

# Leukocyte-Derived Myeloperoxidase Amplifies High-Glucose–Induced Endothelial Dysfunction Through Interaction With High-Glucose–Stimulated, Vascular Non–Leukocyte-Derived Reactive Oxygen Species

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**Vascular non-leukocyte-derived reactive oxygen species (ROS), such as superoxide and hydrogen peroxide ( $H_2O_2$ ), have emerged as important molecules in diabetic endothelial dysfunction. In addition, leukocyte-derived myeloperoxidase (MPO) has been implicated in vascular injury, and its injury response is  $H_2O_2$  dependent. It is well known that MPO can use leukocyte-derived  $H_2O_2$ ; however, it is unknown whether the vascular-bound MPO can use high-glucose–stimulated, vascular non-leukocyte-derived  $H_2O_2$  to induce diabetic endothelial dysfunction. In the present study, we demonstrated that MPO activity is increased in vessels from diabetic rats. In high-glucose–incubated rat aortas and in carotid arteries from rats with acute hyperglycemia, vascular-bound MPO utilized high-glucose–stimulated  $H_2O_2$  to amplify the ROS-induced impairment of endothelium-dependent relaxation via reduction of nitric oxide bioavailability. Hypochlorous acid (HOCL)-modified LDL, a specific biomarker for the MPO/HOCL/chlorinating species pathway, was detected in LDL- and MPO-bound vessels with high-glucose–stimulated  $H_2O_2$ . The results suggest that vascular-bound MPO could use high-glucose–stimulated  $H_2O_2$  to amplify high-glucose–induced injury in the vascular wall. MPO/ $H_2O_2$ /HOCL/chlorinating species may represent an important pathway in diabetes complications and a new mechanism in phagocyte- and systemic infection–induced exacerbation of diabetic vascular diseases. *Diabetes* 53: 2950–2959, 2004**

**E**ndothelial dysfunction is a major contributing factor to the pathogenesis of diabetic vascular complications (1–6). Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide ( $H_2O_2$ ), have emerged as important molecules in the

pathogenesis of diabetic vascular complications, including endothelial dysfunction (1,2). Vascular NAD(P)H oxidase, the nonleukocyte oxidase, has proven to be the major source of ROS in diabetic vasculature (3,4).

A growing body of evidence suggests that leukocytes play an important role in the pathogenesis of diabetic vascular complications (7–10). In addition, it is well known that recurrent infection is one of the main clinical characteristics of diabetic patients, and infection-induced inflammation exacerbates diabetic vascular lesions (9,11). Although a strong association exists between leukocytes and the development of diabetic endothelial dysfunction and atherosclerosis (7–12), the molecular mechanisms involved in these processes are still unclear.

Recently, it has been suggested that myeloperoxidase (MPO), a heme protein derived from leukocytes, plays an important role in leukocyte-mediated vascular injury responses in inflammatory vascular diseases such as atherosclerosis (13). The physiological substrate of MPO is  $H_2O_2$ , which is used to oxidize chloride, resulting in the formation of the proinflammatory oxidant, hypochlorous acid (HOCL). HOCL then reacts with various amino acids and proteins to produce chloramines, such as chlorinated L-arginine and chlorotyrosine, and modified proteins, such as HOCL-modified LDL. High levels of MPO, MPO-derived 3-chlorotyrosine, and HOCL-modified LDL are detected in animal and human atherosclerotic lesions (14–17). Furthermore, MPO's possible role as a mediator of coronary artery disease and acute coronary syndromes has recently received considerable attention in clinical studies (18–20). Individuals with an MPO deficiency are reported to experience beneficial effects against cardiovascular damage (21).

Recently, we found that MPO elicits endothelial dysfunction in an  $H_2O_2$ -dependent manner (22,23). The reduced bioavailability of nitric oxide (NO) is responsible for the endothelial dysfunction induced by the MPO system (MPO/HOCL/chloramines/HOCL-modified proteins) (22–26). Our group (23) and Baldus and colleagues (22,27) have further demonstrated that MPO itself is a transcytosable protein. In contrast to leukocyte-released  $H_2O_2$ , which has a very short half-life, vessel-bound MPO can remain in the vascular wall for a significant period of time (23). It is well known that MPO can use leukocyte-derived  $H_2O_2$ . However, it has remained uncertain whether MPO can use vascular non-leukocyte oxidase–derived  $H_2O_2$  in

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cGMP, guanosine 3',5'-cyclic monophosphate; HB-SOD, heparin-binding superoxide dismutase; HOCL, hypochlorous acid; KHB, Krebs-Henseleit buffer; L-NAME, *N*<sup>ω</sup>-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; MPO, myeloperoxidase; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase.

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the vascular wall; our recent report suggests that MPO is able to use the vascular non-leukocyte oxidase-derived  $H_2O_2$  to produce HOCL and its chlorinating species (23). More importantly, MPO-derived HOCL and chlorinating species amplify the ROS-induced injury by impairing the endothelium-dependent relaxation (23).

The presence of increased ROS such as  $H_2O_2$  in diabetic vasculature is well documented, and the activation, adhesion, and infiltration of leukocytes into the vessel wall is a critical component in the development of diabetes complications (8,12); however, the role of MPO in diabetes complications is unknown. Therefore, the objective of the present study was to determine the interaction of MPO with high-glucose-stimulated, non-leukocyte oxidase-derived ROS in the vasculature and its role in diabetic endothelial dysfunction.

## RESEARCH DESIGN AND METHODS

We obtained human MPO and rabbit anti-human HOCL-modified LDL antibody from Calbiochem, 4-aminobenzoic acid hydrazide from Aldrich Chemical, Amplex Red from Molecular Probes, rabbit anti-rat neutrophil antiserum and normal rabbit serum from Accurate Scientific, and biotinylated anti-rabbit IgG antibody, Vectastain ABC kit, and DAB kit from Vector Laboratories. A guanosine 3',5'-cyclic monophosphate (cGMP) enzyme immunoassay kit was purchased from Biotrak Amersham. All the other materials were from Sigma.

Age-matched male diabetic obese, nondiabetic obese, and nondiabetic lean Zucker rats were obtained from Genetic Models. Age-matched male Sprague-Dawley (SD) rats were obtained from Harlan Breeding Laboratories. Female C57BL/6 mice (age 6 weeks) and NADPH oxidase subunit gp91<sup>phox</sup>-deficient mice (B6.129S6-Cybb<sup>tm1Din</sup>, homozygote) were obtained from The Jackson Laboratories. The animals were anesthetized with ketamine (60 mg/kg)/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 Publ. no. 85-23, revised 1985).

**Measuring of MPO activity.** MPO activity in aortas was measured using the previously described method (23). MPO activity was determined by dividing the absorbance change per min at 460 nm by the molar extinction coefficient for *o*-dianisidine [ $\epsilon = 1.13 \times 10^4 \text{ mol} \cdot \text{l}^{-1} \cdot \text{cm}^{-1}$ ] and was normalized to tissue weight (grams).

