Effects of the Reactive Oxygen Species Hydrogen Peroxide and Hypochlorite on Endothelial Nitric Oxide Production

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Abstract—Reactive oxygen species (ROS) hydrogen peroxide (H$_2$O$_2$) and hypochlorite (HOCl) participate in the pathogenesis of ischemia/reperfusion injury, inflammation, and atherosclerosis. Both NO and ROS are important modulators of vascular tone and architecture and of adhesive interactions between leukocytes, platelets, and vascular endothelium. We studied the effect of H$_2$O$_2$ and HOCl on receptor-dependent (bradykinin [10$^{-6}$ mol/L] and ADP [10$^{-4}$ mol/L]) and receptor-independent mechanisms (calcium ionophore A23187 [10$^{-6}$ mol/L]) of NO production by porcine aortic endothelial cells (ECs). Changes in the level of EC cGMP (the second messenger of NO) were used as a surrogate of NO production. EC cGMP increased 300% in response to bradykinin and A23187 and 200% in response to ADP. Exposure of ECs to H$_2$O$_2$ (50 µmol/L) for 30 minutes significantly impaired cGMP levels in response to ADP, bradykinin, and the receptor-independent NO agonist A23187. In contrast, preincubation with HOCl (50 µmol/L) impaired cGMP production only in response to ADP and bradykinin but not A23187. These concentrations of H$_2$O$_2$ and HOCl did not result in increased EC lethality as assessed by lactate dehydrogenase release. Neither H$_2$O$_2$ nor HOCl affected EC cGMP production in response to NO donor sodium nitroprusside, which suggests that guanylate cyclase is resistant to these oxidants. We also demonstrated that neither H$_2$O$_2$ nor HOCl affects endothelial NO synthase (eNOS) catalytic activity as measured by conversion of L-arginine to L-citrulline in EC homogenates supplemented with eNOS cofactors. The present studies show that H$_2$O$_2$ impairs NO production in response to both receptor-dependent and receptor-independent agonists and that these effects are due, at least in part, to inactivation of eNOS cofactors, whereas HOCl inhibits NO production by interfering with receptor-operated mechanisms at the level of the cell membrane. Concentrations of H$_2$O$_2$ and HOCl used in the present studies have been shown to be generated in vivo during inflammation and ischemia/reperfusion. Therefore, we infer that these effects of H$_2$O$_2$ and HOCl on EC NO production may contribute to disregulated vascular tone and altered leukocyte-EC interactions that occur in vascular injury as a result of those causes in which ROS generation is involved. (Hypertension. 2001;38:877-883.)

Key Words: endothelium • nitric oxide • reactive oxygen species • atherosclerosis • inflammation

Vascular injury secondary to processes such as ischemia/reperfusion, inflammation, and atherosclerosis is accompanied by synthesis and release of reactive oxygen species (ROS). Among the ROS generated are hydrogen peroxide (H$_2$O$_2$), hypochlorite (HOCl), hydroxyl, and superoxide (O$_2^-$). These oxidants can induce injury in a variety of mammalian cells, including endothelial cells (ECs).

Although O$_2^-$ can be directly toxic, it has limited reactivity with most biological molecules. However, O$_2^-$ can dismutate either spontaneously or through superoxide dismutase to H$_2$O$_2$. Therefore, H$_2$O$_2$ almost always is formed under any circumstance in which O$_2^-$ is produced. Acting as an electron donor, O$_2^-$ also can lead to generation of hydroxyl radical through an O$_2^-$-driven Fenton reaction, and by interaction with NO, O$_2^-$ can generate highly reactive peroxynitrite. No direct interaction has been described for NO with H$_2$O$_2$ or HOCl.

