Hypochlorous Acid, a Macrophage Product, Induces Endothelial Apoptosis and Tissue Factor Expression

Involvement of Myeloperoxidase-Mediated Oxidant in Plaque Erosion and Thrombogenesis

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**Objective**—Superficial erosion of coronary plaques due to endothelial loss causes acute coronary syndromes (ACS). Macrophages at erosive sites of human coronary atheroma present myeloperoxidase (MPO), an enzyme that produces hypochlorous acid (HOCl).

**Methods and Results**—Activated MPO-positive macrophages or exogenous HOCl promoted detachment of endothelial cells (EC) from “Matrigel” substrata in vitro. Pathophysiologically relevant concentrations of HOCl caused EC death in a concentration-dependent manner: HOCl (≈20 to 50 μmol/L) induced rapid shrinkage of EC with nuclear condensation and disruption of EC monolayers, whereas concentrations >100 μmol/L immediately induced blebbing of the EC plasma membrane without shrinkage. HOCl (≈30 to 50 μmol/L) also induced caspase-3 activation, poly (ADP-ribose) polymerase degradation, and DNA laddering in EC. Increased intracellular glutathione (GSH) levels after treatment with GSH monoethyl ester (GSH-MEE) attenuated HOCl-induced EC apoptosis. Sublethal concentrations of HOCl (≈1.0 to 15 μmol/L) increased tissue factor in EC and GSH-MEE treatment limited this effect of HOCl.

**Conclusions**—HOCl can provoke EC death and desquamation by either apoptotic or oncotic cell-death pathways, and sublethal concentrations of HOCl can increase endothelial tissue factor. These results show that MPO-positive macrophage-derived HOCl in the subendothelium of atheroma may participate in ACS by promoting superficial erosion and increasing thrombogenicity. (Arterioscler Thromb Vasc Biol. 2004;24:1-7.)

**Key Words:** acute coronary syndromes • plaque erosion • myeloperoxidase • apoptosis • oxidative stress • endothelial cells • tissue factor

Thrombosis underlies most acute complications of atherosclerosis, notably the acute coronary syndromes (ACS). Coronary thromboses may arise not only from a fracture in the plaque’s protective fibrous cap, but also from superficial erosion of the luminal endothelium without a rupture extending into the lipid core. In approximately one quarter of cases of sudden cardiac death, fatal thrombosis in the coronary arteries results from superficial erosion of coronary fibrous plaques morphologically recognized as “stable plaques.”

This mechanism of plaque disruption appears more common in women and smokers. Disordered metabolism of interstitial collagen likely sets the stage for fibrous cap rupture in lipid-rich atheroma. However, the molecular mechanisms of endothelial erosion in atherosclerotic plaques are uncertain. The erosive sites of coronary plaques contain activated inflammatory cells and abundant proteoglycan.

Several lines of evidence support the presence of apoptosis of endothelial cells (ECs) in atheroma as well as increased circulating apoptotic EC in patients with ACS, suggesting that EC death atop the atherosclerotic arterial intima participates in endothelial desquamation and subsequent thrombosis.

Inflammation and oxidative stress contribute to the pathogenesis of many human diseases including atherosclerosis. Recent studies have demonstrated the presence of the prooxidant enzyme myeloperoxidase (MPO) and products of MPO-mediated reactions in human atherosclerosis. Stimulated phagocytes can secrete this MPO at inflammatory sites, where it generates a powerful reactive oxygen species, hypochlorous acid (HOCl). HOCl can in turn serve as a...
metal-independent oxidizing agent in vivo. A subpopulation of macrophages in human atheroma can present MPO.\textsuperscript{12,13} Granulocyte macrophage colony-stimulating factor (GM-CSF) can selectively regulate the ability of macrophages to retain MPO and produce HOCl in vitro.\textsuperscript{13} Moreover, MPO and HOCl-modified proteins localize at erosion sites in these lesions.\textsuperscript{13} We therefore tested the hypothesis that HOCl affects endothelial viability and that HOCl modifies thromogenicity in the endothelium, thereby leading to superficial erosion and occlusive thrombosis.