**Determining vascular ROS production.** Vascular ROS production in isolated rat aortas incubated with low (11.1 mmol/l) and high glucose (44 mmol/l) was measured. Superoxide was determined using lucigenin-derived chemiluminescence (23). After a 5-h incubation with low or high glucose, the aorta segments were placed in a Krebs-Henseleit buffer (KHB) and allowed to equilibrate for 30 min at 37°C. Scintillation vials containing 2 ml KHB with 25  $\mu\text{mol/l}$  lucigenin were placed in a scintillation counter switched to the out-of-coincidence mode. To determine the predominant source of ROS in the vessel wall in our model, the vascular NAD(P)H oxidase inhibitors diphenylene iodonium (10  $\mu\text{mol/l}$ ) and apocynin (0.3 mmol/l), the xanthine oxidase inhibitor allopurinol (10  $\mu\text{mol/l}$ ), or the NO synthase inhibitor *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME; 100  $\mu\text{mol/l}$ ) were added to the tissue bath.  $H_2O_2$  production and location were determined by qualitative analysis with transmission electron microscopy, as previously described (23,28). To further analyze the  $H_2O_2$  production quantitatively, the Amplex Red assay was applied (29). Briefly, aortas were incubated with low- or high-glucose solution; the solution was then collected 5 h later. Next, Amplex Red reagent (200  $\mu\text{mol/l}$ ) and horseradish peroxidase (1 unit/ml) were added to the solution.  $H_2O_2$  levels were measured in a microplate reader at an excitation wavelength of  $530 \pm 25 \text{ nm}$  and an emission wavelength of  $590 \pm 35 \text{ nm}$ .

**Assessing vascular function.** Isometric tension was measured in isolated aortic and carotid artery rings, as previously described (23,26,30). The vessels were cut into individual ring segments (2–3 mm wide) and suspended from a force-displacement transducer in a tissue bath. Ring segments were bathed in KHB solution. The vessels were contracted with phenylephrine [ $(3 \times 10^{-8}) - 10^{-7} \text{ mol/l}$ ]. When tension development reached a plateau, acetylcholine [ $10^{-9} - (3 \times 10^{-6}) \text{ mol/l}$ ] was added cumulatively to the bath to evoke endothelium-dependent relaxation (endothelial function). Endothelium-independent relaxation was tested by cumulative addition of the NO donor sodium nitroprusside (SNP).

To determine the effect of exogenous MPO on hyperglycemia-induced vascular dysfunction in vitro, the rat and mouse aortic rings were incubated in

KHB with low glucose (11.1 mmol/l; the standard glucose concentration for vascular function measurement in vitro) or high glucose (44 mmol/l). After a 5-h incubation, the vessels were incubated with or without MPO (400 nmol/l) for 1 h, then extensively washed; vascular function was determined next. We have found that a short incubation (1 h) with MPO (400 nmol/l) followed by an extensive washing increases vascular-bound MPO activity to the levels of MPO in vessels under pathological conditions such as sepsis and diabetes (23). It should be noted that the starting glucose concentration (11.1 mmol/l) in normal KHB solution for the in vitro vascular function assay was higher than normal blood glucose level (<7 mmol/l) to keep the normal vascular function. We used 44 mmol/l glucose, which is higher than blood glucose levels in diabetic patients, for the high-glucose solution for the same reason. We have found that glucose-stimulated activity of vascular NAD(P)H oxidase is concentration and time dependent and that isolated vessel segments are able to keep normal vascular function at a low-glucose concentration (5.5 mmol/l) for at least 12 h (data not shown). To further test the effect of MPO on endothelial function at glucose concentrations comparable with those seen in vivo in diabetes, some rat aortic rings were incubated in KHB with low (5.5 mmol/l) or high glucose (20 mmol/l). After a 5-h incubation, the vessels were then incubated with or without MPO (400 nmol/l) for another 2 h.

Neutrophil is the main source of MPO. To determine the effect of endogenous MPO on high-glucose-induced endothelial dysfunction, rats were injected with a nontoxic dose (0.5 mg/kg, i.p.) of lipopolysaccharide (LPS) to increase endogenous vascular-bound MPO. Vessels from rats with neutrophil depletion were used as controls. Neutrophil depletion was performed 24 h before LPS injection by intraperitoneally injecting 1 ml of rabbit anti-rat neutrophil antiserum (1:10 dilution) (31) and was monitored by measuring MPO activity in the vascular wall. The vessels were isolated after 5 h of LPS injection and incubated with low or high glucose for 5 h, after which endothelial function was measured.

To determine if MPO-induced endothelial dysfunction occurs through the MPO/high-glucose-stimulated  $H_2O_2$ /HOCL/chlorinating species pathway, the vessels were coincubated with the  $H_2O_2$  scavenger catalase (800 units/ml), the HOCL scavenger L-methionine (100  $\mu\text{mol/l}$ ), or vitamin C (200  $\mu\text{mol/l}$ ), which scavenges HOCL and reverses chlorinating species formation (32). In addition, heparin-binding superoxide dismutase (HB-SOD; 20 units/ml), which prevents ROS-induced vascular injury by catalyzing  $O_2^-$  to  $H_2O_2$ , was added to high-glucose-pretreated vessels with or without MPO; vascular function was then determined. Previous studies have shown that vascular NADH oxidase is the main source of high-glucose-stimulated  $O_2^-$  and  $H_2O_2$ . To further test whether MPO is able to use high-glucose-stimulated, NAD(P)H oxidase-derived  $H_2O_2$  to amplify the endothelial dysfunction, aortic rings from the NAD(P)H oxidase subunit gp91<sup>phox</sup>-deficient mice were used.

To determine whether MPO can use hyperglycemia-induced  $H_2O_2$  to amplify the impairment of endothelium-dependent relaxation in vivo, acute hyperglycemia in rats was induced. Briefly, a catheter was inserted in the right femoral vein to infuse 50% dextrose. The infusion rate of dextrose was adjusted to maintain blood glucose at 350 mg/dl. After 4 h of hyperglycemia, the rat right internal carotid artery and caudal origin of the common carotid artery were transiently clipped, and a PE10 catheter was inserted from the right external carotid artery. The inside of the common carotid artery was flushed with 500  $\mu\text{l}$  KHB, then filled with 100  $\mu\text{l}$  KHB without or with MPO (400 nmol/l; vehicle control) into the common carotid artery and incubated for 1 h. Next, the vessel was flushed again, the right common carotid artery was isolated, and vascular function was measured.

