Hypochlorous Acid Impairs Endothelium-Derived Nitric Oxide Bioactivity Through a Superoxide-Dependent Mechanism

Roland Stocker, Annong Huang, Erin Jeranian, Jing Yun Hou, Tina T. Wu, Shane R. Thomas, John F. Keaney, Jr

Objective—To determine how hypochlorous acid (HOCl), the principal product of myeloperoxidase, modulates vascular function.

Methods and Results—Rabbit arterial rings exposed to HOCl (0 to 500 μmol/L) exhibited dose- and time-dependent impairment of endothelium-dependent arterial relaxation to acetylcholine and A23187, but not the NO donor, diethylenetriamine NONOate, suggesting that HOCl targets the endothelium. This effect was not because of cytotoxicity, as HOCl treatment produced no significant change in endothelial cell morphology or lactate dehydrogenase release. We observed HOCl-mediated endothelial cell protein oxidation by immunoreactivity to HOP-1, a monoclonal antibody specific for HOCl-oxidized protein. In support of this notion, known HOCl scavengers, such as methionine and N-acetylcysteine, partially preserved endothelium-derived NO bioactivity in response to HOCl. In an unanticipated observation, HOCl-mediated impairment of NO bioactivity was prevented by manganese superoxide dismutase in a manner dependent on its enzymatic activity. Finally, we found that HOCl reduced endothelial nitric oxide synthase dimer stability, an effect that was also inhibited by superoxide dismutase.

Conclusions—Taken together, these data indicate that HOCl imparts a defect in endothelial NO production due to a superoxide-dependent reduction in endothelial nitric oxide synthase dimer stability. These data provide another mechanism whereby myeloperoxidase-derived oxidants can contribute to the impairment of NO bioactivity that is characteristic of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2004;24:2028-2033.)

Key Words: oxidant stress • nitric oxide • endothelium • superoxide

Atherosclerosis and its associated pathological conditions, such as hypercholesterolemia and diabetes mellitus, are characterized by abnormal endothelium-derived nitric oxide (EDNO) bioactivity. In atherosclerosis, vasodilatation to agonists for EDNO release, such as acetylcholine or aggregating platelets, is impaired or replaced by constriction. Endothelial dysfunction has been directly demonstrated in atherosclerotic human coronary arteries, and there is strong evidence that it contributes to plaque activation and the pathogenesis of myocardial ischemia. In fact, the presence of vascular dysfunction predicts the occurrence of clinical events in patients with atherosclerosis.6

Available evidence suggests that vascular oxidative events play an important role in endothelial dysfunction. In animal models of atherosclerosis and hypercholesterolemia, the vascular production of superoxide anion radical is increased, and this abnormality can decrease NO bioactivity through peroxynitrite formation. Indeed, limiting superoxide production with oxypurinol, a xanthine oxidase inhibitor, partially restores EDNO bioactivity.7 Similarly, in atherosclerotic rabbits, chronic treatment with polyethylene glycolated-superoxide dismutase (SOD) increases vascular SOD activity and partially restores NO-mediated relaxation to acetylcholine.9 Later stages of atherosclerosis, however, are more complex. In Watanabe heritable hyperlipidemic rabbits, impaired EDNO bioactivity is not restored by increasing endothelial SOD levels.10 These observations suggest other oxidative events could contribute to endothelial dysfunction.

Among oxidants produced in atherosclerotic blood vessels, hypochlorous acid (HOCl) is of particular interest. Under physiological conditions, HOCl is the major product of myeloperoxidase (MPO),11 a heme enzyme both present and active in human atherosclerotic lesions.12 The MPO in human atherosclerotic vessels is often closely associated with endothelial cells,13 consistent with reports that MPO binds to the endothelium and is transcytosed to its abluminal side,14 where
it can catalytically consume NO. There is also evidence that HOCI-mediated oxidation occurs within endothelial cells based on immunostaining with a monoclonal antibody specific for HOCl-modified proteins. The purpose of this study was to examine the effect of HOCI on endothelial function determined by the effect of HOCI on endothelial function.