H$_2$O$_2$ diffuses freely into cells to produce several biochemical perturbations, including activation of the hexose monophosphate shunt and glutathione redox cycles, oxidation of intracellular sulfhydryls, depression of intracellular ATP, DNA damage, loss of intracellular β-nicotinamide adenine dinucleotide, activation of poly(ADP-ribose) polymerase, fast rise of intracellular calcium, gross perturbations to the cytoskeleton and plasma membrane, and depression in glycolytic flux. All of these processes occur before loss of membrane integrity as measured by vital stains or before loss of preloaded Cr$^{51}$. Myeloperoxidase, a heme protein secreted by phagocytes, amplifies the oxidative potential of H$_2$O$_2$ by generating cytotoxic oxidants and diffusible radical species. The major oxidant generated by the myeloperoxidase-H$_2$O$_2$-Cl$^-$ system at physiological concentrations of Cl$^-$ is HOCl. Toxic properties of HOCl stem from its chemical reactivity, including oxidative bleaching of heme groups and iron-sulfur centers and chlorination of amines and unsaturated lipids. However, in contrast to H$_2$O$_2$, HOCl is less diffusible.
and interacts mainly with cellular membrane components, oxidizing plasma membrane sulfhydryl groups and disturbing cellular functions such as glucose and amino acid transporters and potassium pumping capacity. Endothelial NO synthase (eNOS) is located in small invaginations of the plasma membrane called caveolae and produces NO in response to a variety of agonists. Acetylcholine, adenosine nucleotides such as ADP, and bradykinin (BK) stimulate NO synthesis through receptor-operated mechanisms, whereas calcium ionophore A23187 leads to NO synthesis by stimulating calcium fluxes through receptor-independent mechanisms. NO activates guanylate cyclase in an autocrine fashion within the endothelium and in a paracrine fashion in vascular smooth muscle (VSM), which results in increased intracellular cGMP. The amount of cGMP generated is proportional to the amount of NO and, therefore, is an indirect measurement of NO bioactivity. Although increased cGMP in VSM is linked to relaxation, the role of endothelial cGMP has remained more obscure.

Experimentally, ischemia/reperfusion of the heart and kidney results in increased generation of ROS and decreased endothelial NO bioactivity as evidenced by impaired endothelium-dependent relaxation to acetylcholine and BK, decreased endothelial NO bioactivity as evidenced by impaired endothelium-dependent vasodilatory response to acetylcholine in cerebral circulation. After ischemia/reperfusion, decreased NO bioactivity is not accompanied by a reduction in eNOS as demonstrated by histochemical methods. In addition, infusion of angiotensin (Ang) II into rats results in hypertension accompanied by increased production of O$_2^-$ in the aorta.

We used several strategies to study the effect of nonlethal concentrations of the ROS H$_2$O$_2$ and HOCl on EC NO synthesis. First, we studied the effect of H$_2$O$_2$ and HOCl on EC cGMP production. In our laboratory, it has been difficult to obtain consistent measurements of small quantities of NO generated from cultured ECs. Therefore, we used cGMP production within the same ECs as a surrogate of NO generation. This approach also permitted us to evaluate the effect of ROS on guanylate cyclase. Second, we studied the effect of H$_2$O$_2$ and HOCl on eNOS catalytic activity by measuring the conversion of C$^{14}$ L-arginine to C$^{14}$ L-citrulline in homogenates of ECs previously exposed to these ROS. Third, we determined the effect of these ROS on eNOS catalytic activity in homogenates of ECs and fourth, the effect of these ROS on individual eNOS cofactors.

**Methods**

**EC Culture**

ECs were isolated from fresh porcine aorta by incubating the lumen of the vessels in type II collagenase (2 mg/mL; Sigma Chemical Co) at 37°C for 20 minutes. Aortas were rinsed with medium 199 (Cellgro, Mediatech) supplemented with 10% FCS (HyClone) and cells plated directly into 6-well culture dishes (Costar). Cells were identified as endothelial by their morphology and positive uptake of fluorescent acetylated LDL.