**Measuring tissue guanosine 3',5'-cyclic monophosphate contents.** To determine if NO signaling is involved in the MPO-induced vascular injury response in high-glucose-incubated vessels, rat aortic rings were incubated in KHB with low (11.1 mmol/l) or high glucose (44 mmol/l) for 5 h. The vessels were then incubated with or without MPO (400 nmol/l) for 1 h and then washed. To be consistent with the vascular function study, the vessels were contracted with phenylephrine [ $(3 \times 10^{-8}) - 10^{-7} \text{ mol/l}$ ]. When tension development reached a plateau, either acetylcholine ( $3 \times 10^{-6} \text{ mol/l}$ ) or SNP ( $3 \times 10^{-6} \text{ mol/l}$ ) was added for 1 min. Vessel samples were collected at the basal condition (without any reagents) and after the addition of acetylcholine or SNP. Measurement of tissue cGMP was performed with a cGMP enzyme immunoassay kit (Biotrak; Amersham), according to the manufacturer's recommendation. Values for cGMP were standardized by tissue protein (mg). **Immunohistochemistry.** To obtain further direct evidence that vessel-bound MPO used high-glucose-stimulated  $H_2O_2$  to produce HOCL and chlorinating species in the vascular wall, we detected HOCL-modified LDL by immunohistochemistry (23). MPO is the only enzyme known to generate HOCL in vivo; thus, HOCL-modified LDL is a specific biomarker for the MPO/HOCL/chlorinating species pathway (23). In this experiment, the vessel segments from rat aortas were treated according to one of the following methods: 1) incubated with 1 mg/ml human native LDL and high glucose (44 mmol/l) for 4 h to allow

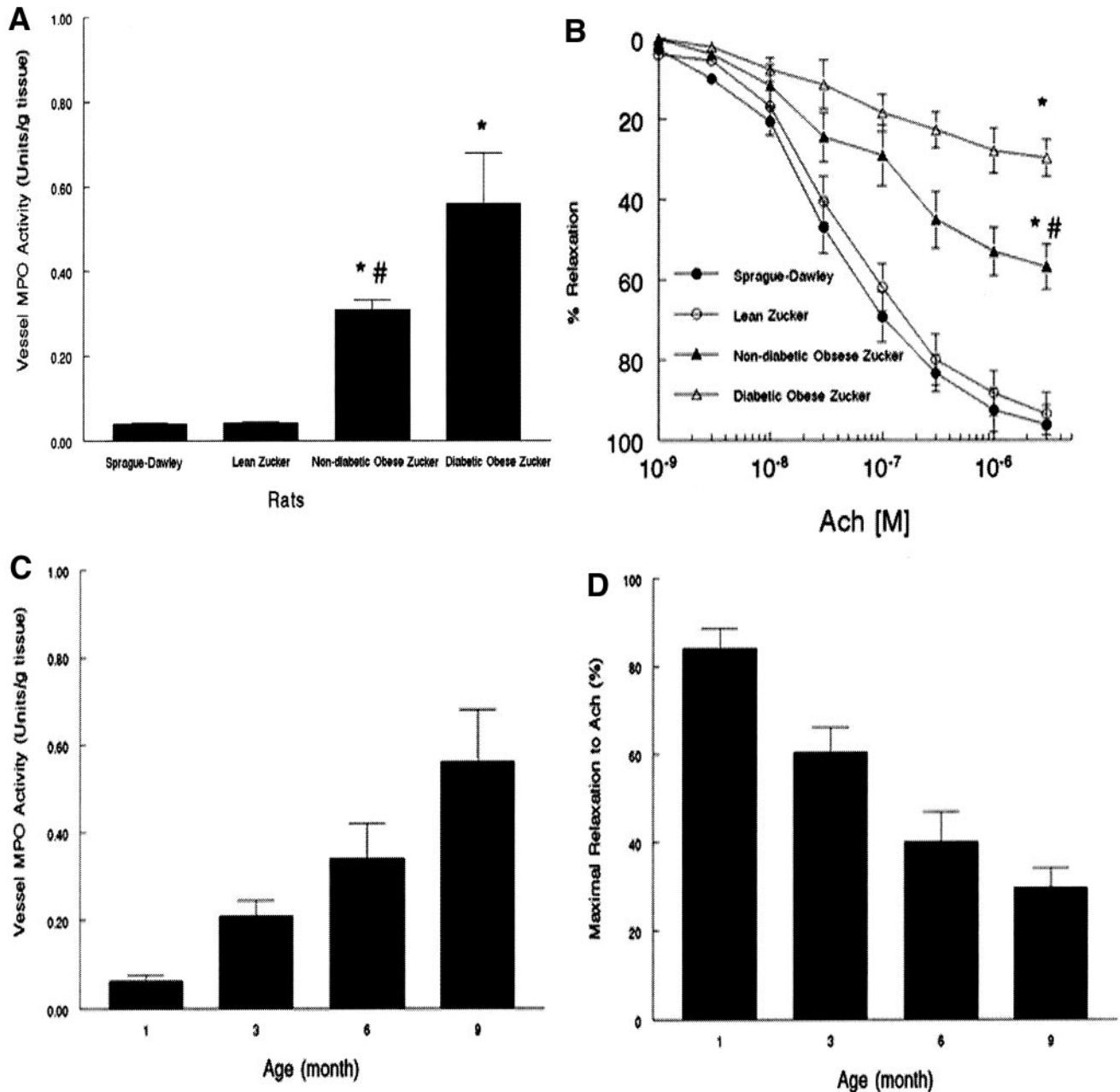


FIG. 1. MPO activity and endothelial dysfunction in diabetic vessels. Shown are the MPO activity (A) and endothelial function (B) in aortas from 9-month-old SD rats and nondiabetic lean, nondiabetic obese, and diabetic obese Zucker rats. The time course changes of MPO activity (C) and endothelial function (D) in aortas from diabetic Zucker rats are also shown. \* $P < 0.01$  vs. SD rats; # $P < 0.05$  vs. diabetic rats ( $n = 5$  for each group).

native LDL uptake into the vascular wall and to increase the production of high-glucose-stimulated  $H_2O_2$ , after which MPO (400 nmol/l) was added for 1 h; 2) not preincubated in any experimental reagents to serve as vehicle controls; 3) incubated with LDL alone for 5 h; 4) incubated with LDL and high glucose for 5 h; 5) incubated with MPO alone for 1 h; 6) incubated with LDL and low glucose (11.1 mmol/l) for 4 h, after which MPO was added for 1 h; 7) incubated directly with HOCL-modified LDL (1 mg/ml) for 1 h and used as positive controls (HOCL-modified LDL was prepared by reacting human native LDL [1 mg/ml] and HOCL [1 mmol/l]); or 8) incubated with MPO and exogenous  $H_2O_2$  (10  $\mu$ mol/l) to serve as additional positive controls. In addition, vessels without the primary antibody during the immunohistochemistry were used as negative controls. HOCL-modified LDL immunostaining was performed in paraffin-embedded vessel sections (5  $\mu$ mol/l) using the primary HOCL-modified LDL antibody (1:200 dilution for 30 min). A biotinylated anti-rabbit IgG secondary antibody was then applied. Immunostaining was detected using a Vector ABC kit. Sections were counterstained with hematoxylin.

**Statistics.** All data are presented as means  $\pm$  SE. Dosage-response profiles for different experimental conditions were analyzed using the Sigma Stat statistical analysis program. Unpaired observations were assessed by one-way ANOVA and multiple range tests.  $P < 0.05$  was required for significance.

