Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins

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Recruitment and activation of polymorphonuclear neutrophils (PMNs) reflects a primary immunological response to invading pathogens and has also emerged as a hallmark of vascular inflammation. One of the principal enzymes released upon PMN activation is myeloperoxidase (MPO), a heme protein that not only generates cytotoxic oxidants but also impacts deleteriously on nitric oxide-dependent signaling cascades within the vasculature. Because MPO also associates with the membrane of PMN, we evaluated whether MPO could also function as an autocrine modulator of PMN activation. The extent of PMN membrane-associated MPO was elevated in patients with acute inflammatory vascular disease compared with healthy individuals. Isolated PMNs bound free MPO by a CD11b/CD18 integrin-dependent mechanism. PMNs exposed to MPO were characterized by increased tyrosine phosphorylation and p38 mitogen-activated protein kinase activation. Also, nuclear translocation of NFκB in PMN was enhanced after incubation with MPO, as was surface expression of CD11b. Binding of PMN to MPO-coated fibronectin surfaces amplified PMN degranulation, as evidenced by increased release of MPO and elastase. MPO also augmented PMN-dependent superoxide ($O_2^{\bullet -}$) production, which was prevented by anti-CD11b antibodies, but not MPO inhibitors. Collectively, these results reveal that binding of MPO to CD11b/CD18 integrins stimulates PMN signaling pathways to induce PMN activation in a mechanism independent of MPO catalytic activity. These cytokine-like properties of MPO thus represent an additional dimension of the proinflammatory actions of MPO in vascular disease.

atherosclerosis | cytokine | endothelium | leukocyte | nitric oxide

Recruitment and activation of polymorphonuclear neutrophils (PMNs) is considered one of the principal defense mechanisms of innate immunity. Activation of PMNs in response to inflammatory mediators such as bacterial lipopolysaccharides, cytokines, and complement factors leads to the adherence of these cells to the vessel wall before exiting the blood vessel in an attempt to eliminate pathogens in affected tissues (1, 2).

Activation of PMNs not only imply changes in the adhesive and cytotoxic properties of the leukocyte itself but also may have profound systemic effects, given the intraluminal release of enzymes harbored within cytoplasmatic vesicles of PMNs (3–5). One of the principal enzymes released from the neutrophil's azurophilic granules is myeloperoxidase (MPO), a heme protein abundantly expressed in PMNs and accounting for up to 5% of total cell protein (6). MPO has long been considered a key constituent of the neutrophil's cytotoxic armament by catalyzing the formation of hypochlorous acid, a potent oxidant that displays bactericidal activity *in vitro* (7). However, recent observations expand this view and show that MPO-derived oxidants are critically involved in a more subtle modulation of signaling pathways. For example, low levels of MPO-derived hypochlorous acid has been demonstrated to activate mitogen-activated protein (MAP) kinases (8), induce nuclear translocation of transcription factors (9), regulate cell growth by activating tumor-suppressor proteins (10), or modulate the activity of metalloproteinases (11). Also, vascular cell glycosaminoglycan-associated MPO and MPO-derived free radical intermediates interfere with vascular signaling pathways by oxidizing endothelial derived nitric oxide (NO) (12, 13).

Interestingly, MPO not only proved to adhere to endothelial cells but has also been implicated in PMN membrane association. Indirect evidence derives from observations revealing the prevention of PMN-binding to MPO-coated surfaces in the presence of CD11b antibodies (14). Importantly, the CD11b/CD18 integrin is not only a critical mediator for PMN surface adherence but also communicates signaling events evoked by various cytokines, which ultimately modulate the activation state of PMNs (15, 16).

Given the above premises, we hypothesized that MPO may alter intracellular signaling pathways in PMNs upon adhering to integrins on the neutrophil membrane. Here, we demonstrate that MPO binds to CD11b/CD18 integrins on PMNs, leading to induction of intracellular signaling cascades and translating into up-regulated PMN degranulation, CD11b surface expression, and NADPH oxidase activity in an autocrine manner. These properties of MPO add to the growing body of evidence characterizing MPO as a proinflammatory mediator and reveals alternative functions of this enzyme, which are irrespective of its bactericidal and enzymatic activity.

Materials and Methods

Materials. Purified MPO was obtained from Planta Natural Products (Vienna), fibronectin (FN) was from GIBCO Invitrogen (Karlsruhe, Germany); MPO inhibitor was from Calbiochem; and Sepharose A CL-4B was obtained from Pharmacia Biotech (Freiburg, Germany). Mouse anti-elastase antibody was from Research Diagnostics (Flanders, NJ); anti-Myc antibody (clone 9E10) and mouse anti-p65 antibody were from Santa Cruz Biotechnology; rabbit anti-histone H4 was from Serotec; rabbit polyclonal anti-MPO antibody was from Calbiochem; phycoerythrin (PE)-conjugated anti-MPO monoclonal antibody and FITC-conjugated anti-CD66b antibody were both from Acris Antibodies (Hiddenhausen, Germany); rabbit anti-p38 activated MAP kinase (MAPK) antibody was from Promega (Mannheim, Germany); rabbit anti-MAP kinase p38 (p38 MAPK) antibody was from Calbiochem; and mouse anti-phosphotyrosine antibody was purchased from Oncogene (San Diego). Secondary antibodies Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit were obtained from Molecular Probes. Chambered slides (four-chamber slides from the Permanox LabTek chamber slide system) and 96-well plates were from Nunc; the MPO ELISA kit was from Calbiochem; the elastase ELISA kit was from IBL (Hamburg, Germany);

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Abbreviations: PMN, polymorphonuclear neutrophil; MPO, myeloperoxidase; FN, fibronectin; MAP, mitogen-activated protein; MAPK, MAP kinase; p38 MAPK, MAP kinase p38; PE, phycoerythrin; OZ, opsonized zymosan.

