Interaction of myeloperoxidase with vascular NAD(P)H oxidase-derived reactive oxygen species in vasculature: implications for vascular diseases

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Myeloperoxidase (MPO) is a heme protein derived from neutrophils, monocytes, and macrophages. It is well known that the defense of the organism through production of hypochlorous acid (HOCl) is the traditional role of MPO; however, there is a growing body of evidence suggesting that MPO might play important roles in noninfectious diseases, especially in the pathogenesis of the above-mentioned inflammatory vascular diseases (7, 33, 39). In this regard, MPO- and HOCl-modified low-density lipoprotein (LDL) are highly expressed in animal and human atherosclerotic vessels (19, 28, 29). Malle et al. (28) first found that HOCl-modified LDL existed in atherosclerotic plaques of rabbits in response to dietary cholesterol. MPO is the only enzyme known to generate HOCl in vivo, thus HOCl-modified LDL is a stable and specific biomarker of MPO-catalyzed oxidation in vivo. Colocalization of immunoreactive MPO- and HOCl-modified-LDL in serial sections of rabbit lesions provided convincing in vivo evidence for the MPO-HOCl-chloride system mediated oxidation of (lipo)proteins. The same group also found that MPO- and HOCl-modified LDL were highly expressed in human atherosclerotic lesions but not in normal control vessels (29). MPO and HOCl-modified LDL were located both in vascular cells and extracellular spaces (29). Furthermore, Hazen et al. (19) first found that 3-chlorotyrosine, another specific marker of MPO-catalyzed oxidation, was markedly elevated in LDL isolated from human atherosclerotic intima. In addition, one recent report showed that a significant positive correlation existed between the intima-to-media area ratio (I/M) and MPO and HOCl-modified protein present in human atherosclerotic vessels (18). To further investigate the possible role of MPO in the development of atherosclerosis, two clinical studies were performed. One study showed that elevated levels of leukocyte and blood MPO are associated with the presence of coronary heart disease (47). Another study demonstrated that people with MPO deficiency had beneficial effects against cardiovascular damage (27).
In contrast to the above clinical studies, disruption of the MPO gene in LDL receptor-deficient mice resulted in increased atherosclerosis (6). The mouse and human difference is an important distinction with regard to the role of MPO in the pathogenesis of atherosclerosis (31). Normally, rodent leukocytes may not secrete active MPO. Indeed, there is no detectable MPO in the atherosclerotic vascular wall of LDL receptor-deficient mice (6).

Leukocyte activation leads to the release of MPO from storage granules into the extracellular space. Leukocyte NAD(P)H oxidase-derived H₂O₂ is the physiological substrate of MPO. MPO utilizes H₂O₂ to oxidize chloride, resulting in the formation of the proinflammatory oxidant HOCl and chlorinating species. Up to 40% of the H₂O₂ generated by activated leukocytes is used to form HOCl, and the local concentration of HOCl potentially exceeds 100 μmol/l under pathological conditions (3, 12, 22, 35). We (10, 44, 46) and other investigators (1, 23, 32) confirmed that there are at least three mechanisms involved in MPO-induced vascular injury responses: 1) consumption of nitric oxide (NO), 2) oxidation of LDL into the form that is taken up by macrophages in an uncontrolled manner, and 3) reaction with l-arginine and protein to produce endogenous NO synthesis inhibitors (Cl-l-Arg and HOCl). All three mechanisms are H₂O₂ dependent (4, 10, 19, 29, 32, 44, 46).

We further demonstrated that MPO is a transcytosisable protein. Not only can infiltrated leukocytes release MPO into the vascular wall, but blood-derived MPO can also bind and infiltrate into the vascular wall directly (4, 10, 44). Our in vitro study showing the short-time (2 h) in vitro exposure to MPO (10–400 nM) resulted in a dose-dependent increase of vessel-bound MPO activity (0.054–2.967 U/g tissue) (44). This is comparable to the levels of MPO in vessels under pathological conditions such as sepsis and atherosclerosis (1.66 ± 0.43 U/g tissue and 0.53 ± 0.12 U/g tissue separately, our unpublished data). More interestingly, the vascular-bound MPO (0.054–2.967 U/g tissue) inhibited endothelium-dependent relaxation in a dose-dependent manner in the presence of H₂O₂ (44).

