Nitric oxide is crucial for maintaining normal vascular function, and inactivation of NO by excessive production of reactive oxygen species (ROS) is implicated in several cardiovascular diseases. There is increasing evidence that NADPH oxidase expressed in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) is a major source of superoxide. Five isoforms of the Nox subunit of NADPH oxidase have been identified (named Nox1 to Nox5), of which Nox1, Nox2, and Nox4 are found in vascular tissues. Increased NADPH oxidase activation is associated with conditions such as endothelial dysfunction, intimal hyperplasia, atherosclerosis, and hypertension and has been linked to impairment of endothelial NO function in patients with coronary artery disease.

Recently, we found that treatment of human microvascular ECs (HMECs) with NO donors inhibited the superoxide-generating capacity of NADPH oxidase, which may involve S-nitrosylation of the p47phox subunit. In addition, previous studies have revealed that exposure of ECs to exogenous NO induces Nrf2-dependent expression of heme oxygenase-1 (HO-1), the rate-limiting enzyme in heme degradation. Several lines of evidence have indicated that induction of HO-1 has remarkable antioxidant effects, because: (1) bilirubin is a free radical scavenger and has chain-breaking antioxidant activity which may prevent lipid peroxidation; (2) HO-1 expression prevents ROS-induced cellular damage in both neuronal cells and VSMCs; and (3) cardiac-specific expression of HO-1 protects against inflammation and oxidative damage in hearts subject to ischemia and reperfusion injury in vivo. Moreover, in human airway smooth muscle, HO-1 induction reduced cellular ROS production, indicating that HO-1 and/or its products may regulate ROS production. We, therefore, set out to test the hypothesis that NO may modulate intracellular ROS production by NADPH oxidase via induction of HO-1 in HMECs.

Methods
An expanded Methods section is available in an online supplement available at http://www.hypertensionaha.org.

Cell Culture
HMECs (a gift from Prof Philip Hogg, University of New South Wales, Sydney, Australia) were cultured in MCDB-131 media (Gibco) containing 10% FCS and hydrocortisone (50 μg/mL) in a CO2/O2 incubator at 37°C. Confluent cells of passages 25 were arrested with low serum medium (0.1%) overnight before experimentation.

ROS Measurement
TNF-α-induced cellular ROS generation was measured by 2',7'-dichlorofluorescein (DCFH) diacetate (DA) fluorescence with a PolarStar (BMG LABTECH) microplate reader at excitation 485 nm.
and emission 520 nm. To eliminate the influence of nonspecific oxidation of the probe by cells on the fluorescence signal, a time control sample (cells and probe only) was included in all of the experiments. The fluorescence signal intensity of the test samples was first normalized as the percentage of the time control and then expressed as the percentage of the value immediately before the addition of TNF-α (baseline). NADPH oxidase activity was assessed by NADPH-stimulated superoxide using lucigenin-enhanced chemiluminescence as described previously.16

Western Blot
Total proteins were separated by 10% SDS-PAGE and transferred to Hybond nitrocellulose membrane (Amersham). The membrane was blocked with 5% nonfat milk powder in Tris-buffered saline (pH 7.5) and hybridized overnight with primary antibodies, which were then detected with horseradish peroxidase–conjugated anti-IgG and visualized with an ECL kit (Amersham).

Real-Time PCR
Total RNA was extracted with RNXigel reagent (Ambion), reverse transcribed to cDNA. Quantitative real-time PCR reactions were performed in an ABI Prism 7700 system (Applied Biosystems) using the TaqMan Universal PCR master mix and the predesigned gene-specific probe and primer sets (TaqMan Gene Expression Assays, Applied Biosystems).

Immunofluorescence
Six ×10⁴ HMECs were resuspended in MCDB-131 medium and seeded in Lab-Tek 2-well chamber slides (Nunc). Indirect immuno-fluorescence staining was carried out using goat anti p47phox antibody (Santa Cruz) 1:50 in blocking buffer at 4°C overnight followed by fluorescein isothiocyanate–conjugated secondary antibody at room temperature for 1 hour.

