Novel model of inflammatory neointima formation reveals a potential role of myeloperoxidase in neointimal hyperplasia

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Yang, Jian, Yunhui Cheng, Ruirui Ji, and Chunxiang Zhang. Novel model of inflammatory neointima formation reveals a potential role of myeloperoxidase in neointimal hyperplasia. Am J Physiol Heart Circ Physiol 291: H3087–H3093, 2006.—Atherosclerosis, which is characterized by neointima formation, is an inflammatory disease. However, there is no inflammatory product-elicited neointimal model to support the causal role of inflammation in atherogenesis. We reported previously that leukocyte-derived MPO induces vascular injury responses such as endothelial dysfunction. We now test the role of MPO in inflammatory neointima formation. We infused temporarily isolated rat common carotid arteries with MPO (200 nM) and incubated for 1 h. We found that although MPO itself did not induce any neointima formation 2 wk after treatment, in the presence of its substrate, hydrogen peroxide, MPO was able to elicit neointimal hyperplasia. We further confirmed that MPO-induced neointimal hyperplasia is mediated by its product, hypochlorous acid (HOCl). HOCl elicited apoptosis both in intima and media followed by vascular proliferative response and resulted in neointima formation with a heterogeneous cell population. Both histological and functional features of HOCl-treated vessels are similar to those in atherosclerotic lesions. To our knowledge, this is the first direct in vivo demonstration of neointimal formation induced by a product of the inflammatory cascade. The results suggest that MPO may be a mediator for pathological neointima growth. This novel neointimal model could be useful for studying inflammation and atherosclerosis.

atherosclerosis; inflammation; leukocyte

ATHEROSCLEROSIS is a pathological process characterized by vascular neointima formation and lipid accumulation (15, 25). According to statistics from the American Heart Association, atherosclerosis is the leading cause of death in the United States and the cause of more than half of all mortality in the developed countries of the world. Over the past century, the pathogenetic views of atherosclerosis have evolved substantially. Before the 1970s, the link between lipids and atherosclerosis had been well established. In the 1970s and 1980s, additional studies focused on growth factors and the proliferation of vascular smooth muscle cells (VSMCs). Since the 1990s, the role of inflammation has been considered and has now been confirmed by many clinical studies and experimental data (14, 24). Indeed, inflammation accompanies all the stages of atherosclerosis from initiation to athromatous neointima progression and to plaque disruption and complication formation. Inflammation is reported to be involved in endothelial cell injury and endothelial dysfunction, inflammatory cell recruitment, VSMC proliferation, and lipid accumulation. Therefore, atherosclerosis is actually an inflammatory vascular disease (14, 24). However, there is no inflammatory product-elicited neointimal model to support the causal role of inflammation in atherogenesis.

Leukocytes are the primary inflammatory cells. There is growing evidence of the significance of leukocytes in the development and exacerbation of atherosclerosis and its complications, such as myocardial infarction and stroke. First, leukocytes are the main cellular components in atherosclerotic lesions (15, 25). The interaction between endothelial cells and leukocytes and the resulting endothelial injury and dysfunction are believed to be the initial step of atherogenesis (24). Second, leukocytes accumulating in the vascular wall are able to take up lipids, resulting in the formation of foam cells (15). Third, leukocytes may interact with VSMCs and result in increased VSMC proliferation (14, 15, 24, 25). In addition, leukocytes may also play an important role in plaque destabilization and rupture (14). Indeed, increased leukocyte count and activity are associated with both clinical and experimental atherosclerosis (4, 8, 17). Furthermore, infection, which increases leukocyte count and activity in both circulation and the vascular wall, contributes to atherogenesis and the development of atherosclerotic complications (1, 7). Although a strong association exists among leukocytes, inflammation, and atherosclerotic lesion formation, the molecular mechanisms involved in this process remain unclear.