**Materials and Methods**

**Cell Culture**

We isolated and cultured human saphenous vein ECs (HSVECs) in Medium-199 (GIBCO) with 10% FCS and EC growth supplement (complete Medium-199). We used these ECs at low passage numbers (passages 2 to 3). For experimental use, we cultured HSVECs on 6-well plates (7×10\(^4\) per well) with coated “Matrigel” (a soluble basement membrane extract, CollaborativeBiomedical). In some experiments, we treated HSVECs with a cell permeant analog of an endogenous antioxidant glutathione (GSH)-monooethyl ester (GSH-MEE; \(\cong 1.0\) to 10.0 mmol/L) for 4 hours, or we cultured HSVECs with a statin (3-hydroxy-3-methylglutaryl coenzyme-A reductase inhibitor, cerivastatin, a gift from Bayer AG, at clinically achievable concentrations: \(\cong 1.0\) to 10 mmol/L) for 3 days. We also isolated human peripheral blood mononuclear cells and neutrophils from platelepheresis byproducts from normal donors by Ficoll density gradient centrifugation.\textsuperscript{13} We obtained human monocyte-derived macrophages that express MPO by treatment with GM-CSF and MPO-negative macrophages by culture with human serum (5% to 10%) as described.\textsuperscript{13} Macrophages were harvested by trypsinization with gentle scraping and resuspended in Hanks balanced salt solution (HBSS). Macrophage-viability was assessed by trypan blue exclusion test (viability >90%).

**Assay of Macrophage-Mediated Endothelial Detachment**

We determined macrophage-induced disruption of endothelial monolayers by quantitating fluorescently labeled HSVECs that remained attached after various treatments by fluorescence microscopy compared with control EC monolayers.\textsuperscript{15} We labeled HSVECs with the PKH26-green fluorescent Cell Linker Kit (Sigma). We labeled isolated monocytes and cultured macrophages with the PKH76-red fluorescent Cell Linker Kit (Sigma). We washed EC monolayers (7×10\(^4\) per well) twice with HBSS, and then layered suspensions of monocytes or macrophages in HBSS (4×10\(^6\) per well) over the EC monolayer. In some experiments, we activated the monocytes and macrophages with a combination of opsonized zymosan (OZ: 0.2 mg/mL) and phorbol myristate acetate (PMA: 500 nmol/L). After a 4-hour co-incubation, we washed the EC monolayers, which were exposed to the monocytes or macrophages, with PBS, and the remaining adherent HSVECs (green cells) were counted under fluorescent microscopy by two independent observers.

**Cell Viability Assay and HOCl Production Assay**

We exposed HSVECs to various concentrations of HOCl (total volume 1.5 mL of HBSS per well), with or without antioxidants, for 15 minutes. After the HOCl exposure, HSVECs were further incubated for 15 minutes and further incubated in the complete Medium-199 for 24 hours. After incubation, HSVECs were lysed in a DNA extraction solution and the DNA was collected and analyzed as described.\textsuperscript{22}

**Assay of Caspase-3 Activity**

We treated HSVECs with or without HOCl for 15 minutes and further incubated them for 6 hours in the complete Medium-199. The cells were lysed in the Cell Lysis Buffer included in the Casapse-3 Colorimetric Assay Kit (R&D Systems). The enzymatic reaction for caspase-3 activity was carried out using the p-nitroanilide conjugated DEVD peptide substrate.