## RESULTS

**Myeloperoxidase activity increases in diabetic vessels.** As shown in Fig. 1A, MPO activity in aortas from 9-month-old normal SD rats and nondiabetic lean Zucker rats was fairly low, suggesting there is almost no MPO in normal vessels. However, MPO activity in aortas from diabetic obese Zucker rats was significantly higher ( $P < 0.001$ ). In addition, MPO activity in vessels from nondiabetic obese Zucker rats was also higher than that from



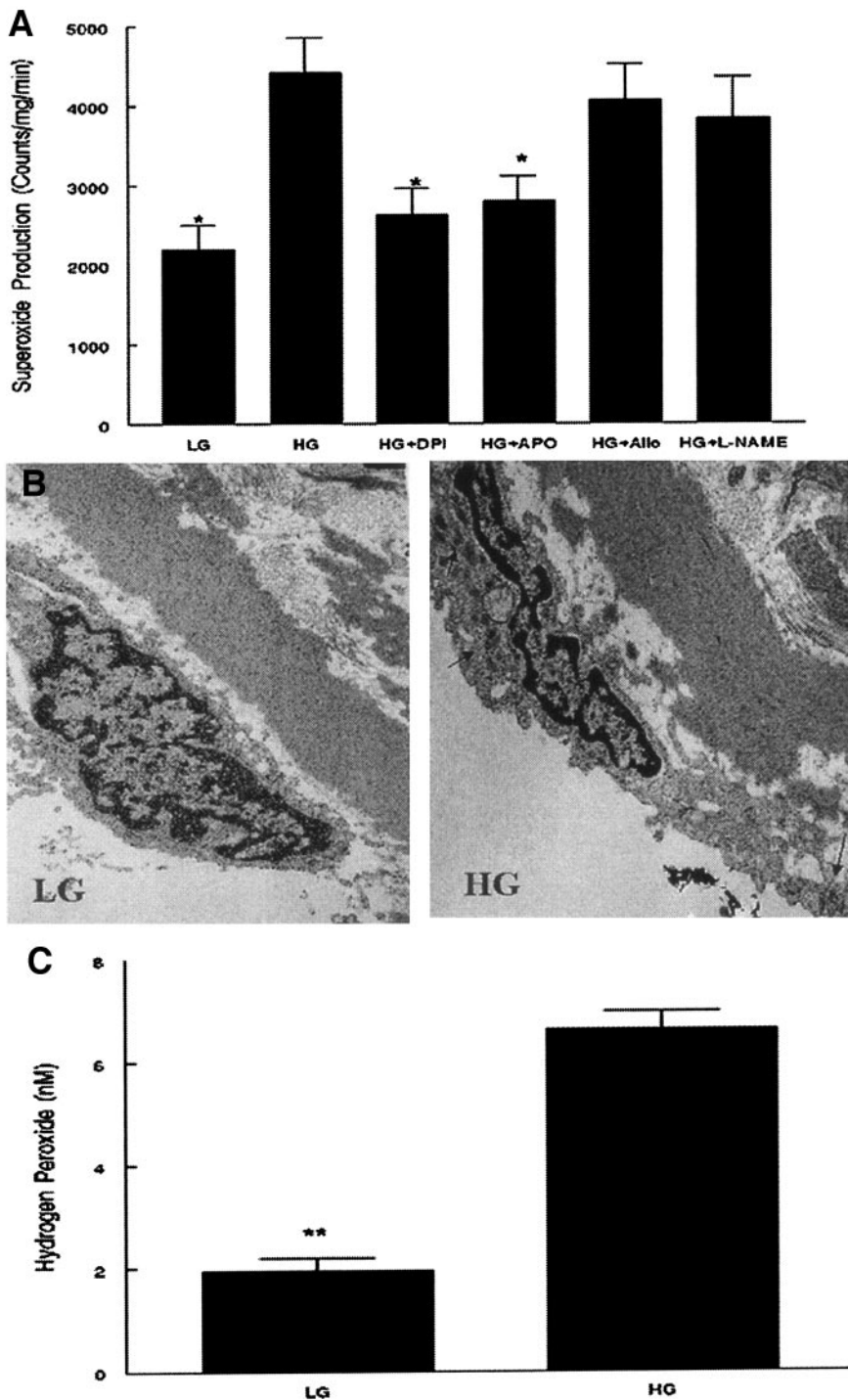


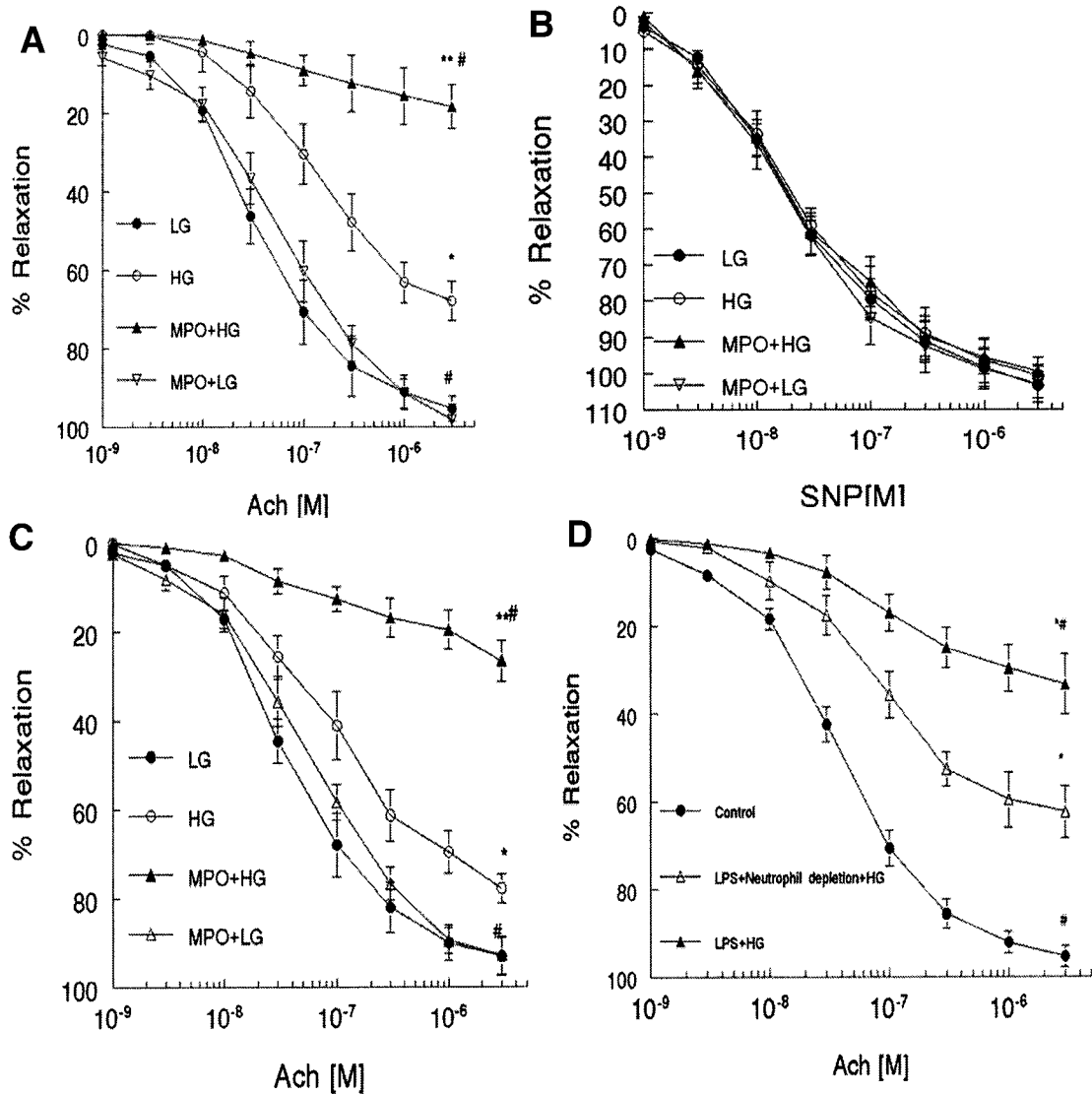
FIG. 2. Effect of high glucose on ROS production in the vascular wall. **A**: Superoxide production in rat aortic ring segments. Allo, allopurinol. **B**:  $H_2O_2$  production determined by transmission electron microscopy. **C**:  $H_2O_2$  production determined by Amplex Red assay. HG, high glucose; LG, low glucose. \* $P < 0.01$ , \*\* $P < 0.001$  vs. high-glucose group ( $n = 5$  for each group).