**Materials and Methods**

Cell culture reagents were obtained from Life Technologies Inc. Reagent HOCI was obtained from Fischer Scientific. Diethylylamine NONOate (DEANO) was purchased from Cayman Chemical. [3H]-l-arginine (1 mCi, 53 mCi/mmol) was obtained from New England Nuclear, and cGMP assay kits were from Cayman Chemical. All other compounds were purchased from Sigma Chemical Co. Physiological salt solution (PSS) contained 118.3 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 1.2 mmol/L MgSO₄, 1.2 mmol/L KH₂PO₄, 25 mmol/L NaHCO₃, 11.1 mmol/L glucose, 10 μmol/L indomethacin, and 0.026 mmol/L Na₂EDTA. PBS consisted of 10 mmol/L NaH₂PO₄, 0.15 mol/L NaCl, pH 7.4.

**In Vitro Assay of Vascular Function**

New Zealand White rabbits (2.5 to 3.5 kg) of either sex (12 male, 8 female; Pine Acres Rabbity, Norfolk, Mass) were used for this study, and all animal studies were approved by the Boston University Medical Center Institutional Animal Care and Use Committee. The thoracic aorta was isolated from rabbits, and vessel segments were prepared and vascular function assayed as described previously. To determine the effect of HOCI on vascular function, equilibrated (90 minutes) vessels were incubated in glucose-free PSS with HOCI. After incubation, vessels were washed with glucose-containing PSS and relaxation tests as above. Glucose-free PSS was used to simplify the experimental system and control for any reaction between extracellular glucose and HOCI. Preliminary experiments demonstrated that incubation for up to 60 minutes in glucose-free PSS had no effect on arterial relaxation (data not shown). Later experiments subsequently demonstrated that similar results were observed with and without glucose (data not shown). Antioxidants such as SOD, N-acetylcysteine, uric acid, ascorbic acid, and methionine were added to organ chambers as a 1:1000 dilution just before HOCI exposure and replaced after washing away the HOCI.

**Scanning Electron Microscopy**

Segments of thoracic aorta were harvested as above and incubated in glucose-free PSS (1 mL) or glucose-free PSS containing 500 μmol/L HOCI for 5 to 60 minutes under conditions of continuous oxygenation. After incubation, vessels were washed with PBS, fixed in 10% PBS-buffered formalin, and prepared for electron microscopy as described. Samples were then coated with gold and palladium and observed in an AMR 1000Å scanning electron microscope (AMRAY).

**Assessment of Lactate Dehydrogenase Release**

Segments of thoracic aorta were isolated as above, and placed with an open end down in a Petri dish so as to form a "well" with endothelium lining the sides of the well. The vessel segments were then filled with PSS (50 μL) with or without HOCI for various periods of time. After incubation, the PSS was collected and analyzed for lactate dehydrogenase (LDH) content using a spectrophotometric assay as described. Preliminary experiments indicated that HOCI had no effect on the assay at concentrations <1 mmol/L.

**Assessment of HOCI-Modified Proteins**

Segments of thoracic aorta were isolated and incubated in PSS containing HOCI with or without antioxidants as described above. After incubation, vessels were either snap-frozen in liquid nitrogen or fixed in 10% buffered formalin as described. Immunostaining for HOCl-modified proteins was performed using HOP-1 essentially as described.

**Cell Culture and Assessment of Endothelial Nitric Oxide Synthase Activity**

Porcine aortic endothelial cells (PAECs) were harvested and cultured essentially as described. For experiments, PAECs were used between passages 3 to 10 and were washed twice in HEPES-buffered physiological salt solution (HPSS) as described and incubated in HPSS with HOCI as indicated in the text.

**Statistical Analysis of Data**

All values are presented as a mean ± SEM. The vascular responses to acetylcholine, A23187, and DEANO are reported as the percent reduction in tension (relaxation) compared with the contraction produced by 1 μmol/L phenylephrine. Dose responses to these agents were compared within treatment groups with repeated-measures ANOVA, and responses between treatment groups were compared by 2-way ANOVA with a post hoc Dunn or Dunnet test as appropriate. Statistical significance was accepted if the null hypothesis was rejected with a P < 0.05.