It is well known that MPO can use leukocyte NAD(P)H oxidase-derived H₂O₂; however, it is unknown whether MPO can use vascular NAD(P)H oxidase-derived H₂O₂ in the vascular wall. Our hypothesis is that vascular-bound MPO derived from both circulating blood and infiltrated phagocytes can use vascular NAD(P)H oxidase-derived H₂O₂ to produce HOCl and chlorinating species. This MPO-HOCl-chlorinating species pathway in the vascular wall plays an important role in vascular injury and development of vascular diseases.

**MATERIALS AND METHODS**

**Materials.** Human MPO, rabbit anti-human MPO, and polyclonal anti-HOCl-modified LDL antibody were purchased from Calbiochem. Mouse anti-rat CD45 (leukocyte common antigen, clone OX-1) was obtained from Pharmingen. 4-Aminoazobenzoic acid hydrazide (ABAH) was from Aldrich Chemical. Biotinylated anti-rabbit IgG antibody, Vectastain ABC kit, and DAB kit were from Vector Laboratories, and the osmotic minipump (ALZET model 2002) was obtained from Durect. All the other materials were from Sigma.

**Animals.** Ten-week-old male Sprague-Dawley rats (Harlan Breeding Laboratories; Indianapolis, IN) were used in the study. The animals were anesthetized with ketamine (60 g/kg per xylazine 5 mg/kg). All protocols were approved by the Institutional Animal Care and Use Committee at the University of Tennessee and were consistent with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, Revised 1985).

**Vascular function assessments.** Isometric tension was measured in isolated aortic and carotid artery ring segments of control and experimental rats as described (45, 46). At the time the rat was euthanized, the rat thoracic aortas were excised and cleansed of fat and adhering tissue. The vessels were cut into individual ring segments (2–3 mm in width) and suspended from a force-displacement transducer in a tissue bath. Ring segments were bathed in Krebs-Henseleit (K-H) solution. The vessels were contracted to 50–60% of maximum capacity with 100 nM phenylephrine (3 × 10⁻⁶–10⁻⁷ M). When tension development reached a plateau, ACH (10⁻⁹–3 × 10⁻⁸ M) was added cumulatively to the bath to stimulate endothelium-dependent relaxation. Endothelium-independent relaxation was tested by cumulative addition of the NO donor sodium nitroprusside (SNP).

To compare the vascular injury response caused by H₂O₂, HOCl, and Cl-l-Arg, the vessel segments were preincubated with a subtoxic dosage (5–50 μM) of these three compounds for 1 h, followed by extensive washing, and then endothelium-dependent and -independent relaxation were tested.

To test whether MPO utilizes vascular NAD(P)H oxidase-derived H₂O₂ as a substrate to produce HOCl and chlorinating species in the vascular wall, we incubated rat aortic ring segments with ANG II (100 nM) for 4 h to increase the activity of vascular NAD(P)H oxidases and the production of H₂O₂. Vessels incubated with vehicle were used as controls. After a 4-h incubation, the vessels were then incubated with MPO (400 nM) for another 1 h, followed by washing, and then the vascular function was determined. To determine whether MPO-induced vascular dysfunction is through the MPO-vascular NAD(P)H oxidase-H₂O₂-HOCl-chlorinating species pathway, the vessels were coincubated with either H₂O₂ scavenger catalase (800 U/ml), HOCl scavenger l-methionine (100 μM), or anti-oxidant vitamin C (Vit C) (200 μM), which scavenges HOCl and reverses chlorinating species formation (8). Because l-methionine and Vit C may also scavenge other free radicals, MPO inhibitors, 5-fluorouracil (5-FU) (2 mg/ml) (2), and ABAH (100 μM) (24, 25) were coincubated with MPO to further test whether the MPO-induced injury response is via HOCl generation. Heparin-binding superoxide dismutase (HB-SOD) prevents ROS, and ANG II induced vascular injury by catalyzing O₂⁻ to H₂O₂, whereas the later is catalyzed to H₂O and O₂ by catalase. In the presence of MPO, if MPO uses H₂O₂ to produce HOCl and chlorinating species, the SOD-induced protective effect might attenuate. To test this, HB-SOD (20 U/ml) was added into ANG II-pretreated vessels with or without MPO, and vascular function was determined.