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microcons YM3 were from Millipore, and enhanced chemiluminescence (Femto SuperSignal) was purchased from Pierce. All other materials were from Sigma.

Isolation of Human PMNs. For isolation of human PMNs, blood was taken from six healthy volunteers in the morning as described (17). Neutrophils were suspended in PBS^+ [PBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mg/ml glucose (pH 7.4)].

FACS Analysis of MPO on the Membrane of PMNs. Peripheral blood was prepared for two-color flow cytometry. After lysis of erythrocytes with hemolytic buffer (155 mM NH_4Cl/12 mM $NaHCO_3$/0.1 mM EDTA, pH 7.2, for 2 min), 10^6 cells were incubated with PE-conjugated anti-MPO and FITC-conjugated anti-CD66b antibody (30 min at 4°C). Isotype-matched mouse IgG served as control. Two-color flow cytometric analysis was performed by using a FACSCalibur flow cytometer and CELLQUEST software (Becton Dickinson, Heidelberg). Each analysis included at least 10,000 events. The percentage of $CD66b^+$ cells was assessed after correction for the percentage of cells that were reactive by using an isotype-matched control. Gates for phenotypic analysis of $CD66b^+$ cells were set so that the lower left panel contained at least 98% of the total cells analyzed.

Determination of MPO Plasma Levels. Plasma was analyzed for MPO by using an ELISA technique following procedures recommended by the manufacturer.

Immunohistochemistry. For immunohistochemistry, nonactivated, freshly isolated PMN from healthy donors (5×10^5 cells) were incubated in PBS^+ (20 min), which was supplemented with MPO in some cases (5.2, 52, and 130 nM), at room temperature and with gentle shaking. Cells were exposed to FN-coated surfaces of chambered slides for 5 min. Nonadherent cells were discarded, followed by washing with PBS^+. Adherent cells were fixed (acetone/methanol) for 4 min at −20°C and washed repeatedly thereafter. Subsequently, PMNs were blocked with goat serum (10% in PBS for 2 h). Incubation with primary antibodies, rabbit polyclonal anti-MPO (1:1,000), and mouse monoclonal anti-CD11b (1:400) were performed overnight at 4°C. Secondary antibodies were Alexa Fluor 594 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit (1:100). Nuclei were stained with DAPI (1 μg/ml). Images were acquired on a Leitz fluorescence microscope (Leica, Wetzlar, Germany).

Immunoprecipitation of CD11b from PMN Lysates. PMNs (4×10^6) were lysed (100 mM NaH_2PO_4 and 0.1% Triton X-100, containing protease inhibitors) and cleared by centrifugation (for 10 min at $1{,}000 \times g$ at 4°C). Precleared lysates were incubated with either anti-CD11b or anti-elastase antibodies for 2 h on ice, followed by precipitation with Sepharose A (125 mg/ml at 50 μl for 30 min). Adherent proteins were eluted with 1× protein sample buffer (for 5 min at 95°C) and analyzed by Western blot.

MPO-Binding Studies, Peroxidase Activity Measurements, and Determination of Elastase Release. Nonactivated PMNs from healthy donors (5×10^5), suspended in PBS^+, were bound to MPO-, FN-, or FN/MPO-coated surfaces for 5 min. In some cases, PMNs were preincubated with anti-CD11b (0.4 μg/ml for 40 min). After repeated washing to remove nonadherent cells, adherent PMNs were incubated for 30 min at room temperature in PBS^+ and supplemented with opsonized zymosan (OZ) (0.5 mg/ml) when indicated. MPO activity was determined by photometrically assessing the oxidation of tetramethylbenzidine as described (13). For assessment of elastase release, supernatants were concentrated with microcons YM3 2-fold before being analyzed by ELISA following the manufacturer's instructions.

Assessment of PMN NADPH Oxidase Activity. The generation of superoxide anion ($O_2^{\bullet -}$) by nonactivated PMNs was determined as the linear rate of superoxide dismutase-inhibitable reduction of cytochrome *c* as described (18, 19). When indicated, PMNs were exposed to 52 nM MPO, 0.5 mg/ml OZ, and 10 μM formyl-Met-Leu-Phe, respectively.

Western Blot Analysis. For analysis of cellular tyrosine phosphorylation, MAPK activation, and translocation of NFκB by Western blot, nonactivated PMN (10^6 ml^{-1}) were preincubated with or without anti-CD11b antibody (200 ng/ml for 40 min) in PBS^+.