MPO is a heme protein derived from leukocytes (neutrophils, monocytes, and macrophages) (11, 13). Under physiological conditions, MPO catalyzes the reaction between hydrogen peroxide (H2O2) and chloride and results in the production of hypochlorous acid (HOCl). HOCl is the only source of HOCl in vivo. HOCl is highly reactive, reacting with amino acids and proteins to produce chloramines such as chlorinated L-arginine (Cl-L-Arg) (29) and protein derivations such as HOCl-modified LDL (19). Recent reports (22, 23) have suggested that MPO may play an important role in vascular injury and atherogenesis. In this regard, MPO and HOCl-modified protein are highly expressed in both human and experimental atherosclerotic neointima (10, 18). Elevated levels of MPO are associated with the presence of coronary heart disease and predict risk in patients with acute coronary syndromes (3, 33). Recently, we found that MPO is a transcytosable 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 (2, 32). We also found that vessel-bound MPO exhibits activity in the vascular wall for a significant period of time (32). Vascular-bound MPO can use only

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leukocyte-derived H$_2$O$_2$ but also nonleukocyte-derived H$_2$O$_2$ within the vascular wall to produce HOCl and chlorinating species (32). Our recent findings suggest that MPO is able to induce endothelial cell injury and endothelial dysfunction (28, 29, 31, 32). The current study tests the hypothesis that MPO may induce neointimal hyperplasia via its chlorinating product.

**Materials and Methods**

**Carotid artery nonmechanical injury model.** We hypothesized that MPO may induce neointima formation via its chlorinating product. To test the hypothesis, we applied a carotid artery nonmechanical injury model (Fig. 1A), which we used in a recent study (27). Briefly, Sprague-Dawley rats (250 to 300 g) (Harlan) were anesthetized with ketamine (60 mg/kg ip) and xylazine (5 mg/kg ip). The right internal carotid artery and caudal origin of the common carotid artery were transiently clipped, and the common carotid artery segment was infused with treatment solution via a PE-10 catheter inserted into the external carotid artery. After a 1-h treatment, the clips were removed, and circulation in the common carotid artery was restored. Two weeks after treatment, the animals were euthanized for carotid artery morphometric analysis.

**Carotid artery balloon injury model.** Carotid artery balloon injury was induced as described previously (30). Briefly, Sprague-Dawley rats (250 to 300 g) (Harlan) were anesthetized with ketamine (60 mg/kg) and xylazine (5 mg/kg). Under a dissecting microscope, the right common carotid artery was exposed through a midline cervical incision, and blood flow to the site of surgical manipulation was temporarily interrupted by ligation with vessel clips of the left common, internal, and external carotid arteries. A 2-Fr Fogarty catheter (Baxter-Edwards) was introduced through an arteriotomy in the external carotid artery and advanced to the proximal edge of the omohyoid muscle. To produce carotid artery injury, we inflated the balloon with saline and withdrew it six times from just under the proximal edge of the omohyoid muscle to the carotid bifurcation. After injury, the external carotid artery was permanently ligated with a 6-0 silk suture. The clips at the common and internal carotid arteries were released, and blood flow was restored.

All the animals were maintained at constant humidity (60 ± 5%), temperature (24 ± 1°C), and light cycle (6:00 AM to 6:00 PM) and...
were fed a standard rat pellet diet. 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 1996).

**Morphometric analysis for neointimal formation.** Morphometric analysis was performed in hematoxylin-esoin-stained sections by a computerized image analysis system (Scion Image CMS-800) as described in a previous study (30). Six sections (5 μm thick), sectioned at equally spaced intervals of carotid arteries, were used. The medial area was calculated by subtracting the area defined by internal elastic lamina from the area defined by external elastic lamina, and the intimal area was determined by subtracting the lumen area from the area defined by the internal elastic lamina. Finally, the intimal-to-medial area (I/M) ratio of each section was calculated. The average I/M ratio of the six sections was used as the I/M ratio of this animal.