normal controls ( $P < 0.01$ ), although it was lower than in diabetic obese Zucker rats. Consistent with MPO activity, endothelial function was impaired in nondiabetic obese Zucker rats and further damaged in diabetic obese Zucker rats (Fig. 1B). The time course of changes in MPO activity and the maximal endothelium-dependent relaxation in aortas from diabetic obese Zucker rats are shown in Fig. 1C and D.

**High glucose stimulates reactive oxygen species formation in isolated vessel segments.** Our results showed that after vessels were incubated with high glucose for 5 h, vascular superoxide and  $H_2O_2$  were increased (Fig. 2).

There are three main potential sources of superoxide and  $H_2O_2$  in the vascular wall: vascular NAD(P)H oxidase, xanthine oxidase, and NO synthase. Our results indicated that vascular NAD(P)H oxidase is the predominant source of ROS in high-glucose-incubated vessels in vitro, as the xanthine oxidase inhibitor allopurinol or NO synthase inhibitor L-NAME had no significant effect on the increased production of superoxide, whereas the vascular NAD(P)H oxidase inhibitors diphenylene iodonium or apocynin reduced superoxide significantly (Fig. 2A).

**Exogenous and endogenous myeloperoxidase interacts with high-glucose-stimulated  $H_2O_2$  within the**



**FIG. 3.** Effects of MPO on vascular function of rat aortas. **A:** MPO amplified the high-glucose (HG)-induced endothelial dysfunction. **B:** MPO had no effect on endothelium-independent relaxation. **C:** Exposure to 7 h of 20 mmol/l glucose and 2 h MPO induced endothelial dysfunction similar to that of 6 h of 44 mmol/l glucose and 1 h MPO (A). **A–C:** \* $P < 0.01$ , \*\* $P < 0.001$  vs. LG; # $P < 0.05$  vs. high-glucose group. **D:** Endogenous MPO amplified high-glucose-induced endothelial dysfunction. \* $P < 0.01$  vs. control; # $P < 0.05$  vs. neutrophil depletion ( $n = 6$  for each group). LG, low glucose.

**vascular wall in vitro.** As shown in Fig. 3A, high glucose (44 mmol/l) partially impaired endothelium-dependent relaxation through increased ROS formation. Exogenous MPO had no notable effect on low-glucose-treated vessels; however, MPO significantly amplified the impairment effect on endothelium-dependent relaxation in high-glucose-pretreated vessels (Fig. 3A). In contrast, both high glucose and MPO had no injurious effect on endothelium-independent relaxation to SNP (Fig. 3B). A similar inhibitory effect on endothelial function was found after a 7-h exposure to 20 mmol/l glucose and a 2-h exposure to MPO (Fig. 3C). Endogenous MPO also amplified high-glucose-induced endothelial dysfunction, demonstrating that neutrophil depletion attenuated vascular-bound MPO activity (data not shown) and high-glucose-induced impairment of endothelium-dependent relaxation (Fig. 3D).

The  $H_2O_2$  scavenger catalase, the HOCL scavenger L-methionine, and vitamin C prevented the injury response (Fig. 4A). Although coincubation with HB-SOD totally

blocked high-glucose-induced endothelial dysfunction, when present, MPO might compete with catalase in catalyzing their common substrate  $H_2O_2$  to produce HOCL and its chlorinating species, thereby reducing the protective effect of SOD (Fig. 4B). However, HB-SOD still had some protective effect when it was compared with MPO in the high-glucose-treated group without HB-SOD, possibly because of its reduction effect on superoxide (Fig. 4A and C). This result suggests that the mechanism by which MPO induces additional injury on endothelium-dependent relaxation is the reaction with high-glucose-stimulated  $H_2O_2$ , which produces HOCL and its chlorinating species. Because vascular NAD(P)H oxidase is the main source of high-glucose-stimulated  $H_2O_2$ , aortas from NAD(P)H oxidase subunit gp91<sup>phox</sup>-deficient mice were therefore used to confirm the novel injury pathway. As shown in Fig. 4C, MPO and high-glucose-induced endothelial dysfunction were significantly attenuated in vessels without the NAD(P)H oxidase subunit gp91<sup>phox</sup>. It should be noted that