**Results**

**HOCI and Vascular NO Bioactivity**

We observed that HOCI at a plausible pathophysiological concentration of 100 μmol/L produced time- and concentration-dependent inhibition of acetylcholine-mediated arterial relaxation (Figure 1A and 1D; P < 0.001). We observed a similar effect of HOCI when EDNO production was stimulated with the receptor-independent agonist, A23187 (Figure 1B and 1E; P < 0.001). To determine whether this effect was related to smooth muscle cell dysfunction, we examined the effect of HOCI on exogenous NO administered as DEANO, a direct NO donor. As shown in Figure 1C and 1F, HOCI had no material effect on smooth muscle cell responsiveness to NO, suggesting that HOCI selectively impairs endothelial function under these conditions.

**HOCI and Endothelial Cell Viability**

To determine whether the effect of HOCI was related to morphological alterations in the endothelium, we performed scanning electron microscopy from vessels treated as in Figure 1. Incubation of vessel segments with 500 μmol/L HOCI for up to 60 minutes produced no visible change in endothelial cell morphology (Figure 2). We next tested the effect of HOCI cell viability using LDH release from vessel segments. As shown in Figure 3, vessel segments incubated with 50 to 500 μmol/L HOCI did not demonstrate significant
LDH release compared with those incubated with buffer alone. Thus the incubation conditions used in this study were not associated with significant loss of endothelial cell morphology or viability.

Arterial HOCl Exposure Leads to Protein Modification in the Endothelium

To determine the effect of HOCl exposure on protein modification, we examined immunoreactivity to the monoclonal antibody, HOP-1.23,24 We found that control aortic segments demonstrated no HOP-1 immunoreactivity (Figure 4A), whereas segments exposed to 500 μmol/L HOCl produced striking HOP-1 staining in the endothelium with virtually no staining in the media (Figure 4B). Segments stained with irrelevant or without primary antibody demonstrated no HOP-1 immunoreactivity (data not shown). This immunoreactivity was abrogated by the inclusion of HOCl-modified BSA during immunostaining (Figure 4C). Thus aortic segments exposed to HOCl demonstrate HOCl-modified proteins in the endothelium in preference to the medial layer of the arterial wall.

HOCl and EDNO Bioactivity in the Endothelium

To determine the implications of HOCl-mediated protein modification in the endothelium, we incubated PAECs with HOCl and determined both NO bioactivity and eNOS catalytic activity. Exposure of PAECs to HOCl produced a dose-dependent decrement in EDNO bioactivity (Figure 5A) and eNOS activity (Figure 5B) determined as cGMP accumulation and the conversion of [3H]-l-arginine to [3H]-l-citrulline, respectively. These data suggest that HOCl impairs endothelial cell production of NO.

HOCl-Mediated Endothelial Dysfunction and Antioxidants

Because the extracellular milieu normally contains antioxidant enzymes and low-molecular-weight antioxidant species, we sought to characterize effective antioxidant protection against HOCl-mediated endothelial dysfunction. We found that uric acid and ascorbate, the 2 most abundant water-soluble...
antioxidants in plasma and extracellular fluids, afforded little protection against HOCl-mediated endothelial dysfunction (Figure IA and IB, available online at http://atvb.ahajournals.org). In contrast, the sulfur-containing amino acids methionine and N-acetylcysteine offered considerable protection against HOCl regardless of the agonist used to stimulate EDNO (Figure I). These findings are in keeping with the facile reaction of HOCl with the amino acid residues cysteine and methionine as described.17 Vessel incubation and staining involved no HOCl exposure (A), HOCl and stained with HOP-1 (B), and HOCl and HOP-1 staining in the presence of 320 μg/mL HOCl-modified BSA (C).

To determine the effect of enzymatic antioxidants on HOCl-induced endothelial dysfunction, we tested the effect of catalase and SOD in this system. We found that catalase offered no protection against HOCl-mediated impairment of endothelial function (data not shown). In contrast, SOD (150 IU/mL) offered considerable protection against the deleterious effects of HOCl on NO-mediated arterial relaxation (Figure 6A). This protection was dependent on enzymatic activity, as heat-inactivated SOD did not alter the effect of HOCl (Figure 6A). We observed no protection when SOD was added after exposure of the vessel segment to HOCl (data not shown), suggesting a specific role for superoxide rather than nonspecific scavenging of HOCl by the protein during the time of oxidant exposure of the vessel segment. Thus it appears the deleterious effect of HOCl on the endothelium is superoxide-dependent.

