Myeloperoxidase Delays Neutrophil Apoptosis Through CD11b/CD18 Integrins and Prolongs Inflammation

Driss El Kebir,* Levente József,* Wanling Pan, János G. Filep

Abstract—Polymorphonuclear neutrophil granulocytes have a central role in innate immunity and their programmed cell death and removal are critical for efficient resolution of acute inflammation. Myeloperoxidase (MPO), a heme protein abundantly expressed in neutrophils, is generally associated with killing of bacteria and oxidative tissue injury. Because MPO also binds to neutrophils, we investigated whether MPO could affect the lifespan of neutrophils. Here, we report that MPO independent of its catalytic activity through signaling via the adhesion molecule CD11b/CD18 rescued human neutrophils from constitututive apoptosis and prolonged their life span. MPO evoked a transient concurrent activation of extracellular signal-regulated kinase and Akt, leading to phosphorylation of Bad at both Ser112 and Ser136, prevention of mitochondrial dysfunction, and subsequent activation of caspase-3. Consistently, pharmacological inhibition of extracellular signal-regulated kinase, Akt, or caspase-3 reversed the antiapoptosis action of MPO. Acute increases in plasma MPO delayed murine neutrophil apoptosis assayed ex vivo. In a mouse model of self-resolving inflammation, MPO also prolonged the duration of carrageenan-induced acute lung injury, as evidenced by enhanced alveolar permeability and accumulation of neutrophils parallel with suppression of neutrophil apoptosis. Our results indicate that MPO functions as a survival signal for neutrophils and thereby contribute to prolongation of inflammation. (Circ Res. 2008;103:352-359.)

Key Words: apoptosis ■ neutrophil granulocytes ■ myeloperoxidase ■ integrins ■ inflammation

Recruitment and activation of polymorphonuclear neutrophil granulocytes is among the principal defense mechanisms of innate immunity. However, neutrophil-derived proteinases and reactive oxygen species, which are required for the elimination of microbes, are also capable of inflicting tissue damage. Myeloperoxidase (MPO), a heme protein abundantly expressed in the azurophilic granules, plays a central role in these events. MPO catalyzes the formation of hypochlorous acid, a potent oxidant that has been implicated in killing bacteria and tissue destruction through induction of necrosis and apoptosis. MPO, through formation of secondary oxidants and nitration of protein tyrosine residues, can modulate intercellular signaling in the vasculature and affect the activation state of neutrophils.

Neutrophil trafficking into inflamed tissues is intimately linked to prolonged survival. Mature neutrophils have the shortest half-life (~7 hours) among leukocytes and die rapidly via apoptosis. This constitutively expressed cell death program renders neutrophils unresponsive to proinflammatory stimuli and promotes their removal from inflamed areas by scavenger macrophages with minimal damage to the surrounding tissue, thereby facilitating the resolution of inflammation.

Neutrophil survival is contingent on rescue from apoptosis by signals, such as lipopolysaccharide or proinflammatory cytokines, from the inflammatory microenvironment. Markedly suppressed neutrophil apoptosis has been detected in patients with inflammatory diseases, including acute respiratory distress syndrome, sepsis, and acute coronary artery disease, that are also associated with elevated intravascular MPO levels. However, the link between MPO and the fate of neutrophils is unclear. Hydrogen peroxide or tumor necrosis factor triggers apoptosis of neutrophilic HL60 leukemia cells through MPO-dependent downregulation of FLIPs. In mice, MPO deficiency reduces ischemia/reperfusion-induced renal dysfunction and neutrophil accumulation but fails to abrogate apoptosis during early phases of reperfusion. Understanding the role of MPO in regulating neutrophil survival and apoptosis will provide important information regarding development of novel targeted therapies for dampening inflammation underlying a variety of diseases.

In the present study, we investigated whether MPO can influence neutrophil survival and apoptosis in vitro and consequently the inflammatory response in vivo. Here, we report that MPO signals through the β2 integrin CD11b/CD18 to rescue neutrophils from apoptosis by preventing mitochondrial dysfunction and subsequent activation of caspase-3. We also show that acute increases in plasma MPO level lead to...
delayed murine neutrophil apoptosis and that MPO suppression of neutrophil apoptosis prolongs acute inflammation in carrageenan-induced acute lung injury.