**Cell Incubation**

ECs were washed and preincubated with Krebs-Ringer bicarbonate buffer that contained 3-isobutyl-1-methylxanthine (Sigma; 10$^{-4}$ mol/L) for 30 minutes. ECs were preincubated and then incubated for 3 minutes in Krebs buffer that contained the following NO agonists: A23187 (10$^{-6}$ mol/L), ADP (10$^{-4}$ mol/L), and BK (10$^{-7}$ mol/L; Sigma). Sodium nitroprusside (SNP; 10$^{-3}$ mol/L) also was tested. At the end of 3 minutes’ incubation, intracellular cGMP was measured by radioimmunoassay as previously described. cGMP per well was factored for protein content measured by the method of Lowry et al.

The following experiments were performed to confirm that changes in EC cGMP in response to agonists of NO were due to autocrine effects of endothelial NO. (1) ECs were incubated for 10 minutes with NO inhibitor N$^6$-monomethyl-L-arginine (L-NMMA; 10$^{-4}$ mol/L) before incubation with A23187, ADP, or SNP. (2) ECs were preincubated for 24 hours in cell-culture medium free of L-arginine (Selectamine, Gibco). A23187 (10$^{-6}$ mol/L) and SNP (10$^{-3}$ mol/L) were added to cells preincubated in standard and L-arginine-depleted medium; cGMP was measured as described above.

**Effect of Oxidant Injury on EC cGMP Synthesis**

To induce oxidant injury, ECs were preincubated with either H$_2$O$_2$ (50 $\mu$mol/L) or HOCl (50 $\mu$mol/L) (Sigma) for 30 minutes before incubation with NO agonist or corresponding vehicle. After incubation with NO agonist, cGMP was measured as described above.

**Effect of Oxidant Injury on NOS Activity**

To determine the direct effect of oxidant injury on eNOS, ECs were preincubated with either H$_2$O$_2$ (50 $\mu$mol/L) or HOCl (50 $\mu$mol/L) for 30 minutes, and eNOS activity was determined in vitro in homogenates of ECs. In separate experiments, homogenates of ECs were exposed to H$_2$O$_2$ (50 $\mu$mol/L) or HOCl (50 $\mu$mol/L) for 30 minutes and eNOS activity determined in the presence of optimal amounts of NOS cofactors (Figure 1). In other experiments, individual NOS cofactors were exposed to H$_2$O$_2$ (50 $\mu$mol/L) or HOCl (50 $\mu$mol/L) for 30 minutes and then added to homogenates of ECs for eNOS activity determination. NOS activity was measured as previously described (Figure 1).

**Statistical Analysis**

Data are expressed as mean±SEM. For statistical comparison involving 2 groups, an unpaired Student’s t test was used, whereas for comparison involving >2 groups, ANOVA by means of Statview 512 statistical program was used (Abacus Concepts Inc). Significance was considered present when $P<0.05$.

An expanded Methods section can be found in an online data supplement available at http://www.hypertensionaha.org.
Results

NO Agonists Increase EC cGMP Synthesis

ECs exposed for 30 minutes to the NO agonists A23187, ADP, or BK showed increased cGMP compared with that of control (Figure 2). NOS inhibitor L-NMMA (10^{-4} mol/L) significantly inhibited cGMP production stimulated by these agonists (Figure 2).

Arginine Depletion Diminishes EC cGMP Production in Response to NO Agonists

Twenty-four hours of L-arginine depletion lowered basal levels of cGMP slightly but not significantly from 8.5±0.5 fmol/μg of protein (n=3; P=NS). L-arginine depletion significantly blunted cGMP production stimulated by A23187: for cells in standard medium with A23187, cGMP level was 27.4±2.0 fmol/μg of protein (n=3); in L-arginine-depleted medium with A23187, cGMP level was 11.8±0.40 fmol/μg of protein (n=3, P<0.05).

