte infiltration, significant reduction in LV dilation, and marked preservation of LV function [23].

As the locally increased amounts of MPO in our experiments were not due to higher perfusion or an aggregation of RBCs, we have revealed a new mechanism of *in vivo* MPO-distribution. Given the proven reversibility of MPO–RBC interactions, this suggests a new role for RBCs as carriers of MPO. Implicated mechanisms include RBCs picking up locally increased “free MPO”, for example after neutrophil degranulation processes, and delivering and distributing the membrane bound MPO-load to different organs and surface structures, where increased MPO-concentration could be pathogenic.

This is further underlined by observations made by Rudolph *et al.* [7]. They noted a rapid decrease in MPO-concentration (over 50% in 5 minutes) after injection of MPO into an open-chest pig model. The authors assume that the fast drop in MPO-concentration is due to endothelial binding. By taking our observations into account, the drop of MPO concentration in plasma must be considered to be at least in part due to RBC-binding as well. Further studies are therefore needed to elucidate the kinetics of MPO's distribution over the body.

Fig. 6. Local MPO-concentration in organs after administration of MPO-loaded RBCs *in vivo*. Isolated RBCs were incubated for 10 min *ex vivo* with human MPO and infused into C57BL/6J mice after 2 subsequent washing steps in NaCl. Organs had to be harvested 30 min after perfusion with loaded RBCs; local levels of human MPO have been measured using ELISA after homogenization. a: Hepatic tissue, $n = 8$ per group. b: Cardiac tissue, $n = 8$ per group. c: Lung tissue, $n = 7$ per group. d: Splenic tissue, $n = 8$ per group. e: Cardiac vascular MPO concentration. Eluate was collected using Langendorff-perfusion of explanted hearts with 2 ml of a preheated buffer containing heparin (50 IU/ml). MPO-concentration was evaluated via ELISA after speedvac-concentration. $n = 7$ per group. f: Local tissue concentration of hemoglobin. To rule out changing of perfusion levels as an underlying cause for alteration of local MPO-concentration in tissues above, hemoglobin concentration was evaluated in the same samples. $n = 7$ per group. $^{*} = p \leq 0.05$, $^{**} = p \leq 0.01$ versus perfusion without MPO incubation, unless otherwise indicated. Level of significance was determined using a two-sided unpaired *T*-test.