**DNA Isolation and Electrophoresis**

**Fragmentation Analysis**

HSVECs were treated with various concentrations of HOCl (\(\cong 0\) to 50 \(\mu\)mol/L) for 15 minutes and further incubated in the complete Medium-199 for 24 hours. After incubation, HSVECs were lysed in a DNA extraction solution and the DNA was collected and analyzed as described.\textsuperscript{22}

**Assay of Cellular GSH and Protein Content**

We measured endothelial intracellular GSH content by using the colorimetric GSH Assay Kit (GSH-400, OxisResearch) according to the manufacturer’s instructions. The method is based on a chemical reaction between GSH and R-1 (4-chloro-1-methyl-7-trifluromethyl-quinoxilinium methylsulfate) and subsequent \(\beta\)-elimination reaction under alkaline conditions. The total EC protein was measured by using the BCA-Protein Assay Kit (PIERCES).

**Tissue Factor (TF) Activity Assay and Flow Cytometry**

We determined TF activity in HSVECs by chromogenic measurement of generation of the factor Xa by total cell lysates, as described.\textsuperscript{23} HSVEC-lysates were incubated at room temperature in the presence or absence of a neutralizing anti-human TF antibody (No.4510, American Diagnostica); TF antibody inhibitable values were measured at 410 nmol/L. Total cellular DNA was measured as described.\textsuperscript{13} TF activity corresponding to a 30-second clotting time was defined as 1.0 U/mL, and TF activity was expressed as mU/mg of cellular DNA in the present study.

**Flow cytometry**

Fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal anti-human TF antibody (American Diagnostica) was used for fluorescence-activated cell sorter (FACS) analysis using FACSCaliber, as described.\textsuperscript{30}

**RNA Extraction and RT-PCR**

We extracted total RNA from HSVECs and performed RT-PCR as described.\textsuperscript{13} Pairs of primers were as follows. TF: sense, 5'-CAGTGA-TTCCCTCTCG-3'; antisense, 5'-AACACAGCATT-GGCCAGCAG-3'; and human GAPDH: sense, 5'-GGAGCCCAA-A-GGTCATC-3'; antisense, 5'-CCAGTGAGTCCCGCTTC-3'. We conducted PCR for 25 cycles (confirmed to be within the log-linear range).
range of amplification) for TF and GAPDH, and the products were electrophoresed through a 2.0% agarose-gel.

**Statistical Analysis**

We evaluated the difference between means using the unpaired Student’s t test. For statistical analysis of data from multiple groups, we used one-way ANOVA followed by a post-hoc analysis. P values <0.05 were considered significant.

**Results**

**Human Monocytes and MPO-Positive Macrophages Induced Disruption of EC Monolayers**

Human neutrophils, monocytes, and GM-CSF–treated macrophages, but not human serum-treated macrophages, produced HOCl in response to PMA stimulation (Figure 1A and 1B). Either activated human monocytes or MPO-bearing macrophages promoted the detachment of human EC from Matrigel substrata in vitro (Figure 1C). However, activated human serum-treated macrophages (MØ, lower right lane) did not induce EC detachment (data not shown). Taurine, an HOCl scavenger, effectively prevented the disruption of EC monolayers provoked by either the activated monocytes or the MPO-positive macrophages (Figure 1D). When we collected and replated the detached ECs on new culture dishes, none of the ECs adhered to the culture dish or survived. This result verified the lack of viability of the detached ECs.

**Exogenous HOCl Induced EC Death**

Exogenous HOCl at concentrations achieved in vivo at sites of inflammation evoked EC death in a concentration-dependent manner (HOCl: ~1.0 to 100 μmol/L, Figure 1A, available online at http://atvb.ahajournals.org). HOCl (~25 to 50 μmol/L) produced rapid EC death within 4 hours (Figure 1B). HOCl (~20 to 50 μmol/L) immediately induced EC shrinkage (Figure 1C), followed by nuclear condensation (a morphological change characteristic of apoptosis), and finally, disruption of the EC monolayers (Figure 1C and red nuclei in Figure 1D). Meanwhile, HOCl concentrations >100 μmol/L rapidly induced blebbing of the EC plasma membrane without shrinking or nuclear condensation, morphological findings of oncosis (nonapoptotic cell death; Figure 1E [7×10⁵ cells per well]). Anti-oxidants (500 μmol/L), vitamin C, N-acetyl-cysteine, or taurine reduced HOCl (40 μmol/L)-induced EC death (data not shown).