Gene Silencing With Small Interfering RNA
HMECs were subcultured into 24-well plates (1×10⁴ cells per well) in antibiotic-free MCDB-131 medium containing 10% serum 24 hours before transfection. Cells were transfected with 30 pmol per well of specific small interfering RNA (siRNA) against human HO-1 or control RNA according to the manufacturer’s instructions. Cells were used 48 hours after transfection. For Nox4, cells in 100-mm culture dish were transfected with 1500 pmol Nox4 specific or control siRNA for 72 hours.

Data and Statistical Analysis
Data are expressed as mean ± SEM. The mean data were analyzed with Student t-test or 1-way ANOVA followed by Newman–Keuls t test as appropriate. A value of P<0.05 was regarded as statistically significant.

Results
NO Donor Induces HO-1 Expression in HMECs
To examine the effects of NO on HO-1 expression in HMECs, we treated the cells with DETA-NONOate (NONOate), a long-lasting NO donor at physiological pH with a half-life of ~20 hours at 37°C.17 NONOate from 100 μmol/L reproducibly enhanced HO-1 protein expression at 6 and 24 hours, whereas the Cu-Zn superoxide dismutase level was not altered (Figure 1a). The effect of NONOate was concentration dependent with the maximal HO-1 induction being produced at 1000 μmol/L (Figure 1b). Therefore, this treatment condition (1000 μmol/L for 6 hours) was used throughout the following experiments.

Figure 1. Western blots showing induction of HO-1 expression by NONOate. HMECs were treated with either 0.01 N NaOH (vehicle control) or NONOate (100 μmol/L) for different periods as indicated in (a) or for 6 hours with NONOate at different concentrations (b). The expression of superoxide dismutase was not changed by NO (a).

NO Donor Suppresses NADPH Oxidase–Derived Superoxide in HMEC Through HO-1 Induction
To assess NADPH oxidase activity in HMECs, we measured NADPH-stimulated superoxide production by lucigenin-enhanced chemiluminescence. Similar to our previous findings in VSMCs,16 NADPH-stimulated lucigenin chemiluminescence was blocked by the NADPH oxidase inhibitor diphenyleneiodonium (DPI) but not affected by the xanthine oxidase inhibitor allopurinol, the NO synthase (NOS) inhibitor N⁵-nitro-L-arginine methyl ester hydrochloride (l-NAME), the cyclooxygenase inhibitor indomethacin, or the mitochondrial toxin rotenone (data not shown). To confirm that this assay measures NADPH oxidase function, we transfected HMECs with specific siRNA against Nox4, which has a major role in superoxide production in ECs.4 One predesigned siRNA molecule (No. 2) efficiently decreased Nox4 protein expression in HMECs (Figure 2a) and significantly reduced the NADPH-stimulated lucigenin signal (Figure 2b), confirming that this assay reflects the superoxide-generating capacity of endothelial NADPH oxidase.

Treatment of HMECs with NONOate significantly suppressed NADPH oxidase activity (Figure 2c). This effect cannot be because of a direct interaction between NO and superoxide, because the NO donor was removed before assay. Cotreatment of the cells with NONOate plus the HO-1 inhibitor tin protoporphyrin IX (SnPP) abolished the inhibitory effects of NO on NADPH oxidase–dependent superoxide generation (Figure 2c), indicating that this action of NO depends on HO-1 activity. Moreover, the inhibitory effect of NONOate was blocked by actinomycin D (Figure 2d), an RNA synthesis inhibitor, indicating that the action of NO depends on de novo gene transcription. To exclude the possibility that the protoporphyrin compounds may have NO scavenging effects, we performed organ bath experiments using isolated rat aorta. Neither SnPP nor zinc(II) protoporphyrin IX (ZnPp) had any effects on DETA NONOate-induced vasorelaxations (see online supplemental results), indicating that the observed effects of the HO-1 inhibitors were not because of NO scavenging.
Because SnPP seemed to reduce the chemiluminescence signal, we also used siRNA to block HO-1 expression to exclude the possibility that the effect of SnPP was nonspecific. Western analysis demonstrated that specific siRNA against HO-1 totally blocked NONOate-induced HO-1 expression (Figure 4a) and abolished the inhibitory effect of NONOate, whereas control RNA had little effect (Figure 4b).