**Vascular function assessment.** Isometric tension was measured in isolated carotid artery ring segments of rats as described (30, 32). Ring segments were bathed in Krebs-Henseleit solution. The vessels were contracted to 50–60% of their maximal capacity (50–60% of KCl response) with phenylephrine (3 × 10⁻⁵–10⁻⁷ M). When tension development reached a plateau, ACh (10⁻⁶–3 × 10⁻⁴ M) was added cumulatively to the bath to stimulate endothelium-dependent relaxation. Endothelium-independent relaxation was tested by a cumulative addition of the NO donor sodium nitroprusside. Real-time data were collected for all experiments and downloaded to an IBM PC for later analysis using commercially available software (Workbench for Windows).

**Transferase-mediated dUTP nick-end labeling and proliferating cell nuclear antigen immunostaining for apoptosis and proliferation analysis.** The transferase-mediated dUTP nick-end labeling (TUNEL) method was used as described (6). An apoptosis detection kit was used. Cell proliferation was detected immunohistochemically with proliferating cell nuclear antigen (PCNA) labeling on arterial sections as described (6). Positive staining was displayed by DAB kit as either red (apoptosis) or brown (PCNA). All the sections were counterstained with hematoxylin. Positive cells in the vascular wall were quantitatively analyzed by a computerized image system and were expressed as a percentage of cells occupied by positive staining.

**Immunohistochemistry analysis of cellular components in neointimal lesions.** A panel of immunohistochemical markers for endothelial cells (von Willebrand factor), VSMCs (α-actin), and leukocytes (CD45) were used as primary antibodies. Immunohistochemistry was performed in paraffin-embedded vessel sections as described (30). Immunostaining was detected using a Vector ABC kit. Sections were counterstained with hematoxylin.

**Statistical analysis.** All data are presented as means ± SE. Two-tailed unpaired Student’s t-tests and ANOVA were used for statistical evaluation of the data. Based on the power analysis of the VSMC proliferation, apoptosis, and neointima formation, at least six animals were used per time point per group. A value of P < 0.05 was regarded as significant. All statistical work was done with SPSS software (Microsoft).

**RESULTS**

**MPO elicits neointima formation via its product HOCI.** Two weeks after treatment, the animals were euthanized for carotid artery morphometric analysis. Unexpectedly, there was no neointima formation in MPO-treated (200 nM) arteries (Fig. 1B). Similarly, no neointima formation was found in vehicle-treated vessels (Fig. 1C).

Our previous studies have shown that MPO-induced endothelial cell injury and endothelial dysfunction are substrate (H₂O₂) dependent and are related to its product HOCI (29). We therefore considered whether MPO is able to induce neointima growth in the presence of its substrate H₂O₂. MPO (200 nM) was mixed with H₂O₂ (1 mM) in PBS (pH = 7.4) at 37°C for 15 min before it was infused into the rat carotid arteries. H₂O₂ (1 mM) was used as a control. Interestingly, in the presence of H₂O₂, MPO elicited substantial neointima formation at 2 wk after treatment (Fig. 1D). On the contrary, no neointima growth was found in only H₂O₂-treated vessels (Fig. 1E). To further confirm that MPO-elicited neointimal hyperplasia was the result of its product HOCl, the following three approaches were used. First, MPO inhibitor 4-aminobenzoic acid hydrazide (100 μM) was added to the MPO solution before adding H₂O₂. We confirmed that, under this condition, HOCI formation was blocked (data not shown). Second, HOCI scavenger t-methionine (1 mM) was applied just before the addition of H₂O₂. Third, HOCI (1 mM) in PBS at pH 7.4 was infused into carotid arteries and incubated for 1 h. As shown in Fig. 1, F and G, MPO plus H₂O₂-induced neointimal growth was abolished by MPO inhibitor and HOCI scavenger, whereas HOCI elicited neointima formation similar to MPO plus H₂O₂-treated vessels (Fig. 1H).