Materials and Methods
Myeloperoxidase purified from human neutrophils was obtained from Athens Research and Technology (purity >98% by SDS-PAGE, RZ of 0.82, no detectable eosinophil peroxidase contamination). Lactoferrin and elastase prepared from human leukocytes were from Sigma.

Neutrophil Isolation and Culture
Neutrophils were isolated24 from the venous blood of healthy volunteers who had denied taking any medication for at least 2 weeks. The Clinical Research Committee approved the experimental protocols. Neutrophils (5×10⁶ cells/mL, purity >96%, viability >98%) were cultured in Hanks’ balanced salt solution supplemented with 10% autologous serum on a rotator. Neutrophils were preincubated for 20 minutes with PD98059 (50 μmol/L), LY294002 (1 μmol/L), SB203580 (1 μmol/L), z-VAD-fmk (z-Val-Ala-d-Asp-fluoromethylketone, 20 μmol/L), or z-F-FA-fmk (20 μmol/L, Calbiochem), an anti-CD11b antibody (20 μg/mL, clone ICRF44, BD Biosciences) or 4-aminobenzoic acid hydrazide (20 μmol/L) and then challenged with MPO (10 to 160 nmol/L) at 37°C in 5% CO₂ atmosphere. In some experiments, MPO pretreated with 4-aminobenzoic acid hydrazide (20 μmol/L) or 3-amino-1,2,4-triazole (5 μmol/L) plus H₂O₂ (100 μmol/L) was used. At the designated time points, cells were processed as described below.

Assessment of Apoptosis
Apoptosis was assessed with flow cytometry using fluorescein isothiocyanate (FITC)-conjugated annexin V (BD Biosciences) in combination with propidium iodide (Molecular Probes) and the percentage of cells with hypoploid DNA.24 The presence of morphological changes characteristic of apoptosis was confirmed by light microscopy following staining of neutrophils with hematoxylin/eosin. DNA cleavage was shown by detection of cytoplasmic histone-associated DNA fragments with Cell Death ELISA (Roche) and gel electrophoresis.24

Caspase-3 Activity
Release of 7-amino-4-methyl-coumarin from N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methyl-coumarin (BD Biosciences) by human neutrophil lysates, prepared from 10⁶ cells, was measured using a CytoFluor microplate reader (PE Biosystems) with excitation and emission wavelengths of 340 nm and 460 nm, respectively.24 In CytoFluor microplate reader (PE Biosystems) with excitation and emission wavelengths of 340 nm and 460 nm, respectively.24 In some experiments, MPO treated with 4-aminobenzoic acid hydrazide (20 μmol/L) or 3-amino-1,2,4-triazole (5 μmol/L) plus H₂O₂ (100 μmol/L) was used. At the designated time points, cells were processed as described below.

Mitochondrial Transmembrane Potential (ΔΨₘ)
Neutrophils (5×10⁵ cells) were incubated for 15 minutes at 37°C with the lipophilic fluorochrome chloromethyl-X-rosamine (CMXRos) (200 nmol/L, Molecular Probes),23 and the fluorescence was analyzed in a FACScan flow cytometer.26

Cytochrome c Release
Mitochondrial and cytosolic fractions were prepared using the Mitochondria Isolation kit (Pierce) and cytochrome c levels were measured by an ELISA (Active Motif).27 The intraassay and interassay coefficients of variation were <10%.

Western Blotting
Protein extracts prepared from 10⁶ neutrophils were resolved by SDS-PAGE and transferred to poly(vinylidene difluoride) membranes. Blots were blocked with 5% skimmed milk powder in Tris-buffered saline plus Tween and probed with antibodies to phosphorylated extracellular signal-regulated kinase (ERK)1/2, Akt, p38 mitogen-activated protein kinase (MAPK), Bad(Ser112), and Bad(Ser136) (all from Cell Signaling Technologies) or β-actin (Sigma-Aldrich).24