**Measurement of MPO activity.** Previously, we have shown that MPO can bind to the vascular wall in vitro (10, 44). To measure vessel-bound MPO activity over time, rat right internal carotid artery and the caudal origin of the common carotid artery (CA) were transiently clipped, and a polyethylene (PE-10) catheter was inserted from the right external...
carotid artery. The inside of the CA was flushed with 500 μl K-H buffer and then filled with 100 μl K-H buffer containing MPO (400 nM). After a 60-min incubation period, the vessel was flushed again, the external carotid artery was ligated, and then the CA blood flow was restored. The rats were euthanized at 0, 1, 3, 5, and 7 days after incubation, and MPO activity in CA was measured. Vessel MPO activity was determined as described (41). Rat carotid arteries were isolated, frozen in liquid N2, and pulverized using a mortar and pestle. Samples were resuspended in 1 ml 50 mM potassium phosphate buffer (pH 6) containing 0.5% hexadecyltrimethyl phosphonium bromide. The sample was then homogenized for 3 min, followed by sonication. The suspension was centrifuged at 40,000 rpm for 15 min. An aliquot (0.1 ml) of the supernatant was added to 2.9 ml potassium phosphate buffer (pH 6) containing 0.53 mM o-dianisidine and 0.15 mM H2O2. Absorbance of the sample was monitored at 460 nm every 15 s for 5 min. Potassium phosphate buffer served as a blank. MPO activity was determined by dividing the absorbance change per minute by the molar extinction coefficient for o-dianisidine (ε = 1.13 × 10^4 M⁻¹ cm⁻¹) and was normalized to protein concentration. One unit of MPO was defined as that of degrading 1 μmole peroxide per minute.

**Determination of vascular NAD(P)H oxidase activity and ROS production.** Vascular NAD(P)H oxidase activity and its product O2 were measured using lucigenin-derived chemiluminescence in vehicle-treated control and ANG II-treated (100 nM for 4 h) rat aorta segments (34). Briefly, after preparation, the vessel ring was placed in a K-H buffer and allowed to equilibrate for 30 min at 37°C. Scintillation vials containing 2 ml Krebs-HEPES buffer with 25 μM lucigenin were then placed into a scintillation counter switched to the out-of-coincidence mode. Lucigen counts were expressed as counts per minute per milligram dry weight. For NAD(P)H oxidase activity measurement, either NADH or NADPH was required for significant production. The vessels were then homogenized, frozen in liquid N2, and pulverized using a mortar and pestle. Samples were resuspended in 1 ml 50 mM potassium phosphate buffer (pH 6) containing 0.53 mM o-dianisidine and 0.15 mM H2O2. Absorbance of the sample was monitored at 460 nm every 15 s for 5 min. Potassium phosphate buffer served as a blank. MPO activity was determined by dividing the absorbance change per minute by the molar extinction coefficient for o-dianisidine (ε = 1.13 × 10^4 M⁻¹ cm⁻¹) and was normalized to protein concentration. One unit of MPO was defined as that of degrading 1 μmole peroxide per minute.

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**Immunohistochemistry.** To obtain further direct evidence that vessel-bound MPO can use vascular nonleukocyte oxidase-derived H2O2 to produce HOCl and chlorinating species in the vascular wall, we detected HOCl-modified LDL, a specific biomarker for the MPO-HOCl-chlorinating species pathway, by immunohistochemistry. The vessel segments from rat aortas were incubated with 1 mg/ml human native LDL and ANG II (100 nM) for 4 h to allow native LDL into the vascular wall and increase the production of vascular nonleukocyte oxidase-derived H2O2. The vessels were then incubated with MPO (400 nM) for another 1 h. Vessel segments without preincubation of any experimental reagents, preincubated with LDL alone, LDL, and ANG II but without MPO, MPO alone, or LDL and MPO without ANG II were also detected. Vessels preincubated directly with HOCl-modified LDL (1 mg/ml), which was prepared by the reaction of human native LDL (1 mg/ml) and HOCl (1 mM), were used as positive controls. In addition, vessels incubated with MPO and exogenous H2O2 (10 μM) were also used as positive controls. All the vessel segments were washed extensively before fixation to remove any possible unbound protein and reagents. Artery ring segments were preserved in 10% buffered formalin and embedded in paraffin. HOCl-modified LDL immunostaining was performed in thin sections (5 μm) of tissue. Before incubation with the primary HOCl-modified LDL antibody (1:200 dilution for 30 min), tissue sections were treated with H2O2 to quench endogenous peroxidase activity. A biotinylated anti-rabbit IgG secondary antibody was then applied. Immunostaining was detected using a Vector ABC kit. Sections were counterstained with hematoxycin.