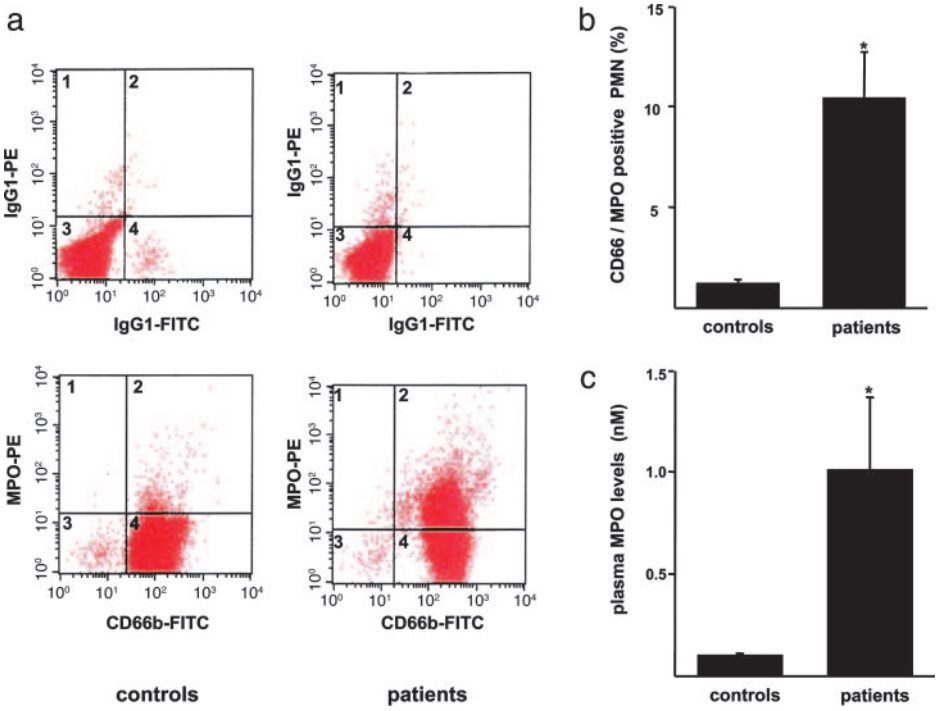

Fig. 1. Increased extracellular deposition of MPO on PMNs in inflammatory vascular disease. MPO burden on PMNs from patients with acute inflammatory disease ($n = 10$) was compared with that of healthy controls ($n = 9$) as evaluated by FACS. Neutrophils were labeled by using anti-CD66b antibodies, and MPO was detected by anti-MPO antibodies. (*a*) Representative scattergrams from a patient (*Right*) and a healthy volunteer (*Left*). (*a Upper*) Isotype controls. (*a Lower*) CD66b and MPO expression. 1, MPO-positive cells; 2, MPO-positive cells coexpressing CD66b; 3, negative cells; 4, CD66b-positive cells. (*b*) Quantification of MPO burden on PMNs in patients and controls (*, $P < 0.05$). (*c*) MPO plasma levels in patients ($n = 10$) vs. controls ($n = 9$) (*, $P < 0.05$).

In some cases, 65 nM MPO or 0.5 mg/ml OZ was added for 30 min. Cells were pelleted and frozen in liquid nitrogen. Pellets were processed in cold buffer containing 100 mM NaH_2PO_4, 0.1% Triton X-100, and protease inhibitors. The membrane fraction-free protein content was concentrated 10-fold by trichloroacetic acid precipitation. Nuclear extracts from PMNs were prepared as described (20).

Western blotting was performed as described (13). Primary antibody concentrations used were rabbit polyclonal anti-MPO (1:10,000), anti-phosphotyrosine (1:1,000), anti-p65 (1:1,000), anti-histone H4 (1:500), anti-p38 MAPK (1:1,000), and anti-p38 MAPK active (1:2,000). p38 MAPK activation is given as ratio for p38 MAPK active/total, and NFκB p65 nuclear translocation is given as ratio for p65/histone H4.

CD11b Fluorescence Assay. Nonactivated, freshly isolated PMNs from healthy donors (1×10^3 μl^{-1}) were incubated in PBS^+ and PBS^+ was supplemented with MPO (5.2–130 nM for 20 min). PMNs were allowed to bind on FN-coated surfaces of 96-well plates (5 min). Nonadherent cells were removed by gentle washing with PBS^+. Cells were fixed with acetone/methanol for 4 min at −20°C and blocked for 2 h at room temperature with 10% goat serum in PBS. Primary antibody (anti-CD11b IgG; 1:400) incubation was performed overnight at 4°C (PBS plus 10% goat serum). The fluorescence intensity of the secondary antibody (Alexa Fluor 488 goat anti-mouse IgG; 1:100) was measured by using a Berthold Fluorometer (Twinkle LB 970, Berthold Technologies, Bad Wildbad, Germany).

Statistics. All data represent the mean ± SD. Statistical analyses were performed by using the paired Student *t* test (STATVIEW 4.5, Abacus Concepts). Results with $P < 0.05$ were considered significant.