To compare with neointima formation after angioplasty, balloon injury was performed on rat carotid arteries. We found that neointima formation induced by MPO-derivd product was less than that induced by balloon injury (Fig. 1I). I/M ratio was used to quantitatively analyze neointima growth, and the results from different groups are shown in Fig. 1, J and K.

**Dose- and time-dependent effects of MPO-derived product on neointima formation.** To further characterize HOCI-induced neointimal growth, dose-response and time-course studies were performed. For the dose response, rat carotid arteries were incubated with 10 μM, 100 μM, 500 μM, 1 mM, or 5 mM HOCI for 1 h, and the animals were euthanized at 2 wk after treatment for morphometric analysis. For the time-course study, rat carotid arteries were incubated with 1 mM HOCI for 1 h, and the animals were euthanized at 7, 14, or 28 days after treatment for morphometric analysis. As shown in Fig. 2, A and B, no neointima formation was found in the 10 μM and 100 μM HOCI-treated groups. Mild neointimal growth was demonstrated in the 500 μM dose group, whereas the 1 mM and 5–mM HOCI-treated groups had major neointima formation. Mild neointima began to form at 7 days after treatment and continued to grow until 4 wk after treatment (Fig. 2, C and D). Therefore, dose- and time-dependent effects existed in HOCI-treated vessels.

**Histological features of neointima formation elicited by MPO-derived product.** We next determined the cellular components in MPO product, HOCI-induced neointimal lesions. To detect the cytological features, we used a panel of immunohistological markers for VSMCs (α-actin), leukocytes (CD45), and endothelial cells (von Willebrand factor); immunohistochemistry was performed in paraffin-embedded vessel sections treated with HOCI (1 mM). As shown in Fig. 3, both VSMC (Fig. 3A) and leukocyte (Fig. 3B) biomarkers are shown in HOCI-induced neointimal lesions. These immunocytochemical markers revealed a heterogenous cell population within MPO-derived neointima, which is similar to the cellular components in atherosclerotic neointimal lesions. Interestingly, at 2 wk after treatment, all the neointimal lesions were covered by endothelial cells, and the expression of von Willebrand factor in neointima was restricted to the endothelium (Fig. 3C). On the contrary, there were still areas not covered by endothelial
cells at the same time point in vessels after angioplasty, and the endothelial cell maker was expressed in both the neointima and endothelium in these balloon-injured vessels (6). Thus histological features of HOCl-treated vessels were similar to those of atherosclerotic arteries, whereas those of balloon-injured arteries were not (26). It is well established that atherosclerotic lesions are covered by endothelial cells. However, the lesion-covering endothelial cells are dysfunctional. Indeed, endothelial dysfunction is an important biomarker for the development and progression of atherosclerosis. To test the functional feature of HOCl-treated vessels, we used the method described previously (30) in isolated carotid arteries at 2 wk after HOCl

![Fig. 2. Dose- and time-dependent effects of MPO-derived HOCl on neointima formation in rat carotid arteries. A and B: neointima formation in vessels 2 wk after treatment with different concentrations of HOCl. A: representative photomicrographs. B: I/M ratio. C and D: neointima formation in vessels at 7, 14, and 28 days (d) after treatment with HOCl (1 mM). C: representative photomicrographs. D: I/M ratio.](image)

![Fig. 3. Histological and functional features of HOCl-treated rat carotid arteries. A: vascular smooth muscle cell marker (α-actin) immunostaining. B: leukocyte marker (CD45) immunostaining. C: endothelial cell maker (von Willebrand factor) immunostaining. D: endothelium-dependent relaxation to ACh. E: endothelium-independent relaxation to sodium nitroprusside (SNP). Untreated, untreated normal vessels (n = 8); Vehicle, vehicle (PBS)-treated vessels (n = 8); HOCl, HOCl (1 mM)-treated vessels (n = 8). *P < 0.05 and **P < 0.01 vs. untreated group.](image)
(1 mM) treatment. The concentrations of phenylephrine used to induce 50–60% of their maximal contractive capacity (50–60% of KCl response) before addition of ACh were (8.57 ± 0.78) × 10^{-8} M, (8.42 ± 0.81) × 10^{-8} M, and (7.88 ± 1.09) × 10^{-8} M in uninjured carotid arteries, vehicle-treated group, and HOCl-treated group, respectively. There were no significantly different among the three groups (P > 0.05). There were also no differences in tension generated by phenylephrine at these experimental concentrations among these three groups (53.02 ± 4.42%, 52.64 ± 5.01%, and 55.02 ± 6.92% of KCl response, respectively, P > 0.05). As shown in Fig. 3D, endothelial function was significantly impaired in HOCl-treated vessels. In contrast, endothelium-independent function was unchanged (Fig. 3E). Therefore, not only the histological features but also the functional features are similar to those in atherosclerotic lesions.