To confirm that the MPO activity in our time-course study was induced by vessel-bound MPO and to exclude any possible MPO activity induced by leukocyte accumulation, MPO and leukocyte immunostaining were performed in thin sections (5 μm) of rat right common carotid arteries 3 days after local short-time (1 h) incubation with 400 nM MPO. Before incubation with either the rabbit anti-human MPO antibody (1:1,500 dilution for 1 h) or mouse anti-rat CD45 (leukocyte common antigen, clone OX-1), tissue sections were treated with H2O2 to quench endogenous peroxidase activity. Either A biotinylated anti-rabbit IgG secondary antibody (for MPO staining) or A biotinylated anti-mouse IgG secondary antibody was then applied. Sections were treated with vehicle as vehicle controls, and sections of rat spleen were used as positive controls for CD45 staining. Immunostaining was detected using a Vector ABC kit. Sections were also counterstained with hematoxycin.

**Statistics.** All data are presented as means ± SE. Dose-response profiles for different experimental conditions were analyzed and tested to determine differences in relaxation responses using the SigmaStat statistical analysis program. Unpaired observations were assessed by one-way analysis of variance and multiple-range tests. A P value of <0.05 was required for significance.

**RESULTS**

**MPO binds to and exhibits activity in the vascular wall for a significant period of time.** The results showed that MPO could bind to and exhibit activity in the vascular wall in vivo (Fig. 1). The activity of MPO in vessels after short-time (1 h) in vivo exposure to high concentration of human MPO (400 nM), followed by extensive washing was 1.61 ± 0.35 U/g tissue, which is 10 times higher than the normal control level (Fig. 1). In sharp contrast, the MPO activity was reduced to 0.35 U/g tissue, which is also 10 times lower than the normal control level (Fig. 1). To confirm that the sustained MPO activity is from the vascular-bound MPO but not from infiltrated leukocytes, both MPO and leukocyte immunostaining...
were performed in the carotid arteries 3 days after local MPO incubation. As shown in Fig. 2, the vascular-bound MPO was located in the intima, and, to a lesser extent, in the media near the intima. In contrast, no leukocyte CD45 immunostaining was observed in the MPO-treated sections, suggesting that the sustained MPO activity is induced by vascular-bound MPO but not by the infiltrated leukocytes.

*MPO-derived* HOCl and chlorinating species (Cl-L-Arg) impaired vascular function and were more potent than *H₂O₂*. To compare the effects of *H₂O₂*, HOCl, and Cl-L-Arg on vascular injury, we incubated vessels with the above three different agents (5–50 μM) for 1 h, followed by washing. Vascular function was then measured. We found *H₂O₂* at 5 and 10 μM had no injury effect on the vascular function. *H₂O₂* at 50 μM inhibited the endothelium-dependent relaxation only by 30.70%, whereas HOCl and Cl-L-Arg badly impaired the vascular function (88.66% and 97.69% inhibition of endothelium-dependent relaxation at 50 μM, respectively) (Fig. 3A). The results indicated that MPO-derived HOCl and chlorinated species amplify *H₂O₂*-induced vascular injury. In contrast, all three compounds had no inhibitory effects on the endothelium-independent relaxation to SNP at the experimental concentrations (Fig. 3B), suggesting the endothelium is the target of the MPO-induced acute injury response.

**Interaction between MPO and vascular NAD(P)H oxidase-derived reactive *H₂O₂* within the vascular wall.**

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**Fig. 1.** Myeloperoxidase (MPO) activity changes in the carotid artery after in vivo incubation of MPO. Activity of MPO was measured in the carotid artery wall over a period of 7 days after in vivo local incubation with 400 nM human MPO (*n* = 6) or vehicle (*n* = 6) for 60 min. Data are means ± SE. *P* < 0.05 and **P* < 0.001 compared with the day 0 group. *P* < 0.05 and **P* < 0.001 compared with MPO-treated group.