Results

Increased intravascular levels of MPO are a hallmark of inflammatory vascular diseases such as sepsis, acute coronary disease, and ischemia and reperfusion, with levels reported to exceed 50 nM (21). Given the indirect evidence for MPO associating with the neutrophil membrane (14), MPO binding to PMN was evaluated by means of flow cytometry (FACS) in patients ($n = 10$) diagnosed for inflammatory disease such as sepsis, ischemia and reperfusion, and acute coronary syndromes. Compared with healthy controls ($n = 9$), the percentage of MPO-positive PMNs was significantly greater (10.45 ± 2.24% vs. 1.2 ± 0.19%, *, $P < 0.01$; Fig. 1 *a* and *b*). Increased MPO association with the PMN membrane in patients was also reflected by enhanced MPO plasma levels as compared with controls (1.01 ± 0.36 nM vs. 0.10 ± 0.01 nM, *, $P < 0.05$; Fig. 1*c*), suggesting not only up-regulated export of MPO toward the outer membrane of PMN but also a potential for binding of free MPO to the PMN surface.

Because binding of MPO to PMN was shown to be inhibitable by anti-CD11b (14), the interaction between MPO and CD11b/CD18 integrins on the PMN membrane was investigated. Immunohistochemical analysis of PMNs from healthy individuals incubated with MPO revealed colocalization of MPO (green) and CD11b (red) and corroborated by the merged image (Fig. 2*a*, yellow). Colocalization of MPO and CD11b was confirmed by coimmunoprecipitation of CD11b and MPO and by using anti-CD11b antibodies, whereas an isotype-matched antibody directed against neutrophil elastase did not precipitate MPO (Fig. 2*b*).

CD11b/CD18 integrins on the PMN membrane not only mediate cell adhesion, but they also translate conformational changes into the initiation of intracellular signaling cascades such as activation of protein tyrosine kinases (16). Thus, CD11b/CD18 are critically linked to PMN activation. Accordingly, it was determined whether MPO modulates the PMN activation state upon binding to CD11b/CD18. Nonactivated, suspended PMNs from healthy donors were

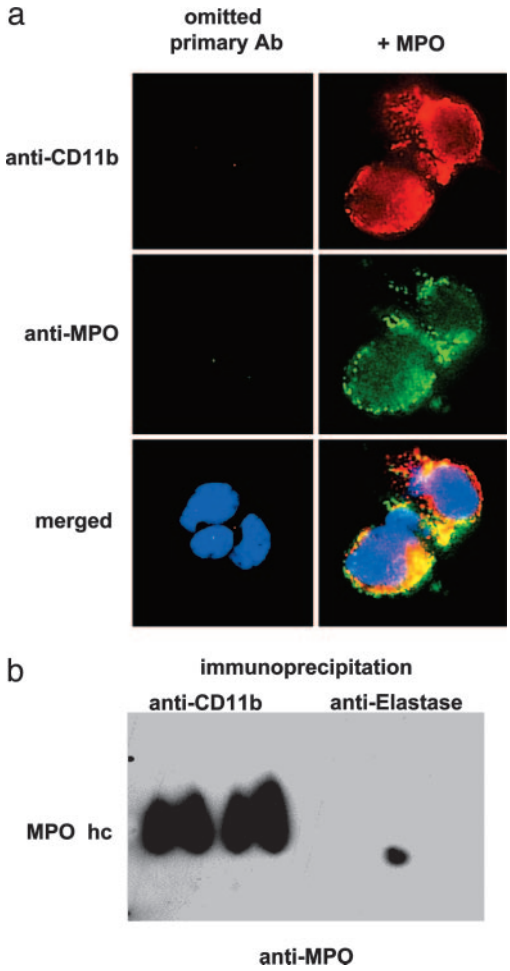

Fig. 2. Colocalization of MPO and CD11b on the PMN membrane. (*a*) Isolated PMNs from healthy volunteers were subjected to MPO (5.2 nM) and immunohistochemically analyzed for extracellular CD11b (red) and MPO (green) deposition. As revealed in the merged image, there was prevailing colocalization for MPO and CD11b (yellow). Control experiments were performed omitting the primary antibodies (*Left*). (Magnification, ×63.) (*b*) Immunoprecipitation of CD11b (*Left*), but not elastase (*Right*), from PMN exposed to MPO resulted in coprecipitation of MPO, as shown by Western blot analysis using anti-MPO antibody. MPO hc, MPO heavy chain.

exposed to MPO (65 nM), and cell lysates were analyzed for protein tyrosine phosphorylation. Exposure of resting PMNs to MPO resulted in markedly increased phosphorylation of multiple cellular proteins (Fig. 3*a*) as compared with untreated PMN (Fig. 3*a*, +34 ± 6.61% increase in PMN plus MPO, *, $P < 0.0001$). Preincubation of PMNs with anti-CD11b prevented the MPO-induced increase in tyrosine phosphorylation (Fig. 3*a*, #, $P < 0.01$), implying that binding of MPO to CD11b/CD18 integrins activates protein tyrosine kinases in PMNs.