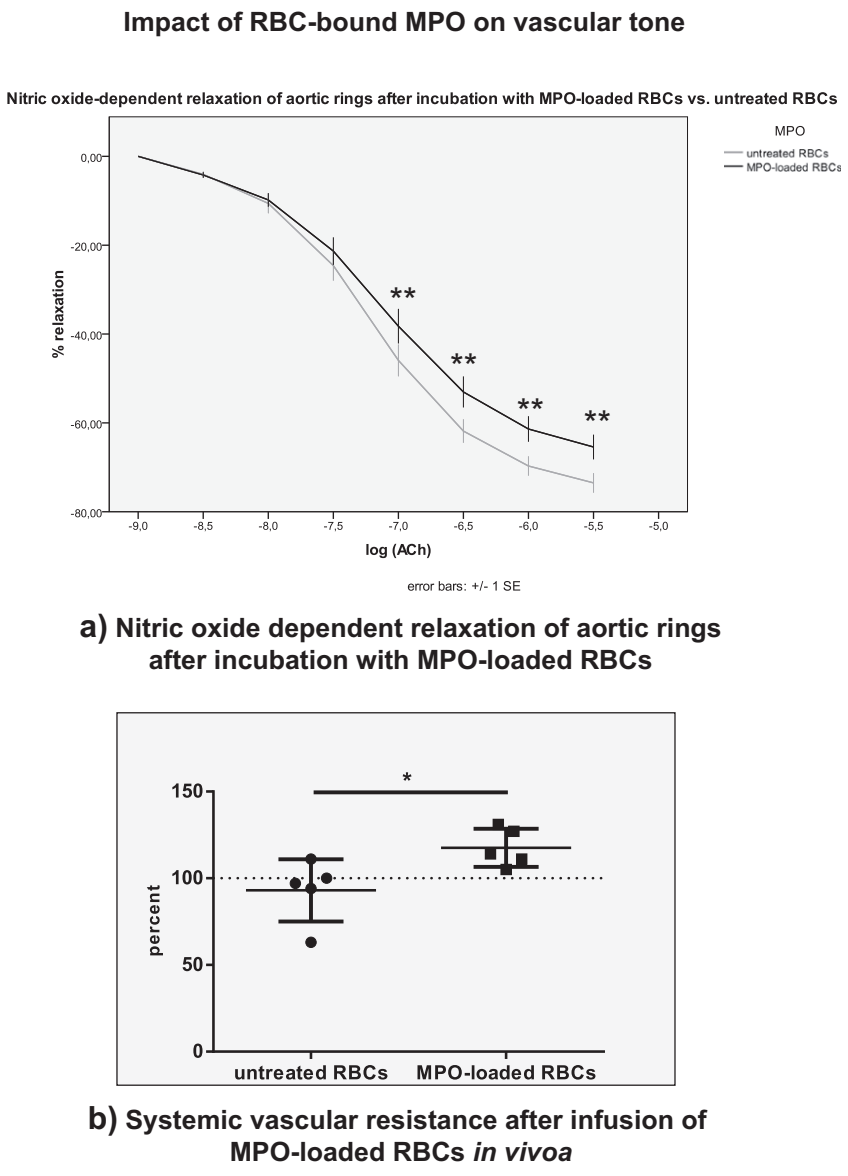


Fig. 8. Impact of RBC-bound MPO on vascular tone. a: Nitric oxide dependent relaxation of aortic rings after incubation with MPO-loaded RBCs. Isolated erythrocytes were exposed to PBS + 20 μg/ml of MPO or PBS alone and subsequently washed twice. After explantation of thoracic aortic segments derived from C57BL/6J mice, aortic rings were incubated in MPO-loaded RBCs or PBS-treated RBCs at a concentration of 1.5 Mio/μl for 30 min. Afterwards, endothelium-dependent relaxation was determined in response to increasing doses of acetylcholine (ACh, 10^{-9}–5×10^{-6} mol/l). No differences could be shown for endothlelial-independent relaxation, induced with NTG. $n = 10$ for controls, $n = 14$ for MPO-treated group. $^{*} = p \leq 0.05$, $^{**} = p \leq 0.01$ versus same ACh-concentration in the corresponding group. Level of significance was determined using a one-way ANOVA with posthoc LSD test. b: Systemic vascular resistance after infusion of MPO-loaded RBCs *in vivo*. MPO-loaded murine RBCs were injected in littermate control mice. Cardiac output and carotid pressure were simultaneously measured via microtip conductance pressure–volume catheters, and systemic vascular resistance was calculated before and 10 min after infusion. The ratio between baseline measurement and post-infusion SVR is shown. $^{*} = p \leq 0.05$ versus other group. Level of significance was determined using a two-sided unpaired *T*-test.

RBCs cannot only deposit MPO in peripheral organ tissue, as our results indicate. They can deliver MPO to cardiac vascular endothelium. MPO release into the coronary circulation by PMNs has been shown before in patients with unstable angina [24]. Furthermore, coronary plaque rupture triggers PMN activation with subsequent binding of MPO to the coronary vessel wall [25]. Additionally, MPO has been shown to impair epicardial, microvascular, and extramyocardial blood flow in anaesthetized pigs [7]. A role for RBCs in distributing MPO to the cardiac vascular bed has not yet been considered. Our organ bath experiments (Fig. 8a) and our hemodynamic measurements in mice (Fig. 8b) indicate that MPO-loaded RBCs impair endothelium-dependent vascular function in general and can mediate vasoconstriction in the microcirculation. Therefore, the increasing amount of vascular bound MPO in cardiac vasculature after perfusion with MPO-loaded RBCs might contribute to the regulatory processes in cardiac vascular homeostasis and NO-bioavailability as well as local vasoconstriction. These findings have mechanistic implications for pathobiological processes such as acute coronary syndrome, and need to be evaluated in future studies. Moreover, given our data, MPO bound to RBCs could contribute to the increase of vascular tone in heart failure, as dysregulation of vascular tone and afterload is a major pathobiological pattern in heart failure [26–28], and MPO is significantly elevated in these patients.

Our results indicate that increased MPO plasma levels in patients were coincident with higher MPO concentrations on their RBCs. It is known that MPO plasma levels add prognostic information in all stages of coronary artery disease and in heart failure [29–34]. Therefore, integrating our results, the RBC-bound fraction of MPO might be of clinical relevance as well.

A recently published clinical study [35] may be supportive for this concept. The investigators looked at size and composition of intracoronary

thrombi in patients with ST-elevation myocardial infarction. Plasma MPO-levels were significantly higher in patients with RBC-rich thrombi. Most interestingly, in patients with RBC-rich thrombi, thrombus size was increased. Furthermore, the number of MPO-positive cells was significantly higher in the RBC-rich thrombi compared to thrombi with a low amount of RBCs.

As a caveat, due to the design of patient sample collection and further sample processing, we did not obtain further details regarding patient characteristics, e.g. age, gender, anticoagulation status. In reference to previous studies [33,36], patient were chosen to be likely to have higher MPO plasma levels in contrast to controls, but patients and controls were not matched. Even had we ruled out severe infections, the difference in MPO levels could be caused by other reasons than ACS or heart failure.

Taken together, our work suggests that MPO is bound to RBCs *in vivo*. We could provide first evidence that this contributes to MPO-distribution and vascular dysfunction in the body. The amount of MPO found adherent to RBCs in patients with elevated MPO plasma levels is significantly increased. Given the important role for MPO in local endothelial function and in atherosclerosis, in promoting atherosclerotic plaque formation, in plaque destabilisation and disease progression [37] and in its role in inflammatory processes, this may indicate an additional new mechanism for vascular inflammation.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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