**HOCl Activates the Apoptotic Cascade in EC**

HOCl (~30 to 50 μmol/L) induced molecular signatures of apoptosis including DNA laddering (Figure 2A) and increased caspase-3 activity in ECs (Figure 2B). Immunoblot
analysis established that HOCl provoked caspase-3 activation and PARP degradation in ECs (Figure 2C and 2D). HOCl also induced cytochrome-C release from the mitochondria into the cytoplasm, indicating that HOCl promotes activation of an apoptotic EC death cascade at least in part by mitochondrial damage (Figure 2E). Furthermore, HOCl rapidly decreased immunoreactive Bcl-2 levels in ECs after 15 minutes (Figure 2F).

**Increased Intracellular GSH Prevents HOCl-Induced EC Death**

HOCl rapidly decreased the levels of intracellular GSH in HSVECs within 30 minutes and was sustained for at least 120 minutes (Figure IIA, available online at http://atvb.ahajournals.org). HOCl lowered EC-GSH content in a concentration-dependent manner (HOCl; 10 to 100 μmol/L, Figure IIB). Treatment with GSH-MEE (0.1 to 10.0 μmol/L) significantly increased the intracellular GSH content (Figure IIC) in HSVECs and significantly limited HOCl-induced EC death (Figure IID). Treatment with a cell-permeant statin, cerivastatin, for 3 days at clinically achievable concentrations produced a statistically significant increase in EC intracellular GSH levels (Figure IIE). Statin treatment substantially attenuated HOCl-induced EC death (Figure IIF).

**Sublethal Concentrations of HOCl Induced Endothelial Tissue Factor Expression**

Lower sublethal concentrations (1.0 to 15 μmol/L) of HOCl significantly increased endothelial TF activity at 16 hours (Figure 3A). HOCl (15 μmol/L) also induced TF antigen on EC surface as determined by FACS analysis (Figure 3B). RT-PCR analysis demonstrated that HOCl increased the levels of TF mRNA expression in HSVECs at 8 hours (Figure 3C and 3D). Exogenous free radical scavengers, vitamin C or taurine, prevented HOCl-induced increases in TF activity in HSVECs (Figure 3E). Treatment with a statin (cerivastatin) or GSH-MEE but not aspirin also significantly attenuated HOCl-induced TF expression in HSVECs (Figure 3E).

**Discussion**

The present in vitro study demonstrates that HOCl can provoke human EC death and detachment by both apoptotic and oncocytic cell death pathways, and that sublethal concentrations of HOCl augment TF expression by human EC. Several clinical investigations point to MPO as a potential marker for cardiovascular risk and activity of coronary artery disease. Macrophages in the sub-endothelium of human atheroma can retain MPO, an enzyme that produces HOCl; we have localized MPO and HOCl-modified proteins in these lesions at sites of erosion in coronary arteries. Local production of HOCl by macrophages containing MPO in the sub-endothelium of human coronary plaques may thus contribute to the pathogenesis of ACS by promotion of superficial erosion and hence increased thrombogenicity. Particularly in women, sudden cardiac death caused by fatal thrombosis in coronary arteries may result from the superficial erosion of fibrous plaques. The present data suggests that MPO-bearing macrophages but not MPO-
negative macrophages can disrupt the EC monolayers and thereby induce EC death. Furthermore, exogenous HOCl, within physiologically achievable concentrations at sites of active inflammation, mimicked the injurious effects of the activated MPO-positive macrophages on human ECs, suggesting that MPO-derived HOCl within inflamed plaques can evoke EC-death and/or desquamation. HOCl can directly induce endothelial dysfunction, decrease adhesivity of extracellular matrix proteins for ECs, and convert latent metalloproteinases into active forms, which could impair the integrity of the luminal endothelium. Because MPO-positive macrophages exist at sites of erosion of coronary plaques and MPO can deposit within sub-endothelium via endothelial transcytosis, local production of HOCl by MPO in the sub-endothelium of human coronary plaques may promote EC desquamation and precipitate ACS.