Phosphorylation of p38 MAPK is considered a central event affecting the activation state of PMN (22). For example, activation of p38 MAPK induces phosphorylation of $p47^{phox}$, the cytoplasmic, regulatory subunit of the NADPH oxidase (23), which induces activation of the NADPH oxidase-dependent respiratory burst. When PMNs were exposed to MPO, phosphorylation of p38 MAPK was elevated (+ 95 ± 32%, *, $P < 0.01$; Fig. 3*b*). Activation of p38 MAPK has not only been linked to NADPH oxidase activation but also to transcription of genes involved in acute inflammatory responses that are primarily initiated by nuclear translocation of NFκB (24). Upon PMN activation, free NFκB translocates to the nucleus and induces transcription of genes

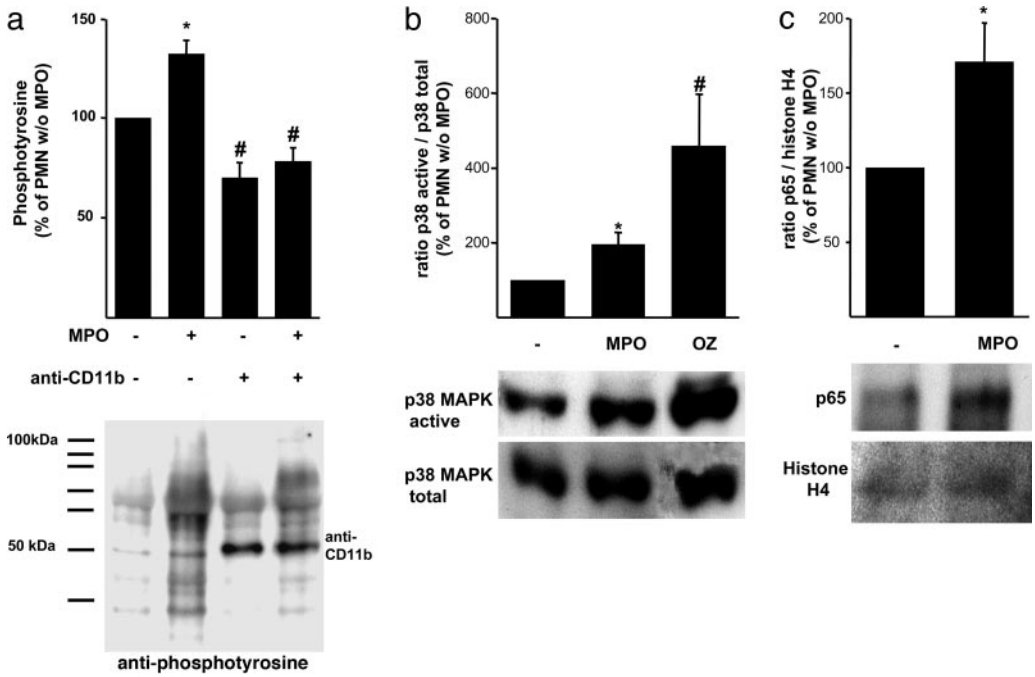

Fig. 3. CD11b/CD18-mediated outside-in signaling initiated in response to MPO. (*a*) Increased intracellular tyrosine phosphorylation. (*Upper*) Nonactivated PMNs from healthy controls exposed to MPO (65 nM) revealed increased tyrosine phosphorylation (+34 ± 6.61% vs. PMN alone; $n = 19$, *, $P < 0.0001$), which was prevented upon preincubation with CD11b antibodies (#, $P < 0.01$). (*Lower*) Representative Western blot probed for anti-phosphotyrosine. (*b*) Activation of p38 MAPK upon MPO binding. (*Upper*) Cell lysates from PMN, treated as described in *a* and probed for active p38 MAPK, revealed increased levels of active p38 MAPK (+95 ± 32% for PMN plus MPO as compared with PMN alone; $n = 6$, *, $P < 0.01$). OZ-stimulated PMNs served as references (+460 ± 217% for OZ-stimulated PMN compared with PMN alone; $n = 4$, #, $P < 0.17$). (*Lower*) Representative Western blots for p38 MAPK active and p38 MAPK total. (*c*) MPO-dependent increase in nuclear translocation of NFκB. (*Upper*) PMNs treated as described in *a* were lysed and nuclear fractions were isolated as described in *Materials and Methods*. Nuclear fractions displayed an increased anti-p65 signal for cells treated with MPO (+70 ± 26%; $n = 3$, *, $P < 0.05$). (*Lower*) Representative Western blots for p65 and anti-histone H4.

encoding for cytokines such as IL-8 or TNF-α (25). Nuclear translocation of NFκB, assessed by probing nuclear fractions for the NFκB subunit p65, was significantly increased in PMNs exposed to MPO (+70 ± 26%, *, $P < 0.05$; Fig. 3*c*), whereas treatment of PMN with OZ did not enhance nuclear translocation of NFκB (data not shown)