Thus, elimination of L-arginine, the substrate of eNOS, from the culture medium or inhibition of NO synthesis with L-NMMA significantly prevented increases in EC cGMP induced by NO agonists, thereby establishing that observed increases in EC cGMP are a result of increased EC NO.

H2O2 Impairs EC cGMP Production in Response to NO Agonists

As shown in Figure 3, H2O2 preexposure for 30 minutes significantly impaired increases in EC cGMP synthesis in response to all NO agonists tested. These changes in cGMP production were not accompanied by increased EC lethality as assessed by LDH release (0% release). However, H2O2 preexposure did not significantly affect EC cGMP production stimulated by the exogenous NO donor SNP (Figure 3). This occurrence suggests that H2O2 did not affect EC guanylate cyclase levels.

HOCl Impairs EC cGMP Production in Response to NO Receptor–Dependent Agonists ADP and BK But Not Calcium Ionophore A23187

Preincubation with HOCl (50 μmol/L) inhibited EC cGMP synthesis induced by ADP and BK (Figure 4). These changes in cGMP production were not accompanied by increased EC lethality as assessed by LDH release (0% release). However, in contrast to H2O2, A23187-stimulated production of cGMP was not significantly affected by preexposure of EC to HOCl (Figure 4). In a manner similar to H2O2, HOCl did not affect SNP-stimulated cGMP production (Figure 4).
Exposure of ECs to H$_2$O$_2$ or HOCl Does Not Affect Intrinsic Catalytic Activity of eNOS
To explore whether observed effects of H$_2$O$_2$ and HOCl on NO production were due to oxidative damage of eNOS or oxidation of the necessary cofactors for optimal eNOS activity, we determined eNOS activity in cell homogenates from ECs preexposed to H$_2$O$_2$ or HOCl in which all eNOS cofactors were added from exogenous sources. As shown in Figure 5, eNOS activity was similar in control ECs and ECs preexposed to H$_2$O$_2$ (50 μmol/L) for 30 minutes. These findings suggest that effects of H$_2$O$_2$ on NO production are because of a direct effect of H$_2$O$_2$ on eNOS cofactors and not on total eNOS catalytic activity. As expected, eNOS catalytic activity in ECs pretreated with HOCl was also preserved, consistent with the notion that effects of HOCl are at a relatively early point in signal transduction (cell membrane), whereas eNOS and its cofactors are spared from HOCl-induced oxidative damage (Figure 5).

To demonstrate a direct effect of H$_2$O$_2$ on eNOS cofactors, individual eNOS cofactors were exposed to H$_2$O$_2$ (50 μmol/L) and then added to the purified enzyme before eNOS catalytic activity was measured. Prior exposure of individual eNOS cofactors tetrahydrobiopterin (BH4), NADPH, and flavin adenine dinucleotide (FAD) to H$_2$O$_2$ did not significantly modify eNOS activity in homogenates of ECs: control, 36.6±1.4 pmol·mg$^{-1}$·min$^{-1}$; cells treated with BH4 plus H$_2$O$_2$, 32.4±1.9 pmol·mg$^{-1}$·min$^{-1}$; with NADPH plus H$_2$O$_2$, 37.8±2.8 pmol·mg$^{-1}$·min$^{-1}$; with FAD plus H$_2$O$_2$, 34.7±1.7 pmol·mg$^{-1}$·min$^{-1}$ (n=3 per duplicate; P=NS versus control). However, when flavin mononucleotide (FMN) or all eNOS cofactors combined were exposed to H$_2$O$_2$, a significant decrease occurred in NOS enzymatic activity, which suggests that oxidative modification of eNOS cofactors and FMN in particular impairs eNOS activity: cells treated with FMN plus H$_2$O$_2$, 30.3±1.8 pmol·mg$^{-1}$·min$^{-1}$ (n=3 per duplicate; P<0.05 versus control) and with all cofactors plus H$_2$O$_2$, 26.5±1.4 pmol·mg$^{-1}$·min$^{-1}$ (n=3 per duplicate; P<0.05 versus control). In contrast, exposure of eNOS from EC homogenates to H$_2$O$_2$ (50 μmol/L) did not impair eNOS activity, which demonstrated that eNOS is resistant to oxidative effects of H$_2$O$_2$, at least at the concentrations used in the present studies: control, 34.4±1.3 pmol·mg$^{-1}$·min$^{-1}$ and cells treated with H$_2$O$_2$, 37.2±2.7 pmol·mg$^{-1}$·min$^{-1}$ (n=3 per duplicate; P=NS versus control). Similar results were found when EC homogenates were prepared in the absence of dithiothreitol, which suggests that the lack of effect of H$_2$O$_2$ on isolated eNOS was not due to the protective effect of dithiothreitol (not shown).