Figure 4. Effect of HOCl on protein modification in the arterial wall. Segments of rabbit aorta were harvested as in Figure 1 and incubated with vehicle alone (A) or with 500 μmol/L HOCl for 30 minutes (B and C) in glucose-free PSS. After incubation, segments were washed with PSS, fixed in 4% paraformaldehyde, and prepared for immunohistochemistry and light microscopy as described.17 Vessel incubation and staining involved no HOCl exposure (A), HOCl and stained with HOP-1 (B), and HOCl and HOP-1 staining in the presence of 320 μg/mL HOCl-modified BSA (C).

Figure 5. HOCl and endothelial NO bioactivity. Confluent monolayers of PAECs were exposed to the indicated concentrations of HOCl in PSS for 30 minutes. Cells were washed and incubated with 1 μmol/L A23187 for 5 minutes and assayed for NO bioactivity and eNOS catalytic activity as intracellular cGMP accumulation (A) and [³H]-L-citrulline production (B), respectively. A23187 stimulation increased PAEC cGMP from 6±6 pmol/mg protein to 48.3±4.8 pmol/mg protein. Data represent mean±SEM of 3 to 5 experiments, *P<0.05 for the effect of HOCl vs PSS alone by 1-way ANOVA.

Figure 6. Enzymatic antioxidants and HOCl-induced endothelial dysfunction. A, Segments of thoracic aorta were harvested from New Zealand White rabbits as described in Methods. Vessels were suspended in organ chambers and incubated for 30 minutes with glucose-free PSS alone (●) or glucose-free PSS with 200 μmol/L HOCl with no additions (○), 150 IU/mL SOD (≈40 μg/mL protein; ⊹), or 150 IU/mL heat-inactivated SOD (○). After incubation, relaxation was assayed in response to the indicated concentrations of acetylcholine. Data represent mean±SEM of 6 to 8 experiments, *P<0.001 vs no CTL by 2-way ANOVA. B, Confluent monolayers of PAECs were exposed to the indicated concentrations of HOCl in PSS for 10 minutes with or without Mn-SOD (150 IU/mL) or its heat-inactivated control (HIMn-SOD). Cells were washed, lysed at 4°C, and eNOS dimer stability assessed using low-temperature SDS-PAGE and immunoblotting as described in Methods. Blots are representative of 6 to 8 independent experiments. C, Composite densitometric data for cells treated in buffer alone (●), Mn-SOD (○), or HIMn-SOD (△). Data are mean±SEM from 6 to 8 experiments; *P<0.05 by 1-way ANOVA.

Discussion
The data presented here demonstrate that blood vessels exposed to HOCl, the principal product of MPO, exhibit a defect in EDNO bioactivity manifest as impaired endothelium-
dependent arterial relaxation. Calculations of oxygen consumption in activated neutrophils suggest that low millimolar concentrations of HOCl are achievable in vivo.22 Considering that diffusion to nearby endothelial cells will diminish the HOCl concentration, our choice of HOCl concentrations ≤500 μmol/L seems reasonable. Therefore, the data contained in this study are consistent with the notion that HOCl could contribute to impaired NO bioactivity in vivo. A surprising observation of this study was that HOCl-mediated impairment of NO bioactivity could be prevented by manganese SOD. We also found that HOCl induced a reduction in eNOS dimer stability, an effect also inhibited by SOD. Taken together, these data indicate that HOCl imparts a defect in endothelial NO production that is dependent, in part, on superoxide production.

It is plausible that MPO-derived oxidants, such as HOCl, may represent an important source of oxidative stress in atherosclerosis. For example, both MPO protein and activity are present in human lesions,12,21 Atherosclerotic lesions and lesion-derived low-density lipoprotein (LDL) contain epitopes recognized by HOP-1, consistent with HOCl-mediated protein oxidation as a feature of atherosclerosis.33 Human lesion LDL also contains 3-chlorotyrosine34 and p-hydroxyphenylacetaldehyde-modified phospholipids.35 2 chemical markers indicative of HOCl production.14 In addition, MPO is found in endothelial cells in vitro,37 and immunohistochemical evidence indicates that human lesion LDL contains epitopes recognized by MPO.13 Cell-bound MPO becomes transcytosed and the endothelium overlaying human atherosclerotic lesions contain MPO.13 Cell-bound MPO becomes transcytosed and colocalizes abuminally where it remains catalytically active.14 Although MPO is normally activated through H2O2 from the phagocyte NADPH oxidase, atherosclerotic blood vessels exhibit an increased flux of superoxide from vascular NAD(P)H oxidase isoforms.38 This source of H2O2 could serve to activate MPO and drive HOCl formation during atherosclerosis. Consistent with this notion, endothelial cells overlaying atherosclerotic lesions stain intensely for HOCl-modified proteins,17 and this finding is reminiscent of our findings with HOCl-treated vessels (Figure 4) suggesting that our in vitro system approximates events that occur in vivo during atherogenesis. Taken together, there is convincing evidence that human endothelial cells are exposed to HOCl-mediated oxidation, and the data presented here support the notion that eNOS may be a target of HOCl thereby contributing to reduced NO bioactivity.