Intravenous Injection of MPO to Rats
The protocols were approved by the Animal Care and Use Committee. Male Wistar rats (weighing 246 to 316 g, Charles River Laboratories) were first implanted with arterial and venous catheters as described.28 Four days later, after recovery from the surgery, they received MPO (5 nmol/kg body weight) or saline intravenously. Mean arterial blood pressure and heart rate were monitored continuously. At 60 minutes post-MPO injection, under isoflurane anesthesia, blood was collected by cardiac puncture. Total and differential white blood cell counts were determined. Cell viability and apoptosis were assessed in freshly isolated neutrophils and in neutrophils cultured for up to 24 hours by staining with propidium iodide and FITC-conjugated annexin V, respectively. Two-color fluorescence was analyzed in a FACScan flow cytometer.27

Plasma MPO Levels
Rat plasma was analyzed for MPO by using an ELISA according to the instructions of the manufacturer (Assay Design). The intraassay coefficient of variation was <6%.

Carrageenan-Induced Acute Lung Injury
The protocols were approved by the Animal Care and Use Committee. Female BALB/c mice (aged 8 to 12 weeks, Charles River Laboratories) were injected intratracheally with 0.1 mL of 0.25% l-carrageenan (Fluka)29 with or without 10 μL of 16 μmol/L MPO or saline as a control. At 1, 2, 3, 4, and 5 days post-MPO, the mice were killed and the lungs were lavaged 5 times with 1 mL of saline solution containing 0.5% heparin. The supernatant of the first milliliter of bronchoalveolar lavage (BAL) fluid was used for measurements of protein concentration. The remaining pooled BAL fluid was centrifuged, and the cells were resuspended in PBS. Total and differential leukocyte counts in BAL fluid samples were assessed by standard light microscopy techniques. Neutrophil viability and apoptosis were assessed with flow cytometry following staining with propidium iodide and FITC-conjugated annexin V, respectively. In separate groups of mice, the lungs were removed without lavage, fixed in 4% formaldehyde, and embedded in paraffin, and 2-μm-thick sections were stained with hematoxylin and eosin. Lung tissue MPO activity was measured using o-dianisidine as a substrate.30

Statistics
Results are presented as means±SEM. Statistical comparisons were made by ANOVA using ranks (Kruskal–Wallis test), followed by Dunn’s multiple contrast hypothesis test to identify differences between various treatments or by Mann–Whitney U test (2-tailed). P<0.05 was considered statistically significant.

Results
Myeloperoxidase Delays Human Neutrophil Apoptosis by Signaling Through CD11b/CD18 Integrins
To investigate whether MPO can affect apoptosis directly, human neutrophils were cultured up to a 96-hour period with MPO purified from human neutrophils. MPO markedly suppressed neutrophil apoptosis in a time-dependent (Figure 1A) and concentration-dependent (Figure 1B) manner. After 24 hours of culture with MPO, an increased number of annexin V– and propidium iodide–negative cells were present and these cells could be identified morphologically as intact neutrophils (Figure 1C). Electrophoretic analysis confirmed the ability of MPO to attenuate DNA fragmentation (Figure...
1D). Considerable proportions of MPO-treated neutrophils retained a nonapoptotic phenotype even after 48 hours in culture; however, cell viability decreased to <19% and <2% by 72 and 96 hours, respectively. Other azurophilic granule constituents, elastase (1 to 100 milliunits/mL) and lactoferrin (0.01 to 1 mg/mL), were without effects (data not shown).

Because anti-CD11b antibodies were found to block MPO binding to neutrophils and subsequent degranulation and superoxide production,10,31 we established whether the antiapoptosis action of MPO also involved this pathway. Indeed, pretreatment with an anti-CD11b antibody prevented the MPO suppression of neutrophil apoptosis, whereas the MPO inhibitor 4-aminobenzenzoic acid hydrazide was without effects (Figure 2). Pretreatment of MPO with 4-aminobenzenzoic acid hydrazide or the mechanism-based inhibitor 3-amino,1,2,4-triazole plus H2O2, which resulted in >95% and 71% inhibition of catalytic activity, respectively, failed to affect the antiapoptotic action of MPO (Figure 2). MOPC-21, a class-matched irrelevant antibody, did not affect neutrophil responses to MPO (data not shown). These findings corroborate that neutrophil responses to MPO are mediated via binding to CD11b, rather than by secondary generation of reactive oxidants.