Similar experiments were performed in which the eNOS cofactors or EC homogenates were exposed to HOCl. With the exception of NADPH (and in contrast to our results with H$_2$O$_2$), exposure of individual eNOS cofactors to HOCl significantly impaired eNOS activity, which suggests that these cofactors are highly sensitive to the oxidative effects of HOCl: control, 36.6±1.4 pmol·mg$^{-1}$·min$^{-1}$; cells treated with BH4 plus HOCl, 16.1±1.1 pmol·mg$^{-1}$·min$^{-1}$; with flavin adenine dinucleotide plus HOCl, 27.2±1.4 pmol·mg$^{-1}$·min$^{-1}$; with FMN plus HOCl, 22.4±3.0 pmol·mg$^{-1}$·min$^{-1}$ (n=3 per duplicate; P<0.05 versus control); and with NADPH plus HOCl, 38.5±1.8 pmol·mg$^{-1}$·min$^{-1}$ (n=3 per duplicate; P=NS versus control). In addition, when all eNOS cofactors were exposed to HOCl, eNOS activity was almost completely abolished (4.34±1.2 pmol·mg$^{-1}$·min$^{-1}$; n=3;
Discussion

A growing body of evidence implicates NO as an important modulator of vascular tone and architecture as well as of adhesive interactions between leukocytes, platelets, and vascular endothelium. Processes of ischemia/reperfusion and atherosclerosis are characterized by increased adhesion of leukocytes and platelets to the vascular endothelium as a result of increased adhesion-molecule expression. The latter has been attributed to diminished bioactivity of NO and increased production of ROS such as H2O2 and O2. O2 is in the extracellular milieu, given its great affinity for NO, rapidly interacts with this molecule and results in NO inactivation and/or production of toxic peroxynitrite. No direct interaction between H2O2 and NO has been described.

ROS generation may be a common pathway for many types of pathological processes. Several ROS including H2O2 and O2 are produced by leukocytes after perturbation of their membranes as part of the respiratory burst or by vascular endothelium during the reperfusion period after ischemic injury. In addition, O2 is generated by Ang II–mediated activation of NADPH/NADH oxidase by the AT1 receptor in VSM cells, vascular ECs, and glomerular mesangial cells. H2O2 can be generated through dismutation of O2 either spontaneously or by superoxide dismutase. Both O2 and H2O2 have been shown to activate intracellular pathways in VSM cells and mesangial cells, which culminate in cellular hypertrophy or proliferation. These actions of Ang II are believed to be important to explain the role of Ang II in pathological vascular remodeling in hypertension and in atherosclerosis. A study recently reported that antioxidants restore endothelial function and decrease blood pressure in animal models of hypertension. Hyperlipidemia in rabbits is associated with increased AT1 receptor density and increased O2 production derived from NADH/NADPH oxidase. In humans with atherosclerosis, AT1 receptor blockade improves endothelium-dependent relaxation mediated by NO. On the other hand, NO has been shown to have an inhibitory effect on NADH/NADPH oxidase and to downregulate Ang II receptors in VSM cells.