**Effects of HO-1 Induction by NO Donor on TNF-α–Induced Oxidative Stress**

We also examined the effects of NONOate on cytokine-induced ROS generation using DCFH-DNA. As shown in Figure 3a, stimulation of HMECs with TNF-α (500 U/mL) time dependently increased the DCFH fluorescence above the background, which peaked at 20 minutes. Similar to NADPH-stimulated superoxide, the TNF-α–induced response was abolished by DPI (10 µmol/L; Figure 3a) but not altered by other enzyme inhibitors (Figure 3b), confirming previous findings that NADPH oxidase was the major source of intracellular ROS generation induced by TNF-α in HMECs.18

Surprisingly, NONOate alone had no effect on TNF-α–induced ROS generation (Figure 3c). However, in the presence of the HO-1 inhibitor SnPP (50 µmol/L), NONOate increased TNF-α–induced ROS production. A possible explanation is that NONOate, while decreasing NADPH oxidase–derived ROS via HO-1 induction, promotes ROS generation from other source(s). For example, previous studies indicate that NO may promote mitochondrial ROS production19,20 by inhibiting the electron transport chain at complex IV.21 We tested whether mitochondria might be involved in ROS production in NONOate-treated cells. As shown in Figure 3c, rotenone suppressed TNF-α–induced ROS in NONOate-treated cells but had little effect in untreated cells. Consistently, the increase in ROS generation by NONOate in ZnPP-treated cells was blocked by rotenone (Figure 3c). In a separate experiment, we treated HMEC cells with NONOate either alone or in the presence of the NADPH oxidase inhibitor DPI (10 µmol/L) for 6 hours. Similar to the results shown in Figure 3c, NONOate alone did not change TNF-α–induced ROS, whereas DPI alone significantly inhibited the TNF-α–induced response (Figure 3d). TNF-α–induced ROS formation in NONOate- and DPI-cotreated cells was significantly higher than that in cells treated with DPI alone (Figure 3d), indicating that the observed prooxidant component of the NONOate action is unlikely to involve NADPH oxidase.

To further confirm the role of HO-1 in modulating ROS production, we demonstrated that NONOate increased TNF-α–induced ROS generation in cells transfected with HO-1 siRNA but not in control RNA–transfected cells (Figure 4c). Taken together, these data suggest that HO-1 expression may counterbalance the prooxidant action of NONOate by inhibiting NADPH oxidase activity.

**NO Had Little Effect on NADPH Oxidase Expression and Protein Stability**

To clarify whether NONOate treatment has direct effects on NADPH oxidase expression, we examined the mRNA levels of different subunits of NADPH oxidase. Nox1 mRNA could not be detected with real-time PCR in HMECs. The mRNA
levels of Nox4, p22phox, and p47phox in HMECs were not significantly changed by NONOate (Figure 5a). Unexpectedly, the mRNA of gp91phox (Nox2) could not be detected in HMECs, although the message could be readily detected in U937 monocytes, and the protein was detected in HMECs with immunoblotting (see below). To examine whether HO-1 induction by NONOate could affect the protein abundance of the heme-containing Nox subunits as described in leukocytes,22 protein levels of Nox1, Nox4, and gp91phox were analyzed with immunoblotting, showing that none of these subunits was affected by NONOate (Figure 5b). These data indicate that the inhibition of NADPH oxidase function by HO-1 expression is unlikely to be mediated by downregulation of the expression of NADPH oxidase subunits.

Bilirubin Mimics the Effects of NO on NADPH Oxidase Function

To elucidate whether the effects of HO-1 on NADPH oxidase function are mediated by its end product bilirubin, we studied the effects of bilirubin on NADPH-stimulated superoxide and TNF-α–induced ROS formation in HMECs. Acute incubation with bilirubin concentration dependently inhibited NADPH oxidase–derived superoxide production (Figure 6a). Similarly, bilirubin also suppressed TNF-α–induced ROS formation (Figure 6b).

Effect of Bilirubin on p47phox Translocation

To examine whether bilirubin may affect the assembly of NADPH oxidase subunits, we performed immunofluorescence experiments and found that in resting cells, most of the p47phox immunoreactivity concentrates at the perinuclear area (Figure 7). TNF-α induced a redistribution of p47phox from perinuclear regions toward the cell periphery, with the fluorescence signal being more organized in a reticular pattern, which is consistent with the pattern of p47phox redistribution induced by angiotensin II.23 Preincubation with bilirubin for 30 minutes prevented this mobilization of p47phox in HMECs (Figure 7).