Myeloperoxidase Prevents Mitochondrial Dysfunction and Caspase-3 Activation

To assess the downstream intracellular signaling pathways, we studied the activation of several MAPks known to regulate neutrophil survival and monitored mitochondrial function and caspase-3 activation. MPO induced a rapid phosphorylation of ERK1/2 and Akt relative to unstimulated controls (Figure 3A). Culture of neutrophils resulted in time-dependent phosphorylation of p38 MAPK that was further enhanced in the presence of MPO (Figure 3B). To confirm the role of these kinases, we used selective pharmacological inhibitors. Both PD98059 and the phosphoinositide (PI)3-kinase (the up-stream regulator of Akt activation) inhibitor LY294002, which inhibited MPO-induced phosphorylation of ERK1/2 and Akt, respectively (Figure 3C), almost completely blocked the responses to MPO (Figure 3D). By contrast, blockade of p38 MAPK with SB203580 significantly increased the number of viable cells and reduced apoptosis. Cotreatment of neutrophils with MPO and SB203580 resulted in similar changes as those observed with either MPO or SB203580 alone (Figure 3D). MPO induced phosphorylation of Bad at both Ser112 and Ser136, a downstream target for ERK and Akt, respectively (Figure 3E).

Reductions in mitochondrial transmembrane potential (ΔΨm) precede development of apoptotic morphology in neutrophils undergoing constitutive programmed cell death.26,32 Thus, following 24 hours of culture, ~60% of neutrophils exhibited reduced ΔΨm (Figure 4A) compared with ~44% annexin V–positive cells (Figure 1A). Consistent with suppression of development of apoptotic morphology, MPO partially prevented disruption of ΔΨm in a concentration-dependent fashion (Figure 4B) and attenuated subsequent release of cytochrome c from the mitochondrion into the cytosol (Figure 4C). This was associated with...
Acute Elevations of Plasma MPO Delay Neutrophil Apoptosis

To study the impact of acute elevations in plasma MPO on neutrophil survival, we injected human MPO intravenously into conscious chronically catheterized rats. This resulted in plasma MPO levels of 127±41 ng/mL (n=6, measured at 60 minutes post-MPO injection) similar to those detected in patients with inflammatory vascular diseases and known to predict acute coronary events.20,21 No MPO was detectable in the plasma of saline-treated rats. MPO injection did not affect mean arterial blood pressure and heart rate (Figure 5A) or leukocyte and neutrophil counts (Figure 5B). However, the number of annexin V–positive cells (Figure 5C), DNA fragmentation, and caspase-3 activity (Figure 5D) were significantly lower in neutrophils isolated within 3 hours of MPO injection (0 hour) or neutrophils (5×10⁶ cells/mL) cultured for 24 hours in the presence of Ac-DEVD-aldehyde, an inhibitor of caspase-3 activity. Baseline neutrophil apoptosis-suppressing action of MPO. Neutrophils were cultured for 24 hours in the presence of MPO (160 nmol/L), or SB203580 (1 μmol/L) with or without MPO (160 nmol/L). Cell viability and apoptosis were assessed by staining with propidium iodide and annexin V–FITC, respectively. Data represent the means±SEM (n=4). *P<0.05 vs untreated. **P<0.01 vs MPO-treated.

Figure 3. Involvement of ERK and PI3-kinase in mediating the antiapoptotic effect of MPO in human neutrophils. A, B, C, and D, Effects of MPO on phosphorylation of Akt, ERK1/2, p38 MAPK, and the proapoptotic protein Bad. Neutrophils (10⁷ cells/mL) were cultured for 24 hours in the presence of PD98059 (50 μmol/L), LY294002 (1 μmol/L), or SB203580 (1 μmol/L) with or without MPO (160 nmol/L). Cell viability and apoptosis were assessed by staining with propidium iodide and annexin V–FITC, respectively. Data represent the means±SEM (n=5 to 7). *P<0.05; **P<0.01 vs untreated; †P<0.05 vs untreated; ††P<0.01 vs untreated. E, Pharmacological blockade of MPO/Akt and PI3-kinase attenuates the apoptosis-suppressing action of MPO. Neutrophils (5×10⁶ cells/mL) were cultured for 24 hours in the presence of PD98059 (50 μmol/L), LY294002 (1 μmol/L), or SB203580 (1 μmol/L) with or without MPO (160 nmol/L). Cell viability and apoptosis were assessed by staining with propidium iodide and annexin V–FITC, respectively. Data represent the means±SEM (n=5 to 7). *P<0.05; **P<0.01 vs untreated; †P<0.05 vs untreated; ††P<0.01 vs untreated. F, The combination of MPO and z-VAD-fmk alone, indicating that MPO and z-VAD-fmk inhibited the same pathway. Neither z-FAM-fmk (a negative control) nor the vehicle (0.1% dimethyl sulfoxide) elicited any detectable effects (data not shown).