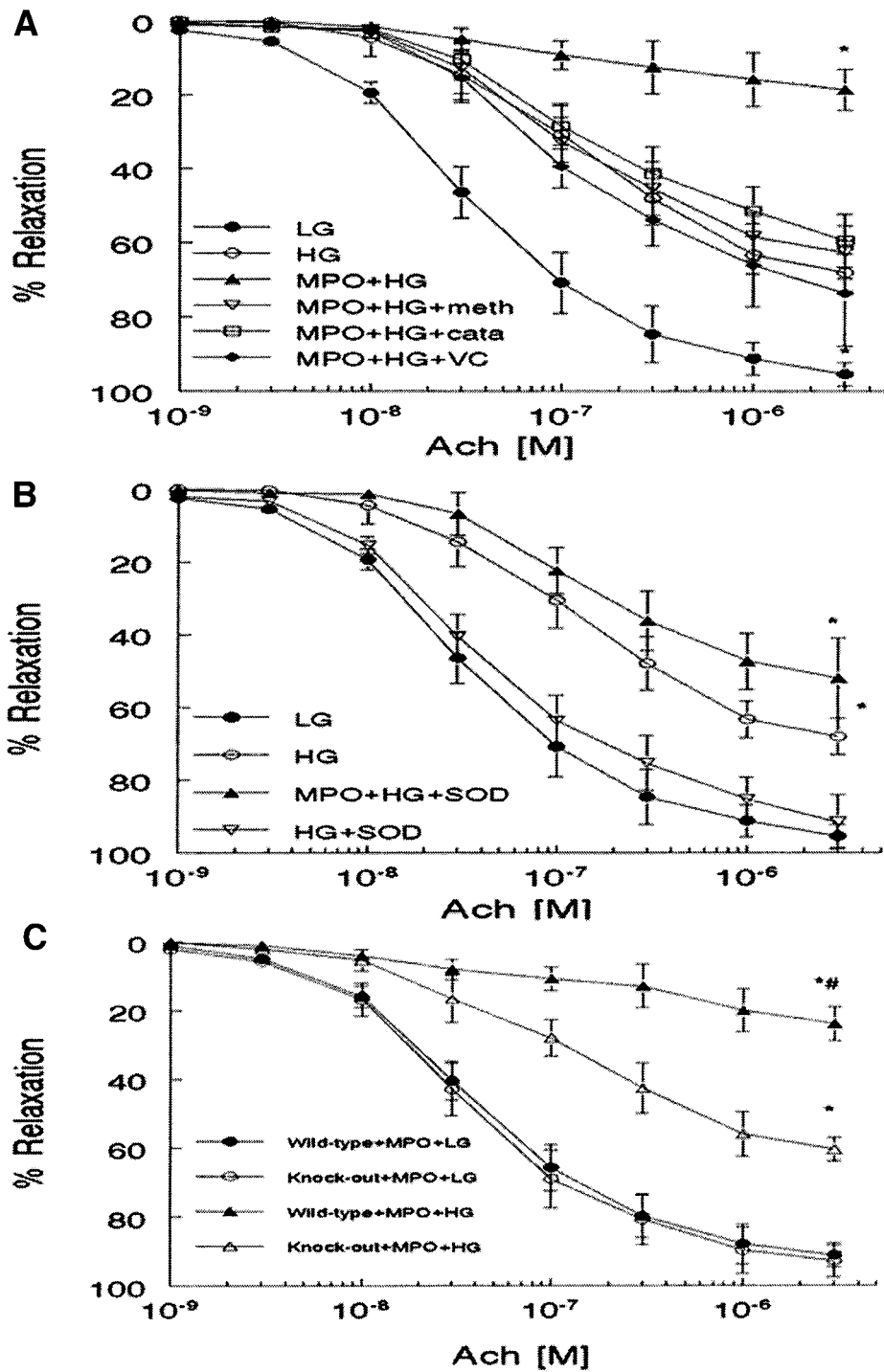


FIG. 4. Role of MPO/H<sub>2</sub>O<sub>2</sub>/HOCl/chlorinating species pathway in MPO-induced vascular impairment. **A:** Catalase (cata), L-methionine (meth), and vitamin C (VC) blocked the MPO-induced endothelial dysfunction. \**P* < 0.01 vs. high-glucose (HG) group (*n* = 6). **B:** Effect of HB-SOD on endothelial function. \**P* < 0.01 vs. vehicle (*n* = 6). **C:** NAD(P)H oxidase deficiency attenuated MPO-induced endothelial dysfunction. \**P* < 0.01 vs. low glucose (LG); #*P* < 0.05 vs. wild-type high-glucose group (*n* = 6).

MPO and high-glucose-induced endothelial dysfunction cannot be completely abolished by NAD(P)H oxidase subunit gp91<sup>phox</sup> deficiency, because the NAD(P)H oxidase subunit gp91<sup>phox</sup> is not the only NAD(P)H oxidase in the vascular wall.

**Myeloperoxidase amplifies high-glucose-induced impairment of endothelium-dependent relaxation by reducing NO bioavailability.** To test whether MPO-induced amplification of high-glucose-elicited impairment of vascular function is related to NO signaling, cGMP production in the vascular wall was measured. As shown in Fig. 5A, a 6-h incubation with high glucose slightly

decreased cGMP production in vessels without agonist stimulation, but the difference did not reach significance (*P* > 0.05). However, high glucose decreased cGMP production by 59% in acetylcholine-stimulated vessels as compared with low-glucose-incubated vessels (*P* < 0.01). The above results were consistent with those from previous reports (33). In acetylcholine-stimulated vessels, MPO amplified the high-glucose-induced inhibitory effect on cGMP production (*P* < 0.01). In contrast, MPO had no significant effect on acetylcholine-stimulated cGMP production in vessels preincubated with low glucose (*P* > 0.05). Consistent with our vascular function measurement,

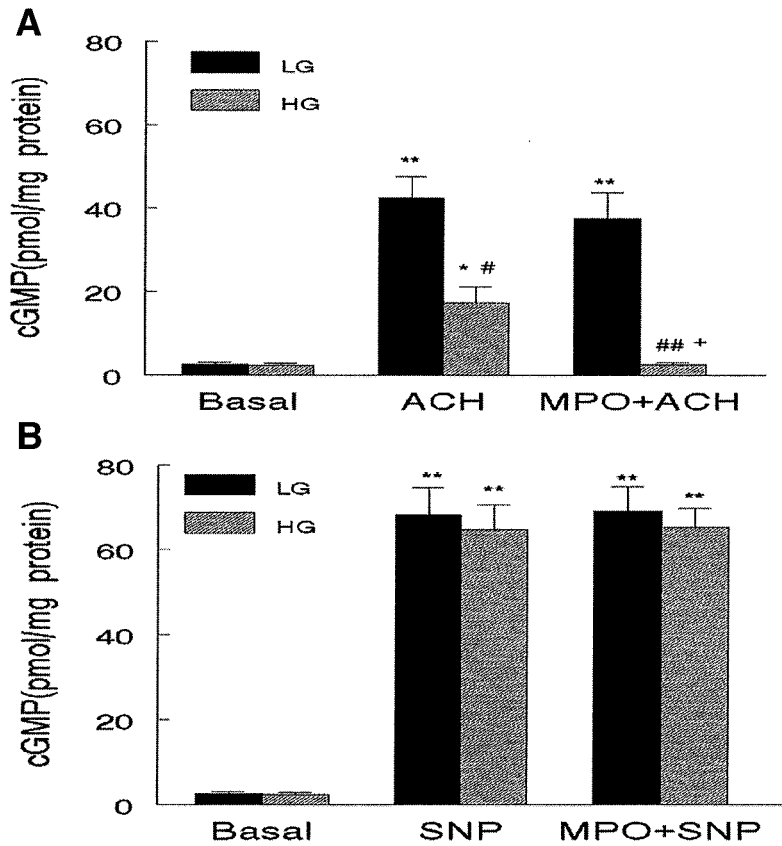


FIG. 5. Effects of MPO on cGMP production of isolated rat aortas incubated with low glucose (LG) or high glucose (HG). *A*: Aorta cGMP production with and without (basal) stimulation with acetylcholine. \* $P < 0.01$ , \*\* $P < 0.001$  vs. basal; # $P < 0.01$ , ## $P < 0.001$  vs. low glucose; + $P < 0.01$  for MPO + ACH + high glucose vs. ACH + high glucose ( $n = 5$ ). *B*: Aorta cGMP production with and without stimulation with SNP. \*\* $P < 0.001$  vs. basal ( $n = 5$ ).

high glucose and MPO had no effect on SNP-stimulated cGMP production (Fig. 5*B*). The results suggest that the MPO-amplified high-glucose-induced impairment on vascular endothelium-dependent relaxation resulted from the reduced NO bioavailability.