**Fig. 2.** Immunostaining of MPO and leukocyte in the carotid artery after in vivo incubation of MPO. Immunohistochemical staining of MPO (A and B) and leukocytes (C and D) were performed in the carotid artery wall 3 days post-in vivo local incubation with 400 nM human MPO (*n* = 3) or vehicle (*n* = 3) for 60 min using specific rabbit anti-human MPO antibody and mouse anti-rat CD45 antibody separately. There was no MPO staining in vehicle-treated vessel (A); however, there was intensive MPO staining in MPO-treated vessel (B). Vascular-bound MPO was located in the intima and, to a lesser extent, in the media near the intima (B). There was no CD45, a common antigen for neutrophils, monocytes, and macrophages, staining in both MPO- and vehicle-treated vessels (C and D). E: positive control for leukocyte (CD45) staining in rat spleen section.
and the endothelium-independent relaxation was tested by addition of 5, 10, and 50 μM H₂O₂, HOCl, or Cl-L-Arg for 1 h followed by washing with Krebs-Henseleit (K-H) buffer. In phenylephrine-contracted aorta ring segments, the endothelium-dependent relaxation was tested by addition of ACh (10⁻⁹-3 × 10⁻⁹ M), and the endothelium-independent relaxation was tested by addition of sodium nitroprusside (SNP) (10⁻⁹-3 × 10⁻⁶ M). Data are means ± SE. *P < 0.01 and **P < 0.001 compared with corresponding dose of H₂O₂.

To test whether MPO utilizes vascular NAD(P)H oxidase-derived H₂O₂ as a substrate to produce HOCl and chlorinating species in the vascular wall, we incubated rat aortic ring segments with ANG II (100 nM) for 4 h to increase the activity of vascular NAD(P)H oxidases and the production of H₂O₂. Our results showed that after incubation with ANG II for 4 h, vascular NAD(P)H oxidase activity and its products superoxide and H₂O₂ were increased (Fig. 4, A–C). There are three potential sources of superoxide and H₂O₂ in the vascular wall without leukocytes: vascular NAD(P)H oxidase, xanthine oxidase, and NO synthase. Our results indicate that vascular NAD(P)H oxidase is the predominant source of ROS in ANG II-treated vessels in vitro because xanthine oxidase inhibitor allopurinol (10 μM) or NO synthase inhibitor L-NAME (100 μM) had no significant effect on the increased production of superoxide, whereas vascular NAD(P)H oxidase inhibitors DPI (10 μM) or Apo (0.3 mM) reduced superoxide significantly (Fig. 4B). This suggests that vascular NAD(P)H oxidases is the main source of H₂O₂ in the vessel wall in our model. The result is consistent with one previous study showing that vascular NAD(P)H oxidase is the predominant source of ROS in vessels from ANG II-induced hypertensive animals (34).

After a 4-h incubation period with ANG II, the vessels were then incubated with MPO (400 nM) for another 1 h, followed by washing, and then vascular function was determined. The results showed that ANG II partially impaired the endothelium-dependent relaxation by a mechanism of increased ROS formation (Fig. 5A). MPO had no notable effect on control vessels; however, MPO significantly amplified the impairment effect on endothelium-dependent relaxation in ANG II pretreatment vessels (Fig. 5A). In contrast, ANG II and MPO had no injury effect on the endothelium-independent relaxation to SNP (Fig. 5B). The mechanism by which MPO induced the additional injury on endothelium-dependent relaxation is by the reaction with vascular NAD(P)H oxidase-derived H₂O₂ and its chlorinating species because the H₂O₂ scavenger catalase, the HOCl scavenger l-methionine, and antioxidant Vit C, which can reverse the formation of chlorinating species, can prevent the injury response (Fig. 5C). Also, coincubation with MPO inhibitors ABAH (100 μM) or 5-fluorouracil (2 mg/ml) blocked MPO-induced impairment of vascular function (Fig. 5D). Furthermore, although coincubation with HB-SOD prevented ANG II-induced endothelial dysfunction, the protective effect of HB-SOD disappeared in MPO-treated vessels (Fig. 5E). The results suggest that MPO uses vascular NAD(P)H oxidase-derived H₂O₂ to amplify the injury response.