Because CD11b/CD18 integrin-mediated "outside-in" signaling is also centrally related to PMN degranulation (16), export of azurophilic granules and release of MPO from PMNs adhering to MPO-coated FN surfaces was evaluated. Nonactivated PMNs were exposed to FN- and FN/MPO-coated surfaces, respectively. Then, PMN degranulation was examined by assessing the enzymatic activity of MPO in the supernatant (Fig. 4*a*) and the extent of elastase release, respectively (+281 ± 32%, *, $P < 0.01$; Fig. 4*b*). The binding of PMN to FN/MPO surfaces profoundly augmented PMN degranulation, compared with FN-coated surfaces (Fig. 4 *a* and *b*, *, $P < 0.001$). Preincubation of PMN with anti-CD11b also prevented the MPO-induced increase in PMN degranulation (Fig. 4 *a* and *b*, #, $P < 0.001$). Interestingly, the extent of MPO release from PMNs bound to FN was only decreased when PMNs were preincubated with anti-CD11b, corroborating previous observations regarding the pivotal role of CD11b for PMN activation upon binding to extracellular matrices (Fig. 4*a*, $P < 0.001$) (15). MPO binding to PMNs resulted in ≈76% of total MPO release, compared with PMN responses to robust inducers of PMN activation such as OZ (Fig. 4*c*, *, $P < 0.01$). Importantly, MPO further increased degranulation of OZ-stimulated PMNs, implying that MPO binding to PMNs not only activates but also primes PMN (+137 ± 21.6%, *, $P < 0.001$; Fig. 4*c*).

Extracellular expression of CD11b/CD18 mirrors PMN activation and facilitates adherence and migratory properties of PMNs (15). To determine whether MPO binding to nonactivated PMNs stimulates CD11b membrane expression, nonpermeabilized PMNs were exposed to increasing concentrations of MPO and CD11b surface expression was evaluated. CD11b/CD18 immunoreactivity was modulated as a function of MPO added; that is, both immunohistochemical analysis of PMNs probed for CD11b (Fig. 5*a*), and fluorometric assessment of surface-exposed CD11b membrane immunoreactivity (Fig. 5*b*) revealed increased CD11b immunoreactivity upon PMN exposure to MPO.

The neutrophil NADPH oxidase is firmly linked to leukocyte activation. Upon activation of PMNs, the cytosolic subunits $p47^{phox}$ and $p67^{phox}$ undergo phosphorylation and associate with the membrane-localized cytochrome b_{558} (26, 27). To assess the effect of MPO binding to CD11b/CD18 on NADPH oxidase activity, superoxide generation from PMNs was assessed by determination of the superoxide dismutase-inhibitable reduction of cytochrome *c*. Superoxide generation by PMNs exposed to MPO was significantly increased (Fig. 6, *, $P < 0.0001$) and comparable with established inducers of NADPH activation such as OZ (0.5 mg/ml) and formyl-Met-Leu-Phe (1 μM). Pretreatment with anti-CD11b prevented the MPO-induced increase in superoxide generation, whereas inhibition of the enzymatic activity of MPO by using the MPO inhibitor 4-aminobenzoic acid hydrazide did not impact on the MPO-induced increase in PMN superoxide generation (Fig. 6, #, $P < 0.01$). This finding further corroborates the notion that MPO mediates PMN activation by primary binding to CD11b, rather than by a secondary generation of reactive species. To exclude the possibility that other cationic or heme-containing enzymes can substitute for MPO-dependent increases in superoxide production, PMNs were also exposed to poly-L-arginine and hemoglobin, both of which did not significantly impact on rates of PMN superoxide production (data not shown).

Discussion

The present data reveal that MPO serves as a powerful autocrine and paracrine stimulator of PMN activation. This property is independent of MPO catalytic activity, and occurs through modulation of intracellular PMN signaling pathways by binding to CD11b/CD18 integrins, thereby provoking degranulation, expression of integrins, and activation of the NADPH oxidase.

Increased intraluminal levels of MPO is a hallmark of systemic inflammatory disease and are viewed as primary host defense mechanism during sepsis, pneumonia, and other pathogen-related diseases (2, 21). Interestingly, increased MPO levels are also observed in states of diseases believed to be unrelated to microbial