Figure 4. MPO prevents loss of mitochondrial transmembrane potential (ΔΨm), cytochrome c release and activation of caspase-3 in human neutrophils. Freshly isolated neutrophils (5×10⁶ cells/mL) were cultured for 24 hours in the presence of PD98059 (50 μmol/L), LY294002 (1 μmol/L), or SB203580 (1 μmol/L) with or without MPO (160 nmol/L) were analyzed. A, Representative dot plots of neutrophils stained with CMXRos. Decreases in CMXRos fluorescence indicate loss of ΔΨm. B, Concentration dependency of the MPO-suppressing action of MPO. Neutrophils (5×10⁶ cells/mL) were cultured for 24 hours in the presence of PD98059 (50 μmol/L), LY294002 (1 μmol/L), or SB203580 (1 μmol/L) with or without MPO (160 nmol/L). Cell viability and apoptosis were assessed by staining with propidium iodide and annexin V–FITC, respectively. Data represent the means±SEM (n=5 to 7). *P<0.05; **P<0.01 vs untreated; †P<0.05 vs untreated; ††P<0.01 vs untreated.
following culture of isolated neutrophils for up to 24 hours (Figure 5C and 5D).

MPO Suppression of Neutrophil Apoptosis Prolongs Acute Inflammation

Having shown in vitro and ex vivo that neutrophil apoptosis was markedly delayed by MPO, we investigated the impact of MPO on the resolution of neutrophil-mediated inflammation in mice. We used a model of acute lung injury induced by low concentration of intratracheal inoculation with carrageenan, which is useful to study resolution of inflammation.29

Carrageenan injection evoked acute pulmonary inflammation characterized by massive infiltration of neutrophils (Figure 6A) and alveolar edema (Figure 6C). Intratracheal injection of MPO alone produced only minimal inflammatory cell accumulation, whereas it prolonged...
carrageenan-induced inflammation (Figure 6A). Thus, the number of neutrophils in the BAL fluid (Figure 6B), BAL fluid protein levels (Figure 6C), and tissue MPO content (an indicator of neutrophil influx into tissues) (Figure 6D) were still markedly elevated at day 5 after carrageenan plus MPO as compared with mice that were injected carrageenan only. The percentage of annexin V–positive neutrophils (Figure 6E), DNA fragmentation (Figure 6F) and caspase-3 activity (Figure 6G) were markedly lower in carrageenan plus MPO-treated mice than in carrageenan-treated mice. Consistently, mice injected with carrageenan or carrageenan plus MPO had marked inflammatory infiltrate at day 1 (Figure 7). By day 5, the lungs appeared to be almost normal in carrageenan-treated mice, whereas the inflammation persisted in mice that had received carrageenan plus MPO (Figure 7).

Discussion

Our results uncovered an unexpected role for MPO to influence the fate of neutrophils and consequently the duration of inflammation. By suppressing the constitutive cell death program, MPO prolonged the life span of neutrophils, thereby delaying the resolution of inflammation. These actions were specific for MPO, because other azurophilic granule constituents lactoferrin and elastase failed to affect neutrophil apoptosis.