**HOCL-modified LDL is detected in LDL and high-glucose-preincubated vascular wall containing myeloperoxidase.** Our results showed that there was no HOCL-modified LDL staining in the negative control (Fig. 6*A*) or vehicle-treated (Fig. 6*B*) vessels or in vessels incubated with LDL alone (Fig. 6*C*), high-glucose + LDL (Fig. 6*D*), MPO alone (Fig. 6*E*), or LDL + MPO + low glucose (Fig. 6*F*). In sharp contrast, the vessels treated with LDL + MPO + high glucose, which showed LDL, high-glucose-stimulated vascular non-leukocyte oxidase-derived  $H_2O_2$ , and MPO in the vascular wall, had strong immunostaining (Fig. 6*G*). The HOCL-modified LDL was located in the intima and, to a lesser extent, in the media (Fig. 6*G*), a situation similar to that seen in the two positive controls (Fig. 6*H* and *I*).

**Myeloperoxidase amplifies the hyperglycemia-induced impairment of endothelium-dependent relaxation in animals with acute hyperglycemia.** After hyperglycemia was induced in vivo and the carotid artery was incubated with MPO, the endothelium-dependent relaxation of the rat carotid artery was determined. Consistent with our in vitro experiment, hyperglycemia partially inhibited acetylcholine-induced, endothelium-dependent relaxation (Fig. 7). Although MPO had no significant effect on vascular function in carotid arteries from rats without hyperglycemia, MPO amplified the impairment of endothelium-dependent function induced by hyperglycemia ( $P < 0.05$ ) (Fig. 7).

## DISCUSSION

Diabetic vascular complication is one of the important causes of atherosclerosis, myocardial infarction, stroke, and limb amputations. Although endothelial dysfunction is well known as a key feature of diabetes and is thought to be a major cause of diabetes-associated vascular complications, the molecular basis of endothelial dysfunction in diabetes is not well understood.

Recent advances in diabetic research suggest that ROS play a key role in hyperglycemia-mediated endothelial dysfunction and vascular complications (34). Both animal and clinical studies have proven that the major source of ROS in diabetic vasculature is the vascular nonleukocyte oxidases such as NAD(P)H oxidases (3,4). Our results confirmed that high glucose increased vascular ROS production and that vascular NAD(P)H oxidase was the main source of that increased production. We also found that high glucose impaired endothelial function by reducing NO bioavailability, a result consistent with those from previous reports (34).

MPO is a leukocyte-derived heme protein. Recent studies have shown that MPO plays an important role in endothelial dysfunction (22–27,30). We found that MPO-mediated endothelial dysfunction is  $H_2O_2$  dependent (23). More importantly, MPO-derived HOCL and chlorinating species amplify  $H_2O_2$ -induced vascular injury (23). It is well known that MPO can use leukocyte NAD(P)H oxidase-derived  $H_2O_2$ ; however, leukocyte-derived NAD(P)H oxidase can be activated to produce  $H_2O_2$  only in respiratory bursts. When the respiratory burst is stopped, the activated leukocyte-derived  $H_2O_2$  disappears quickly (4-min half-life) (35). In contrast, the activated leukocyte-



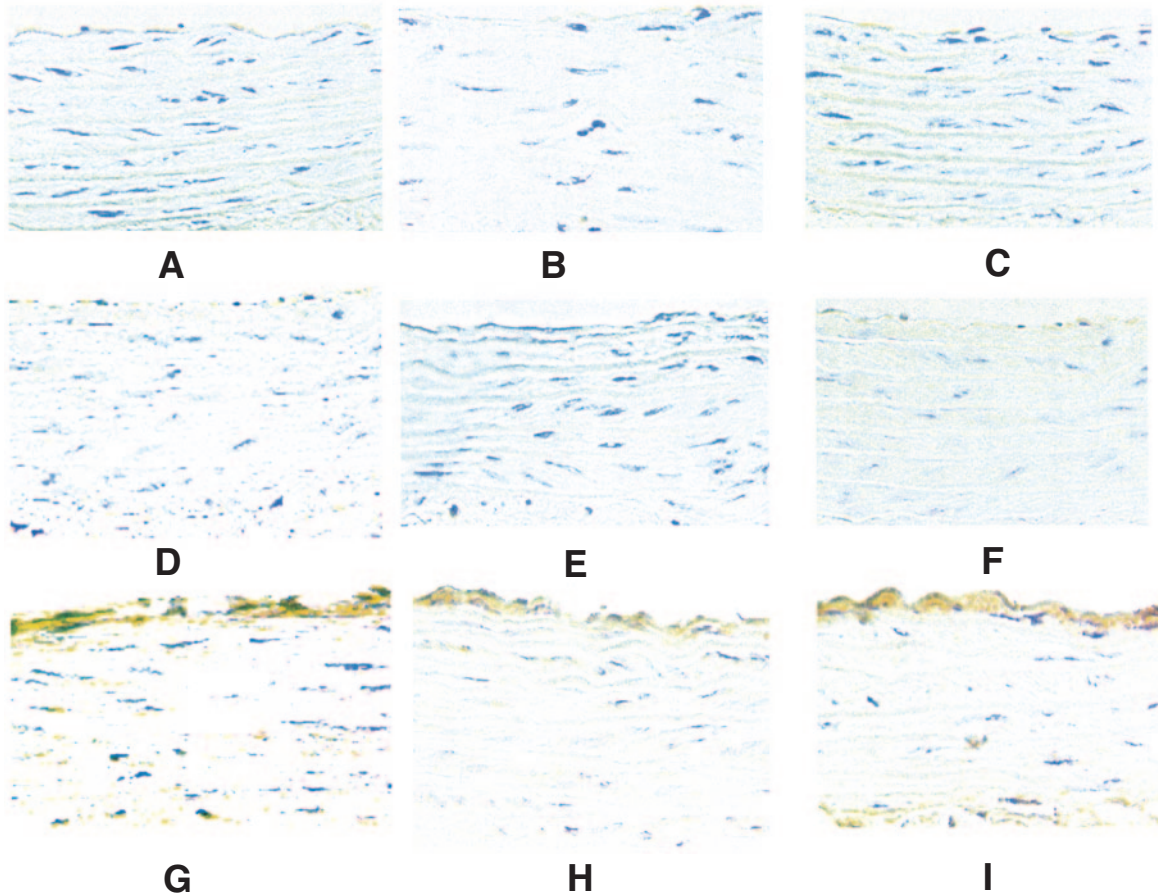


FIG. 6. Immunostaining of HOCL-modified LDL in the vascular wall. Shown are negative control (A); vehicle-treated vessel (B); vessel incubated with LDL (C); vessel incubated with high glucose and LDL (D); vessel incubated with MPO (E); vessel incubated with LDL, low glucose, and MPO (F); vessel incubated with LDL, high glucose, and MPO (G); vessel incubated with MPO and  $H_2O_2$  (positive control; H); and vessel incubated with HOCL-modified LDL (positive control; I).