**Cell apoptosis and proliferation in MPO-mediated neointima formation.** Rat carotid arteries were harvested at 6 h and 1, 3, 7, 14, and 28 days after treatment with HOCl (1 mM) or vehicle (n = 5 animals/time point). Uninjured arteries were used as normal controls. Apoptosis was demonstrated by the TUNEL method (6). Cell proliferation was identified by immunostaining for PCNA (6). As shown in Fig. 4, A and C, apoptosis was not detected in vehicle-treated control vessels. After treatment with HOCl, vascular cell apoptosis within intima (endothelial cell line) and media was quickly induced and peaked at 1 day after treatment. Thereafter, the number of TUNEL-positive cells declined and reached baseline at 14 days within the media. The neointima was distinguishable at 7 days after treatment. Apoptosis in the neointima peaked at 7 days and returned to baseline at 28 days after treatment. In comparison, PCNA staining peaked at 4 days

![Fig. 4. Time-course changes of cell apoptosis and proliferation in rat carotid arteries after treatment with MPO-derived HOCl. A and C: cell apoptosis determined by transferase-mediated dUTP nick-end labeling (TUNEL) method. A: representative photomicrographs. C: quantitative analysis of cell apoptosis in neointima and media. B and D: cell proliferation determined by immunohistochemistry with proliferating cell nuclear antigen (PCNA) labeling. B: representative photomicrographs. D: quantitative analysis of cell proliferation in neointima and media. Note: carotid arteries were isolated after treatment with HOCl (1 mM) or vehicle (uninjured).](http://ajpheart.physiology.org/)

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after treatment within the media and at 7 days within the neointima. PCNA positivity returned to baseline at 28 days after treatment (Fig. 4, B and D).

**DISCUSSION**

Vascular injuries, such as endothelial cell injury, are the earliest events in the development of atherosclerosis and restenosis after angioplasty (1–4). Growing evidence indicates that chronic and acute overproduction of reactive oxygen species (ROS) may be responsible for these early injury responses (16, 21). The first ROS is superoxide (O$_2^-$), which is produced by enzymes such as the nicotinamide adenine dinucleotide (phosphate) [NADH/NAD(P)H] oxidases. Superoxide is dismutated enzymatically to become a less toxic ROS, H$_2$O$_2$, through the action of SODs. Under physiological conditions, H$_2$O$_2$ is further removed via a number of antioxidant mechanisms, both enzymatic and nonenzymatic (21). However, under pathological conditions, H$_2$O$_2$ is able to become other ROS, such as reactive hydroxyl radicals (-OH). In addition, MPO, a heme protein secreted by leukocytes, can also use H$_2$O$_2$ to produce chlorinating species.

Although the role of ROS, such as O$_2^-$, H$_2$O$_2$, and -OH in the development of cardiovascular diseases is well established (9, 16), the role of MPO-derived chlorinating species is still unclear. Recent studies (28, 29, 31, 32) suggest that MPO-derived chlorinating species may play important roles in vascular injury responses. In the current study, we found that although MPO itself did not induce any neointima formation 2 wk after treatment, in the presence of its substrate H$_2$O$_2$, MPO was able to elicit neointimal hyperplasia. Our unpublished data also demonstrated that in balloon-injured rat carotid arteries, which have increased production of H$_2$O$_2$, exogenous MPO increased, whereas inhibition of MPO activity decreased neointima formation. In the current study, we further confirmed that MPO-induced neointimal hyperplasia was mediated by its product HOCl. Both histological and functional features of HOCl-treated vessels are similar to those in atherosclerotic lesions.