Discussion

In this study we confirmed that NO may induce HO-1 expression and provided a novel mechanism by which NO modulates NADPH oxidase–dependent superoxide production. This mechanism of NADPH oxidase suppression is supported by the following findings: (1) an NO donor consistently induced HO-1 expression; (2) the NO donor suppressed superoxide-generating capacity of NADPH oxidase, an effect distinct from a direct neutralizing action of NO; (3) the inhibitory effect of NO was abolished by cotreatment with HO-1 inhibitors; and (4) bilirubin, the metabolic product of HO-1, mimicked the effect of NO. The current results do not exclude the possibility that NO acts via other mechanisms to suppress NADPH oxidase activation, which can be achieved with the NO donor at concentrations <30 μmol/L, as reported previously in neutrophils24 and more recently by our group in HMECs.3 In the present study, however, a direct inhibition of NADPH oxidase function is unlikely to be the predominant mechanism, because the effect of the NO donor was abolished by the RNA synthesis inhibitor actinomycin D, indicating that the action of NO depends on de novo gene transcription.

Under inflammatory conditions, a high level of NO generated by the inducible form of NOS (iNOS) expressed in
leukocytes exerts a cellular stress and, indeed, is involved in cell toxicity and tissue damage.\(^{25}\) Oxidation products of NO, such as peroxynitrite, can induce lipid peroxidation, S-nitrosylation of thiol groups in proteins, and inhibition of enzymes for mitochondrial respiration. It is, therefore, not surprising that treatment of ECs with different NO donors at high concentrations can induce expression of HO-1,\(^{10}\) a heat shock protein that has multiple protective functions against tissue injury.\(^{26}\) We confirmed this protective response in HMECs. Studies in both ECs and macrophages have shown that proinflammatory cytokine–induced HO-1 expression is at least partly dependent on iNOS expression and subsequent NO generation.\(^{27,28}\) In a rat model of adjuvant arthritis, blockade of NO production from iNOS suppressed HO-1 upregulation at the site of inflammation.\(^{29}\) These findings suggest that NO-mediated HO-1 expression may have important roles in preventing tissue damage associated with inflammation.

Here we also confirmed that stimulation of endothelial ROS generation by TNF-\(\alpha\) was mediated by NADPH oxidase activation as reported previously.\(^{18}\) In contrast to the effect on NADPH oxidase function, NONOate did not change TNF-\(\alpha\)-induced ROS production. To elucidate the underlying mechanisms of this discrepancy, we used both a pharmacological inhibitor of HO-1 to block its activity and siRNA to block HO-1 expression. Indeed, in the absence of HO-1 activity or expression, NONOate increased ROS generation in response to TNF-\(\alpha\), suggesting that NONOate has concomitant prooxidant actions. In human umbilical vein endothelium, Corda et al\(^{30}\) reported that mitochondria might contribute to TNF-\(\alpha\)-stimulated ROS generation, and NO could enhance mitochondrial ROS production in ECs.\(^{20}\) In agreement with these findings, we found that inhibition of the mitochondrial electron transport complex I with rotenone inhibited ROS production in ECs exposed to NO donor but not in untreated cells, suggesting that NONOate upregulates mitochondrial ROS production in HMECs. Concomitant induction of HO-1 may counteract this pro-oxidative action of excess NO by inhibiting ROS generated from NADPH oxidase. The enhanced ROS formation by NONOate does not involve NADPH oxidase, because the effect of NONOate was resistant to DPI. Given that both iNOS and HO-1 are upregulated in vascular inflammation, such as atherosclerosis,\(^{31,32}\) we suggest that HO-1 induction by NO could be a physiological compensatory mechanism that may protect ECs from exaggerated oxidative injury during inflammation by modulating NADPH oxidase activity. We also considered whether the increased DCFH fluorescence could be because of altered NO levels, because peroxynitrite rapidly oxidizes DCFH. We concluded, however, that this was an unlikely explanation, because, first, the TNF-\(\alpha\)-induced DCFH signal was not changed by the NOS inhibitor L-NAME (Figure 3b), indicating that altered NO release made little contribution, and, second, in the experiments with exogenous NONOate, the DCFH assay was performed after washing the NO donor from the cells.