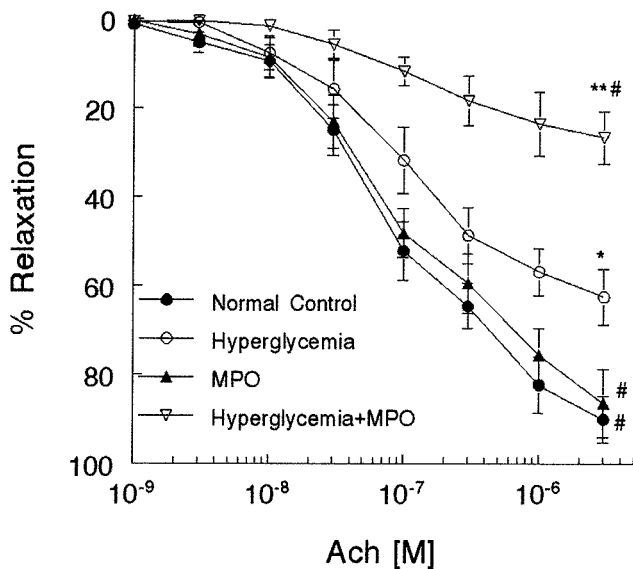


FIG. 7. Effect of MPO on endothelial function in carotid arteries from rats with and without acute hyperglycemia. The endothelium-dependent relaxation was determined in carotid arteries from rats with and without hyperglycemia (control). Some carotid arteries from animals with and without hyperglycemia were incubated with MPO (400 nmol/l) *in vivo* for 1 h. \* $P < 0.05$ , \*\* $P < 0.01$  vs. normal control; # $P < 0.05$  vs. hyperglycemia group.

released MPO is bound to the vascular wall for several days (23). In our study, we observed that MPO activity in diabetic rat vessels is markedly increased and found a positive correlation between MPO activity and endothelial dysfunction in diabetic animals. Recent reports have suggested that vascular nonleukocyte NAD(P)H oxidase-derived ROS such as  $H_2O_2$  are increased in diabetic vessels. If vascular-bound MPO can use vascular nonleukocyte-derived  $H_2O_2$  to produce HOCL and reactive chlorinating species in diabetic vessels, this could be a novel, important vascular injury pathway.

We found for the first time that vascular-bound MPO can use the high-glucose-stimulated  $H_2O_2$  to amplify ROS-induced vascular dysfunction. There are two possible pathways that may be involved in MPO-mediated injury on endothelial function: consumption of NO by MPO in the presence of  $H_2O_2$  (22,24) and reaction with high-glucose-stimulated  $H_2O_2$  to produce HOCL and its chlorinating species (23,25,26,30) and in turn reduce NO bioavailability.

The following observations discount, but do not completely exclude, a role of NO consumption in MPO-induced endothelial dysfunction at the tissue level. 1) Although we tried in earlier research to use Cl-free buffer to test the effect of MPO plus  $H_2O_2$  on endothelial function (22), intra- and extracellular space in the vascular wall still had enough chloride that it was not truly free of Cl. 2)  $H_2O_2$  has



a very short half-life (~4 min) in the vascular wall; however, MPO plus H<sub>2</sub>O<sub>2</sub>-induced endothelial dysfunction cannot be reversed several hours after washing. This finding suggests that some new biomolecules formed from MPO and H<sub>2</sub>O<sub>2</sub> may be responsible for the endothelial dysfunction, whereas HOCL is a physiological product from MPO and H<sub>2</sub>O<sub>2</sub>. HOCL is a highly reactive product that will react with other biomolecules such as amino acids. 3) HOCL scavengers and MPO inhibitors can totally block MPO plus H<sub>2</sub>O<sub>2</sub>-induced endothelial dysfunction, although some effects may be nonspecific (23). 4) MPO plus H<sub>2</sub>O<sub>2</sub> has no effect on another NO donor, SNP-induced relaxation. 5) We found that vitamin C accelerates MPO-dependent NO consumption; however, MPO-induced endothelial dysfunction is inhibited by vitamin C (22,23). 6) HOCL-modified LDL was detected in vessels with MPO and H<sub>2</sub>O<sub>2</sub>. Therefore, vascular-bound MPO/high-glucose-stimulated H<sub>2</sub>O<sub>2</sub>/HOCL/chlorinating species might be the main injury pathway involved in MPO-mediated endothelial dysfunction in diabetic vessels. The main limitation is that HOCL-modified LDL is a biomarker for semiquantitative analysis and localization of MPO-catalyzed chlorination in the vascular wall. Quantification of MPO-mediated chlorinated products using methods such as mass spectrometry in diabetic vessels will be needed to further determine the role of this novel injury pathway in the development of diabetic endothelial dysfunction.

In addition to the role of increased production of ROS, such as H<sub>2</sub>O<sub>2</sub>, and the reduced ability of anti-ROS in diabetic vessels, our results indicate that reaction of vascular-bound MPO with H<sub>2</sub>O<sub>2</sub> to produce a more potent vascular injury species, such as HOCL and chlorinating species, might be an important injury pathway in diabetic vascular damage. This new injury pathway could also be the main link between systemic inflammation and the exacerbation of diabetic vascular diseases.

In the current study, we also found that MPO activity was increased in vessels from nondiabetic obese Zucker rats. This result is consistent with those of a previous study (36) in which the increased leukocyte adhesion to vasculature was found in this kind of rat. Nondiabetic obese Zucker rats are not hyperglycemic; however, these rats are insulin resistant and display endothelial dysfunction. The accurate role of MPO in endothelial dysfunction in nondiabetic obese Zucker rats needs to be further studied.

In summary, vascular-bound MPO could use high-glucose-stimulated H<sub>2</sub>O<sub>2</sub> to amplify high-glucose-induced injury in the vascular wall. Because H<sub>2</sub>O<sub>2</sub> and vascular-bound MPO are increased in diabetic vessels, MPO/H<sub>2</sub>O<sub>2</sub>/HOCL/chlorinating species may represent an important pathway in diabetes complications and a new mechanism in phagocyte- and systemic infection-induced exacerbation of diabetic vascular diseases.

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