**HOCl modified-LDL was expressed in the LDL-preincubated vascular wall containing both MPO and vascular NAD(P)H oxidase-derived H₂O₂.** Our results showed that there is no HOCl-modified LDL staining in the negative control (without primary antibody) (Fig. 6A), normal untreated segment (Fig. 6B), as well as in vessels incubated with LDL alone (Fig. 6C), ANG II and LDL (Fig. 6D), MPO alone (Fig. 6E), and LDL together with MPO (Fig. 6F). In sharp contrast, the vessels treated with LDL, ANG II, and MPO, which had LDL, vascular NAD(P)H oxidase-derived H₂O₂, and MPO in the vascular wall, had strong immunostaining (Fig. 6G). The HOCl-modified LDL was located in the intima and, to a lesser extent, in the media (Fig. 6G), which was similar to those in two positive controls (Fig. 6, H and I).

**DISCUSSION**

There is growing evidence of the significance of MPO in cardiovascular diseases, which includes atherosclerosis, ischemia-reperfusion injury, sepsis, and diabetic vascular complications. Our previous studies (10, 45, 46) have demonstrated that one of the important mechanisms involved in MPO-induced vascular injury is the reduction of NO bioavailability. We also found MPO is a transcytosable protein that can bind and infiltrate into the vascular wall (4, 10, 44). Not only can the...
vascular-bound MPO localize in the extracellular space, but it can also enter vascular cells (10, 42). In the present study, we further demonstrated that the vascular-bound MPO has a relatively long half-life; it can remain in the vasculature for at least 3–5 days. This interesting result points to the importance of understanding the interaction of vascular-bound MPO, both from vascular-infiltrated activated phagocytes and from circulating blood, with vascular ROS such as H2O2, which has a very short half-life.

Our previous studies have shown that MPO-induced vascular injury responses are NAD(P)H oxidase-derived H2O2 dependent (10, 44–46). There are two different kinds of NAD(P)H oxidases: leukocyte-derived and nonleukocyte-derived NAD(P)H oxidase (13, 37). Vascular NAD(P)H oxidases belong to the nonleukocyte-derived NAD(P)H oxidase and are the major sources of ROS in both atherosclerotic and diabetic vasculature (13, 16, 20, 21, 43). Vascular NAD(P)H oxidases-derived ROS such as H2O2 have emerged as important molecules in pathogenesis of inflammatory vascular diseases. Recent reports show that the vascular NAD(P)H oxidases are structurally and genetically distinct from the leukocyte-derived NAD(P)H oxidase (9, 42). In contrast to leukocyte-derived NAD(P)H oxidase, which can only be activated and produce ROS in a respiratory burst manner, vascular NAD(P)H oxidases are low-output, slow-release enzymes and have a moderate constitutive activity, which is absent in phagocytes. In normal vessels, vascular NAD(P)H oxidase-derived H2O2 is catabolized to H2O by catalase and loses any injury effects. However, under pathological conditions such as atherosclerosis, hypertension, and diabetes, the production of H2O2 is much higher than its degradation. Thus the H2O2 content in the vascular wall under the above-mentioned pathological conditions is much higher than in normal vessels, although the absolute tissue concentration needs to be determined (13, 16, 20, 21). One recent report (26) indicates that vascular NAD(P)H oxidase-derived ROS...
might be more important than leukocyte NAD(P)H oxidase-derived ROS in the pathogenesis of atherosclerosis, because a deficiency in leukocyte NAD(P)H oxidase fails to inhibit atherosclerosis in mice with either diet-induced or genetic forms of hypercholesterolemia. Although it is well known that MPO can use phagocyte-derived H2O2, there is a lack of direct evidence showing that the vascular-bound MPO can use vascular NAD(P)H oxidase-derived H2O2 to induce injury in the vascular wall.