Consistent with the commitment of neutrophils to apoptosis, MPO at clinically relevant concentrations delayed, rather than blocked apoptosis, resulting in prolonged survival of human neutrophils in vitro. We confirmed that increasing plasma concentrations of MPO in rats to levels comparable to those detected in patients with inflammatory vascular diseases20,21 was sufficient to retard the apoptotic machinery in neutrophils as assayed ex vivo. Furthermore, MPO also suppressed apoptosis in neutrophils that had emigrated into the airways and delayed resolution of inflammation in a mouse model of carrageenan-induced acute lung injury.

Neutrophils bind to MPO-coated surfaces31 or “free” circulating MPO through CD11b/CD18 integrins,10 leading to degranulation and superoxide production.10 Our results indicate that the apoptosis delaying action of MPO also involves this pathway and is independent of its catalytic activity. Previous studies have shown that MPO upregulates surface expression of CD11b on neutrophils and evokes MPO release from neutrophils,10 implicating an autocrine and paracrine mechanism for perpetuation of the inflammatory response. In addition, following engagement with CD11b/CD18, MPO extends survival and thus the pool of functional neutrophils. Neutrophil-derived MPO may also impact function of other leukocytes, including monocytes and NK cells that express CD11b/CD18.

The β2 integrin CD11b/CD18 is best known for mediating neutrophil adhesion and transmigration across the endothelium and for promoting phagocytosis of microbes.33 CD11b/CD18-mediated “outside-in” signaling provokes neutrophil degranulation34 and contributes to suppression of apoptosis during endothelial transmigration.35,36 However, β2 integrin–mediated neutrophil adhesion per se is not a prerequisite to generate survival signals for antibody cross-linking of CD11b/CD18 in cells in suspension signals survival cues.35 Neutrophil apoptosis is controlled by a complex network of signaling pathways, including the ERK, Akt, and p38 MAPK pathways.10 MPO-induced phosphorylation of p38 MAPK, which mediates activation of NADPH oxidase,10 did not generate a survival signal under our experimental conditions. Indeed, selective pharmacological blockade of p38 MAPK retarded apoptosis both in the absence and presence of MPO. We identified ERK1/2 and Akt-dependent prevention of mitochondrial dysfunction as the key mechanism by which MPO rescued neutrophils from constitutive apoptosis. The delay of neutrophil apoptosis in response to intercellular adhesion molecule-1 adhesion is attributed to Akt activation,37 whereas promotion of neutrophil survival by another CD11b/CD18 ligand fibrinogen requires activation of both Akt and ERK.38 Previous studies also showed that transient activation of PI3-kinase without ERK activation may not be sufficient to delay neutrophil apoptosis.39 Compelling evidence suggests that ERK and Akt-mediated phosphorylation of the proapoptotic protein Bad prevents its association with
the Bcl-2 homolog Mcl-1 expressed in neutrophils. The mechanism of upregulation of inducible NO synthase expression in MPO mice, as well as the impact of acute suppression of inducible NO synthase expression by MPO on the resolution of lung inflammation, remains to be investigated. Absence of MPO-derived oxidant production during E coli septicemia in MPO mice could reduce lung injury and mortality. However, it is not known whether MPO deficiency could affect the longevity of neutrophils, thereby contributing to protection against lung inflammation in this model of sepsis. Recent evidence indicates that prolonged neutrophil survival adversely affect the duration of the inflammatory response. It is intriguing that MPO prolonged the life span of neutrophils, the predominant source of this enzyme. The apoptosis-delaying and neutrophil-activating properties of MPO are reminiscent of those of proinflammatory cytokines and bacterial constituents and indicate an important role for MPO in regulating the inflammatory response, as we showed in a mouse model of acute lung injury. These findings lend strong support to the notion that neutrophil apoptosis is among the critical determinants of the outcome of the inflammatory response in vivo, including models of carrageenan-induced acute lung injury, pleurisy, and arthritis, and, therefore, is a potential target for therapeutic interventions. Thus, suppression of neutrophil apoptosis may ensue prolonged inflammation, whereas induction of neutrophil apoptosis would enhance resolution of inflammation.

In summary, the present study identifies MPO as a survival signal for neutrophils by delaying intrinsic apoptosis, thereby modulating the outcome of acute inflammation. Future studies are, thus, warranted to define whether these actions of MPO represent novel therapeutic targets for dampening inflammation.

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**Disclosures**

None.

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