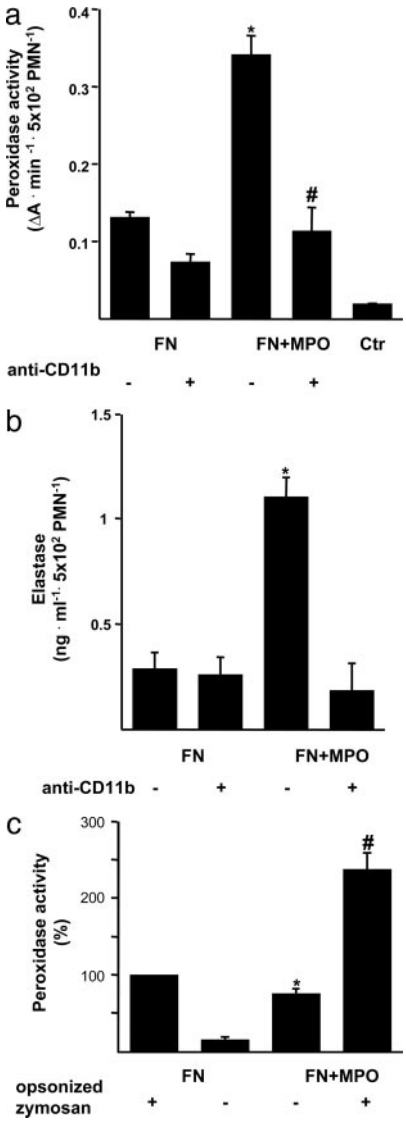

Fig. 4. Adhesion of PMNs to MPO-coated FN increases PMN degranulation. Nonactivated PMNs from healthy controls were exposed for 5 min to FN- and FN/MPO-precoated surfaces. In some cases, PMNs were preincubated with anti-CD11b antibody for 40 min. (*a*) MPO activity. Cell-free supernatants were collected after 30 min, spectrophotometrically analyzed for MPO activity, and corrected for the number of adherent PMNs. MPO-coated FN not exposed to PMNs served as control. MPO release from PMNs was profoundly stimulated on FN/MPO as compared with FN-coated plates only ($n = 8$, *, $P < 0.001$). The increased enzymatic MPO activity in the supernatant was prevented in PMNs preincubated with anti CD11b antibodies (#, $P < 0.001$). (*b*) Elastase release. Supernatants from PMNs bound to FN and FN/MPO were analyzed for elastase by ELISA ($+281 \pm 32\%$; $n = 3$, *, $P < 0.01$). (*c*) When compared with FN-bound PMNs stimulated with OZ, the supernatant of PMNs adherent to FN/MPO-surfaces reached 76% (*, $P < 0.05$). Activation of adherent PMNs was further stimulated by OZ when cells adhered to MPO-coated FN (MPO activity in the supernatant: $+137 \pm 21.6\%$; $n = 6$, #, $P < 0.001$), implying that MPO binding to PMNs profoundly primes the leukocyte.

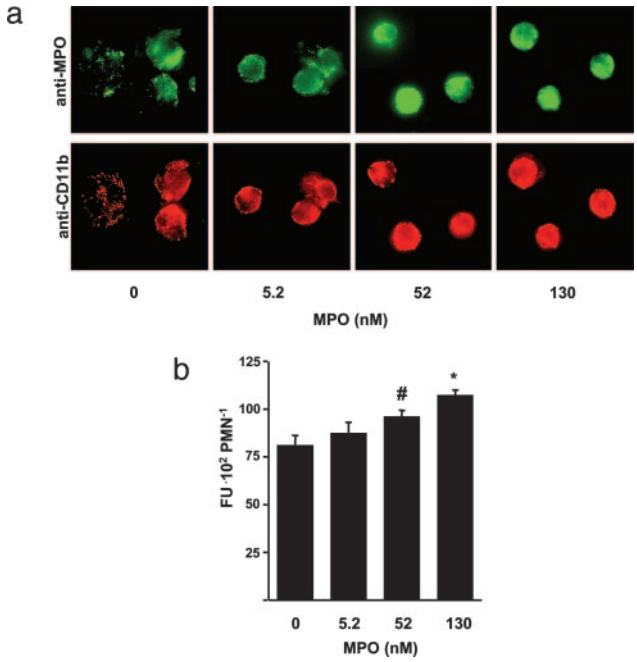

Fig. 5. MPO-dependent expression of CD11b on PMNs. (*a*) Fluorescent labeling of CD11b/CD18 integrin on nonpermeabilized PMNs revealed increased CD11b/CD18 immunoreactivity as a function of MPO addition. Nonactivated PMNs from healthy controls were preincubated with increasing concentrations of MPO (0–130 nM) and exposed to FN-coated surfaces. Extracellular MPO (green) and CD11b (red) of PMNs were visualized by using fluorescently labeled anti-MPO and anti-CD11b antibodies as described in *Materials and Methods*. (Magnification, ×63.) (*b*) Fluorometric evaluation of CD11b immunoreactivity in response to the extent of MPO added. Neutrophils were treated as above, and CD11b fluorescence was assessed as in *Materials and Methods*. CD11b immunoreactivity increased as a function of MPO bound to the surface of PMN. (* and #, $P \leq 0.01$ as compared with omitting MPO.) FU, fluorescence units.

infection, i.e., ischemia and reperfusion injury and acute coronary disease (29–33). In this regard, MPO has long been attributed to tissue injury, primarily through generation of oxidants.

However, the concept that MPO is operative for the bactericidal and toxic properties of leukocytes has been challenged: Individuals with hereditary MPO deficiency are not at increased risk for infections (34). This finding suggests that MPO displays alternative functions. For example, MPO-deficient PMNs are characterized by increased bioavailability of superoxide and hydrogen peroxide, suggesting that MPO may act as a metabolic "sink" for superoxide and hydrogen peroxide (35). Recent observations corroborate this tenet and suggest that neither NADPH-derived superoxide nor MPO-generated hypochlorous acid is essential for killing of bacteria. Instead, it was proposed that MPO evolved as an acceptor for the high concentrations of hydrogen peroxide and superoxide generated in the phagosome needed to activate membrane-immobilized proteases, which ultimately may be responsible for bacterial killing (36, 37).