HOCl can induce cell death by decreasing cellular ATP levels or modifying cell-surface proteins. Recently, Vissers et al demonstrated that HOCl caused apoptosis and growth arrest in human ECs and Wagner et al and Englert et al showed that MPO-derived HOCl and chloramines induced apoptosis in human leukemia cells and human B lymphoma cells. This study shows that sub-lethal concentrations of HOCl rapidly provoke apoptotic EC death, likely caused by Bcl-2 degradation and cytochrome-C release from mitochondria. We originally found that HOCl rapidly decreased Bcl-2 levels in human ECs. The precise mechanisms of HOCl-induced Bcl-2 loss remain uncertain at present. Celli et al demonstrated that intracellular GSH-depletion caused by buthionine sulfoximine could induce degradation of the Bcl-2 protein and promote apoptosis in cholangiocytes. We found that HOCl rapidly decreased the intracellular GSH levels in human ECs, suggesting that GSH-depletion by HOCl might play a key role in the degradation of Bcl-2 protein in EC. Xue et al have shown that locally-generated reactive oxygen species can directly destroy native Bcl-2 protein by a protease-independent mechanism. Thus, Bcl-2 may be a direct or indirect intracellular target of HOCl, triggering the activation of the apoptotic cascade in human EC.

The normal endothelium resists thrombosis. However, increased circulating soluble TF and TF expression by luminal or circulating ECs and the circulating microparticles may promote thrombosis. Activated or apoptotic ECs can release shed-membrane microparticles into the circulation. We demonstrate here that sublethal concentrations of HOCl increase TF activity in human ECs. MPO has recently been shown to serve as a major enzymatic catalyst for initiation of lipid peroxidation in vivo. It has been demonstrated hydroperoxide-dependent activation of latent TF pathway activity and inactivation of TF pathway inhibitor through oxidation, suggesting additional alternative mechanisms of action of MPO-generated oxidants to promote the enhanced thrombogenicity in atherosclerosis.

HOCl activates EC at lower sublethal concentrations but promotes cell death at higher concentrations. This biphasic action of HOCl may contribute to the generation of circulat-
ing TF-bearing EC microparticles in inflammatory diseases. HOCl can activate the transcription factor nuclear factor-kB in T-lymphocytes, and depletion of intracellular GSH can induce proinflammatory gene expression in lung epithelial cells. However, the mechanisms of EC activation by HOCl require further investigation.

Treatment with GSH-MEE or a cell-permeant statin at clinically relevant concentrations increased intracellular GSH content and prevented HOCl-induced cell death and TF expression in cultured human ECs. We have previously demonstrated that GSH administration can improve coronary endothelial vasomotion; moreover, a polymorphism in a gene of γ-GCS, a limiting enzyme of intracellular GSH synthesis, correlates with risk of acute myocardial infarction. GSH contributes importantly to intracellular defenses against oxidative stress, and both atherogenesis and plaque destabilization can involve oxidative stress. Increased intracellular GSH levels in ECs might reduce plaque erosion and be one mechanism by which statins decrease cardiovascular events.

In conclusion, MPO-bearing macrophages and HOCl can provoke human EC death and desquamation by both apoptotic and onotic cell-death pathways, and sublethal concentrations of HOCl can induce TF activity in human ECs. Local production of HOCl by activated monocytes, or MPO-positive macrophages, in the sub-endothelium of human coronary atheroma may participate in the pathogenesis of ACS by promoting superficial plaque erosion and increasing thrombogenicity. These results provide new mechanistic insight into the link between inflammation and the pathogenesis of the acute coronary syndromes.

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