In human umbilical vein ECs, Ago et al\(^{4}\) have reported that the Nox4 was abundantly expressed as compared with other isofoms and might function as the major catalytic component of the oxidase. We confirmed this finding in a different type of human ECs and showed that knocking down Nox4 expression was accompanied by \(\sim50\%\) reduction of superoxide generation. We did not, however, rule out a contribution of other Nox proteins. Indeed, we have found that blockade of Nox2 expression with specific siRNA also significantly suppressed NADPH oxidase activity in HMECs (data not shown), indicating that both isofoms contribute to functional NADPH oxidases in HMECs. It has been demonstrated that increasing HO-1 expression in macrophages decreased the protein abundance of Nox2 and total NADPH oxidase activity, an effect that was reversed by exogenous heme, indicating that HO-1 may modulate NADPH oxidase function by limiting heme availability for maturation of the Nox subunit(s).\(^{22}\) More recently, Pleskova et al\(^{33}\) reported that
in glomerular mesangial cells, Nox1 mRNA was markedly decreased by treatment with DETA NONOate at concentrations over a range from 30 to 500 μmol/L, which peaked at 6 hours and was at least partly mediated by cGMP production, indicating that NO/cGMP signaling may have direct effects on the transcription of Nox genes. In contrast, we did not observe any change in the mRNA or protein levels of NADPH oxidase subunits after HO-1 induction by NO, suggesting that, in vascular ECs, modulation of subunit expression is unlikely to be involved in NO-induced inhibition of NADPH oxidase function.

Finally, we demonstrated that bilirubin, the product of heme metabolism by HO, also exerts a potent suppression of NADPH oxidase activity. At a concentration of 100 nM, bilirubin decreased basal superoxide generation by ~70% and nearly abolished TNF-α–induced ROS generation, consistent with findings in reconstituted neutrophil NADPH oxidase.\(^\text{34}\) Although the underlying mechanism(s) of these actions of bilirubin is not entirely clear, a direct scavenging of superoxide is unlikely to make a major contribution.\(^\text{35}\) In fibroblasts, it has been reported that bilirubin at high concentrations inhibited protein kinase C–dependent protein phosphorylation,\(^\text{36}\) raising the possibility that modifying serine phosphorylation of p47phox and/or its translocation may be a mechanism of inhibition of NADPH oxidase activation. Consistent with this, we demonstrated that bilirubin pretreatment seemed to suppress p47phox intracellular redistribution induced by TNF-α, indicating that bilirubin may impact the activation of endothelial NADPH oxidase. It is noted that inhibition of HO-1 either pharmacologically or genetically did not increase superoxide production from NADPH oxidase in resting cells. This is not surprising, because the basal HO activity in the cultured cells is low, and bilirubin produced constitutively has little impact on NADPH oxidase.

![Figure 5. Effects of NONOate on NADPH oxidase subunit expression. (a) Effects of NONOate on the mRNA levels of Nox4, p22phox, and p47phox measured by quantitative real-time PCR. Results were expressed as 2^{-ΔΔCt} values (n=3). (b) Western blots showing the effects of NONOate (1000 μmol/L for 6 hours) on the protein level of Nox1, Nox2, and Nox4. Control cells were treated with vehicle (0.01 N NaOH). Equal protein load was determined by β-actin in the same blot. The average densitometry data were shown on the right (n=3).](http://hyper.ahajournals.org/)

![Figure 6. Effects of bilirubin on NADPH oxidase function in HMECs. (a) NADPH oxidase activity was assessed by NADPH (100 μmol/L)-stimulated lucigenin chemiluminescence. Cells were preincubated with bilirubin for 30 minutes before assay. Dimethyl sulfoxide was used as vehicle control (con). Data were expressed as count per second (CPS). n=4. (b) TNF-α (500 U/mL for 20 minutes)–induced ROS production was measured by DCFH-DA fluorescence. Bilirubin or dimethyl sulfoxide (con) was added 30 minutes before TNF-α stimulation. Data were expressed as percentage of the baseline level (100%). *P<0.05 vs control, n=6.](http://hyper.ahajournals.org/)
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NO Modulates NADPH Oxidase Function Via Heme Oxygenase-1 in Human Endothelial Cells

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Hypertension. 2006;48:950-957; originally published online September 18, 2006;
doi: 10.1161/01.HYP.0000242336.58387.1f
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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