In the current study, we used ANG II to increase vascular NAD(P)H oxidase and subsequent H2O2 production and vascular dysfunction as a vascular injury parameter. We confirmed that vascular NAD(P)H oxidase is the main source of the increased H2O2 in our vessel model system, which is consistent with previous studies (34). MPO can use the vascular NAD(P)H oxidase-derived H2O2 to amplify the ROS-induced vascular injury as showed by the further impairment of endothelium-dependent relaxation. The mechanism involved in MPO-induced vascular injury is by its reaction with H2O2 to produce HOCl and its chlorinating species. MPO inhibitors, H2O2 scavenger, HOCl scavenger, and antioxidant Vit C can prevent MPO-induced injury response. To further confirm the mechanism, we also used the SOD, which can protect the ROS-induced injury by the following protective pathway: NAD(P)H oxidase-superoxide-SOD-H2O2-catalase-H2O2. Indeed, our results showed that SOD protected against ANG II-induced vascular injury. In the presence of MPO, MPO will compete with catalase in catalyzing their common substrate H2O2 to produce HOCl and its chlorinating species.
chlorinating species, and the protective effect of SOD should be reduced. One recent report (5) also showed that in lipopolysaccharide-injected animals, which have MPO and increased O$_2$/$H_2O_2$ production in the vascular wall, SOD failed to improve ACh-induced relaxation. Our results showed that the protective effect of SOD disappeared in MPO-bound vessels treated with ANG II. Finally, to obtain the direct evidence that vessel-bound MPO can use vascular NAD(P)H oxidase-derived H$_2$O$_2$ to produce HOCl and chlorinating species, we detected HOCl-modified LDL, a specific biomarker for MPO-HOCl-chlorinating species pathway, by immunohistochemistry in the vascular wall (17, 29). Our results showed that HOCl-modified LDL was located in the intima and, to a lesser extent, in the media.

It has been well known for a long time that systemic infection is one of the main inducing factors for the attack of coronary artery disease and stroke, and chronic systemic inflammation exacerbates the atherosclerotic lesions, but the molecular mechanism is still unclear. Under the above inflammatory conditions, the amount of MPO in circulating blood is increased and so is the MPO in the vascular wall. Our results in the present study indicate that MPO may be the main link between systemic inflammation and the exacerbation of vascular diseases.

In summary, vascular-bound MPO could use the vascular NAD(P)H oxidase-derived H$_2$O$_2$ to produce HOCl and its chlorinating species and amplify vascular NAD(P)H oxidase-derived ROS-induced injury in the vascular wall. NAD(P)H oxidase-derived H$_2$O$_2$ is increased in many inflammatory vascular diseases, and leukocyte activation, adhesion and infiltration into the vascular wall are the common events under these conditions. Furthermore, MPO- and HOCl-modified proteins are highly expressed in human atherosclerotic lesions. Thus MPO-HOCl-chlorinating species may represent a common pathogenic pathway in diverse inflammatory vascular diseases and a new mechanism in phagocyte and systemic infection-induced exacerbation of vascular diseases. Blocking the MPO-HOCl-

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**Fig. 6.** Immunostaining of HOCl-modified low-density lipoprotein (LDL) in the vascular wall. Vessel segments from rat aortas were incubated with human native LDL (1 mg/ml) and ANG II (100 nM) for 4 h to let native LDL into the vascular wall and increase vascular NAD(P)H oxidase-derived H$_2$O$_2$. The vessels were then incubated with MPO (400 nM) for another hour. All the vessel segments were washed extensively before fixation to remove any possible unbound protein and reagents. Immunostaining was carried out with the primary HOCl-modified LDL antibody and a Vector ABC kit in paraffin-embedded sections: A, negative control without primary antibody; B, normal untreated vessel; C, vessel incubated with LDL alone; D, vessel incubated with ANG II and LDL but without MPO; E, vessel incubated with MPO alone; F, vessel incubated with LDL and MPO but without ANG II. G, vessel incubated with LDL, ANG II, and MPO. H, vessel incubated with MPO and exogenous H$_2$O$_2$ (10 μM) (positive control); I, vessel incubated with HOCl-modified LDL (1 mg/ml) (positive control). The vessels treated with LDL, ANG II, and MPO (G) and two positive controls (H and I) had strong immunostaining. HOCl-modified LDL was located in the intima and, to a lesser extent, in the media.
chlorinating species pathway might be a new therapeutic approach to vascular diseases.

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DISCLOSURES

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REFERENCES