H2O2 generation is followed by generation of other toxic molecules, including HOCl. When H2O2 is formed by polymorphonuclear cells in vivo, up to 40% is halogenated by myeloperoxidase into HOCl and in acidic conditions into ClO, which also has oxidant activity and may be implicated in several oxidative and cytotoxic reactions at sites of inflammation and vascular disease. Furthermore, the myeloperoxidase-H2O2–ClO system of phagocytes promotes the formation of advanced glycation end products, which play an active role in the pathogenesis of atherosclerosis.

ADP and BK are 2 agonists of NO that bind to receptors located on the EC membrane, whereas A23187 is a calcium ionophore that induces NO synthesis and release, directly promoting a calcium influx into ECs, which activates eNOS. The present studies show that exposure of ECs to nonlethal concentrations of H2O2 and HOCl impairs endothelial NO production in response to several NO agonists, as evidenced by decreased production of cGMP, the intracellular NO second messenger. H2O2 impairs NO synthesis in response to receptor-dependent (BK and ADP) and receptor-independent agonists (A23187), which suggests that H2O2 may affect eNOS catalytic action or eNOS cofactors. However, we found that the catalytic activity of eNOS in extracts from ECs exposed to H2O2 and activated in the presence of exogenously added cofactors is similar to that in control ECs, not exposed to this oxidant. On the other hand, exposure of eNOS cofactors to H2O2 before such cofactors are added to EC homogenates resulted in impaired eNOS activity. These findings suggest that H2O2 affects oxidant-sensitive eNOS cofactors but not the intrinsic catalytic activity of eNOS. Among the eNOS cofactors, FMN was the most sensitive to O2 inactivation and/or production of toxic peroxynitrite. In the aggregate, these findings support our previous studies in a rat model of ischemia/reperfusion acute renal failure that demonstrated preserved eNOS activity in the renal vasculature despite decreased NO bioactivity. Similar findings also have been reported in a model of lead-induced hypertension.

In contrast to H2O2, HOCl impaired responses only to the receptor-dependent agonists BK and ADP. The response was preserved to the calcium ionophore and the eNOS catalytic activity in homogenates of ECs obtained after HOCl exposure. These results with intact cells are consistent with the notion that HOCl affects specific plasma membrane targets and impairs EC production of NO at a relatively early point in receptor-operated signal transduction. eNOS is located in small invaginations of the plasma membrane called caveolae. We speculate that HOCl may be affecting eNOS-caveolae interaction, resulting in impaired receptor-dependent NO production.

On the other hand, exposure of eNOS cofactors or EC homogenates to HOCl resulted in impaired eNOS catalytic activity. Because of the high reactivity of HOCl, its reactions are dependent on the relative concentration and reactivities of compounds in the immediate vicinity of where it is produced. In intact ECs, the closest targets for HOCl to react are the sulfhydryl groups of the cell membrane; however, in the absence of cell membranes, HOCl can react with multiple targets, including eNOS cofactors or eNOS itself, resulting in impaired eNOS activity.

Neither HOCl nor H2O2 blunted SNP-stimulated cGMP production by ECs, which suggests that guanylate cyclase can withstand the oxidative stress imposed by these agents. Moreover, the preserved response to SNP and the lack of LDH release by ECs exposed to H2O2 or HOCl provide strong evidence that cell viability was not affected by exposure to these oxidants.

The present studies suggest that ROS such as H2O2 and HOCl contribute to decreased NO bioactivity observed in vascular ischemia/reperfusion processes and, perhaps, in...
atherosclerosis by interfering with the mechanisms that participate in NO synthesis and release without affecting the intrinsic eNOS catalytic activity. We speculate that this novel effect of H₂O₂ and HOCl on NO production contributes to altered adhesive interactions between leukocytes and endothelium as well as disregulated vascular tone, which occurs during ischemia/reperfusion, vascular inflammation, and atherosclerosis.

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