The mechanism for HOCl-mediated impairment of EDNO bioactivity remains to be determined. Information on indirect consequences of HOCl-mediated oxidation are available, as human umbilical vein endothelial cells exposed to HOCl-modified LDL exhibit time- and concentration-dependent inhibition of NO synthesis compared with unmodified LDL-treated cells.39 In addition, MPO-mediated chlorination of L-arginine can produce a defect in endothelial cell NO synthesis.40 Finally, in the presence of H2O2, MPO can catalytically consume NO and thus theoretically limit its bioactivity.15,16 However, these mechanisms are unlikely to account for our observations as we had no LDL in our experiments, and chlorination of L-arginine could not be expected to be inhibited by SOD.

An unexpected finding of this study was the protective effect of SOD toward HOCl-mediated endothelial dysfunction that was strictly dependent on its enzymatic activity. These findings suggest that exposure of aortic segments to HOCl results in the formation of superoxide that then participates in endothelial dysfunction. A potential source of superoxide, which one might consider is eNOS itself. Previous studies by others have established that uncoupled eNOS generates superoxide,41 and there is precedence for the uncoupling of eNOS through oxidative processes. Specifically, peroxynitrite releases zinc from the zinc-thiolate cluster of eNOS dimer, and this results in monomerization of the enzyme that is accompanied by increased superoxide production.31 As suggested by Zou et al, the zinc-thiolate cluster of eNOS is exquisitely sensitive to oxidative modification,31 and this is consistent with observations that HOCl treatment of PAECs produces an increased proportion of eNOS monomer. However, this process appeared likely to be mediated through superoxide rather than HOCl, as monomerization was inhibited by SOD. Therefore, one must consider whether HOCl induced cellular sources of superoxide other than eNOS and whether there is a mechanism for eNOS monomerization by superoxide. With regard to the former, a recent report indicates that H2O2 induces superoxide production in vascular cells through a nonphagocytic NAD(P)H oxidase.42 Stimulation of this oxidase is dependent on p38 mitogen-activated protein kinase43 that itself is activated by H2O2 in endothelial cells.44 We have recently observed that reagent HOCl activates p38 in aortic endothelial cells (K. Chen and J.F. Keaney Jr, unpublished data, 2002), suggesting that HOCl may induce superoxide generation through activation of NAD(P)H oxidase. Further characterization of superoxide-mediated eNOS monomerization will require continued investigations.

In summary, HOCl impairs endothelial function in isolated segments of blood vessels without appreciable cytotoxicity. This action of HOCl appears superoxide-dependent and is consistent with HOCl-mediated oxidation in the arterial wall. These data suggest that HOCl may yet represent another oxidant relevant to atherosclerosis that has implications for NO bioactivity.

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**Figure I.** Low-molecular weight antioxidants and HOCl-induced endothelial dysfunction.

Segments of thoracic aorta were harvested from New Zealand White rabbits as described in “Methods”. Vessels were suspended in organ chambers and incubated for 30 min with glucose-free PSS alone (closed squares) or containing 200 µmol/L HOCl with no additions (open squares) or with 300 µmol/L urate (diamonds), 150 µmol/L ascorbate (circles), 300 µmol/L methionine (triangles), or 500 µmol/L N-acetylcysteine (inverted triangles). After incubation, vessels were washed with glucose-containing PSS containing the respective antioxidants, contracted with phenylephrine (1 µmol/L), and relaxation assayed in response to the indicated concentrations of acetylcholine (A) or A23187 (B). Data represent mean ± SEM of 5 - 8 experiments, *P<0.001 vs HOCl alone by two-way ANOVA.