The neointimal lesion formation elicited by MPO-derived product is much smaller than that induced by balloon injury (Fig. 1). We think the difference is related to the difference of the injury type and the injury extent. Balloon injury induces quick endothelium denudation and media injury. Therefore, all the endothelial cells in intima are quickly removed after balloon injury. The vascular smooth muscle cells in media are also damaged by mechanical injury. However, the MPO-derived product may only partially induce cell chemical injury. Some endothelial cells still survive. In addition, the endothelial cells lost via inflammatory-based stimuli may be replaced quickly by regenerated endothelial cells due to lack of the large area of denudation. The inflammatory-based, stimuli-induced neointimal formation might resemble the process of atherosclerotic lesion formation. Under atherosclerotic conditions, neointimal lesion formation is a chronic process. Short-term exposure to atherosclerotic stimuli, such as hypercholesterol, can induce only a very small neointimal lesion formation. Also, there is no big area denudation of the endothelium, although the apoptosis rate of the endothelial cells is increased under the hypercholesterol condition.

Our results are consistent with a growing body of evidence suggesting that MPO has a proatherosclerosis effect (3, 10, 18, 22, 23, 33). However, MPO-knockout, LDL-receptor-deficient mice develop atherosclerotic lesions that are larger than those observed in LDL-receptor-deficient mice (5). But the increased atherosclerosis may not be induced by the direct effect of MPO function for the following two reasons: 1) MPO does not exist in these mouse chronic atherosclerotic lesions (5), and 2) expression of exogenous MPO in atherosclerotic mice increases atherosclerotic lesions significantly (20). Even in mouse vessels, the MPO response in acute vessel injury is also different from that in chronic injured vessels such as atherosclerosis. Our unpublished data suggest that MPO is highly expressed in mouse carotid arteries after either guide-wire or air-drying injuries.

Neointimal growth is the balance between cell apoptosis and cell proliferation. To further determine the cellular mechanisms involved in MPO-derived, HOCl-mediated neointima formation, we determined the profiles of vascular cell apoptosis and proliferation at different time points over a 28-day period. The results of these cellular events in HOCl-treated vessels (Fig. 4) indicate that the early cellular event involved in HOCl-mediated neointima growth is apoptosis. Because HOCl is a transcytoseable product, apoptosis or cell injury occurred at both intima and media layers of the vessels. After vascular injury, proliferative or repair response occurred and resulted in the formation of neointima.

The inflammatory response is a common pathological phenomenon, and circulating levels of HOCl are increased under inflammatory conditions. It has been estimated that, during moderate inflammation, the concentration of HOCl within the extracellular space can reach 340 μM (12). However, HOCl may be much higher near the activated leukocytes, such as in the atherosclerotic vascular wall or under severe inflammatory conditions. Our study has demonstrated that HOCl at 100–500 μM is able to induce mild neointima formation. Therefore, the amount of HOCl used for animal treatment is physiologically relevant. MPO may play an important role in vascular proliferation under pathological conditions and may also be a new link between inflammation and exacerbation of atherosclerosis. However, the accurate tissue and cellular levels of HOCl in the vascular wall under these pathological conditions are currently unknown because of the lack of a sensitive and specific method for determining the concentration of HOCl in the vessel tissue. This is the major limitation for the current study.

Our current study demonstrates that MPO is able to induce a neointimal lesion via its product HOCl under our experimental conditions. To our knowledge, this is the first direct in vivo demonstration of neointimal formation induced by a product of the inflammatory cascade. This novel neointima model can be useful for studying inflammation and atherosclerosis.

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