In addition, there is increasing evidence that MPO, by virtue of its catalase-like, H_2O_2-consuming abilities and secondary oxidants, modulates signaling cascades in the vasculature. For example, MPO-dependent oxidation of low-density lipoproteins transforms the lipoprotein into a high-uptake form for macrophages, which in turn propagates foam cell formation (38). Moreover, MPO has emerged as a significant source of nitrating species such as nitrogen dioxide (16). Nitration of tyrosine residues, mediated by MPO-generated nitrogen dioxide, causes inhibition of superoxide dismutase (39) and prostacyclin synthase activity (40), accelerates fibrinogen coagulation properties (41), and modulates migratory properties of matrix proteins such as FN (42). Finally, after endothelial MPO binding, transcytosis and accumulation within the subendothelial matrix, MPO catalyzes NO oxidation, primarily through the generation of secondary radical intermediates, to potently inhibit NO-dependent vascular signaling (12, 13, 43).

The current study extends our understanding of the inflammatory signaling properties of MPO in two ways: First, MPO not only modulates intercellular signaling cascades, but it also modifies intracellular signaling pathways in PMNs. Second, MPO-dependent

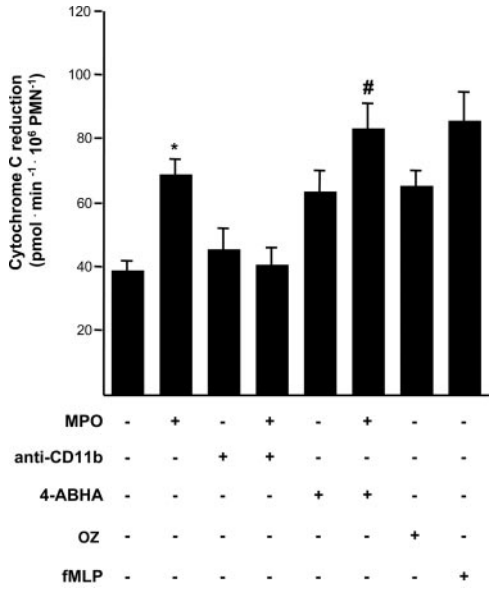

Fig. 6. Increase in NADPH oxidase activity in PMNs in response to MPO binding to CD11b. MPO enhances superoxide production of PMNs. Incubation of nonactivated PMNs from healthy donors (2.5×10^6) with MPO (130 nM) increases superoxide production, as assessed by reduction of cytochrome *c* (*, $P < 0.0001$). This increase was prevented in PMNs preincubated with anti-CD11b antibodies, but not by the MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH) (5 μM; #, $P = 0.02$). Superoxide generation in response to MPO binding to PMNs was slightly lower as compared with established inducers of NADPH activation such as OZ (0.5 mg/ml) and formyl-Met-Leu-Phe (1 μM).

changes in PMN activation is independent of the catalytic activity of MPO, and it more directly depends on its biophysical properties as a ligand for CD11b/CD18. Consequently, binding of MPO to CD11b/CD18 leads to increased tyrosine phosphorylation (Fig. 3*a*), MAPK activation (Fig. 3*b*), translocation of NFκB (Fig. 3*c*), and the modification of signaling events that are redox-sensitive and modulated by superoxide and hydrogen peroxide, respectively (44, 45). Because MPO consumes both superoxide and its principal substrate H_2O_2, at first glance, increased levels MPO would have been expected to further inhibit, rather than augment, the PMN activation state. Instead, a significant increase in neutrophil activation was observed and MPO inhibitors did not prevent this MPO-dependent increase in PMN activation (Fig. 6). In contrast to this expectation, binding of MPO and CD11b/CD18 integrin enhanced MPO-dependent activation of PMNs (Figs. 3, 4, and 6). This integrin is essential for PMN adhesion by binding to extracellular matrix proteins such as FN, fibrin, and collagen, as well as adhesion molecules like intercellular adhesion molecule-1, and also mediates phagocytosis of iC3b-loaded particles (15, 16, 46).

To date, association of MPO with the outer membrane of PMNs has been viewed as a consequence of increased transport of MPO toward the membrane (47). The current results reveal that PMN binds PMN-derived "free" or circulating MPO. Thus, MPO-mediated PMN activation is not limited to the host cell, but it also influences the activation state of remotely located or vicinal PMN, thus amplifying systemic PMN activation by acting as both an autocrine and paracrine proinflammatory mediator. Because CD11b/CD18 integrins are also located on monocytes and natural killer cells (16), neutrophil-derived MPO may also modulate the activation state of other leukocytic cells that are closely linked to the progression of chronic inflammatory vascular disease, and that are localized to the atherosclerotic plaque in stable disease (48). Accordingly, MPO exerts leukocyte-activating functions, an event reminiscent of other PMN-derived potent proinflammatory cytokines such as TNF-α and IL-8.

In summary, MPO not only serves as an index of neutrophil recruitment and activation, but it also displays traditional cytokine-like properties that can serve to modulate the activation state of leukocytes in inflammatory vascular disease. Future studies are thus warranted that aim to modulate the association of MPO with leukocyte integrins and to define whether MPO-dependent leukocyte activation represents a therapeutic target.

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