Transcriptional and Posttranscriptional Regulation of Endothelial Nitric Oxide Synthase Expression by Hydrogen Peroxide

Grant R. Drummond, Hua Cai, Michael E. Davis, Santhini Ramasamy, David G. Harrison

Abstract—Diverse stimuli, including shear stress, cyclic strain, oxidized LDL, hyperglycemia, and cell growth, modulate endothelial nitric oxide synthase (eNOS) expression. Although seemingly unrelated, these may all alter cellular redox state, suggesting that reactive oxygen intermediates might modulate eNOS expression. The present study was designed to test this hypothesis. Exposure of bovine aortic endothelial cells for 24 hours to paraquat, a superoxide ($O_2^{-\cdot}$)–generating compound, did not affect eNOS mRNA levels. However, cotreatment with paraquat and either Cu^{2+}/Zn^{2+} superoxide dismutase or the superoxide dismutase mimetic tetrakis(4-benzoic acid)porphyrin chloride increased eNOS mRNA by 2.3- and 2.2-fold, respectively, implicating a role for H_2O_2. Direct addition of 100 and 150 μmol/L H_2O_2 caused increases in bovine aortic endothelial cell eNOS mRNA that were dependent on concentration (ie, 3.1- and 5.2-fold increases) and time, and elevated eNOS protein expression and enzyme activity, accordingly. Nuclear run-on and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole–chase studies showed that H_2O_2 caused a 3.0-fold increase in eNOS gene transcription and a 2.8-fold increase in eNOS mRNA half-life. Induction of eNOS by H_2O_2 was not affected by the hydroxyl radical scavenger DMSO, mannitol, or *N*-*tert*-*butyl*-α-phenylnitrone, but it was inhibited by the antioxidants *N*-acetylcysteine, ebselen, and exogenously added catalase. Unlike H_2O_2, the 4.0-fold induction of eNOS by shear stress (15 dyne/cm^2 for 6 hours) was not inhibited by *N*-acetylcysteine or exogenous catalase. In conclusion, H_2O_2 increases eNOS expression through transcriptional and post-transcriptional mechanisms. Although H_2O_2 does not mediate shear-dependent eNOS regulation, it is likely to be involved in regulation of eNOS expression in response to other physiological and/or pathophysiological stimuli. **(*Circ Res*. 2000;86:347-354.)**

Key Words: paraquat ■ superoxide dismutase ■ eNOS mRNA stability ■ cultured endothelial cells

Although endothelial nitric oxide synthase (eNOS) was originally thought to be a constitutively expressed enzyme, it has become clear in recent years that its expression can be modulated by a variety of chemical, physical, and developmental stimuli.[1] Regulation of eNOS expression occurs at both the transcriptional and post-transcriptional levels. For example, in cultured human and bovine endothelial cells, cyclic strain,[2] nordihydroguaiaretic acid (NDGA),[3] estrogens,[4] and the oxidized LDL (oxLDL) components 13-hydroperoxyoctadecadienoate[5] and lysophosphatidylcholine[6] have been shown to increase eNOS gene transcription. Likewise, transforming growth factor-β (TGF-β)[7] and laminar shear stress[8] increased the transcriptional activity of the 5′-promoter region of the eNOS gene as assessed by transfection of bovine aortic endothelial cells (BAECs) with chimeric eNOS promoter/luciferase constructs. In contrast, stimuli such as cell growth[9] and the 3-hydroxy-3-methylglutaryl–CoA reductase inhibitor, simvastatin[10] increase eNOS expression by prolonging the half-life of the eNOS mRNA.

A common property of many stimuli that increase eNOS expression is the ability to increase the production of reactive oxygen intermediates (ROIs) in endothelial cells. Thus, laminar shear stress,[11] cyclic strain,[12] oxLDL,[13] high glucose,[14] TGF-β[15] and proliferation[16] have all been associated with an elevated production of ROIs in endothelial cells. Furthermore, for many of these stimuli, this increase in production of ROIs was shown to be important for regulating expression of other genes. For example, induction of heme-oxygenase-1,[17] c-fos,[18] monocyte chemotactic protein-1,[19] and intercellular adhesion molecule-1 (ICAM-1)[20,21] in human umbilical vein endothelial cells by shear stress and cyclic strain was inhibited by the antioxidants *N*-acetylcysteine (NAC) and catalase. Similarly, the phenolic antioxidants probucol and vitamin E have been shown to inhibit oxLDL-mediated induction of ICAM-1 and vascular cell adhesion molecule-1 expression.[22] Finally, transcriptional induction of macrophage-colony stimulating factor by TGF-β_1 was inhibited by catalase but not by superoxide dismutase (SOD), suggesting that H_2O_2, rather than superoxide anions ($O_2^{-\cdot}$), mediates this effect.[23]

These observations raise the possibility that ROIs represent common signaling molecules for modulating eNOS expression. Indeed, the human, bovine, and murine eNOS promoter

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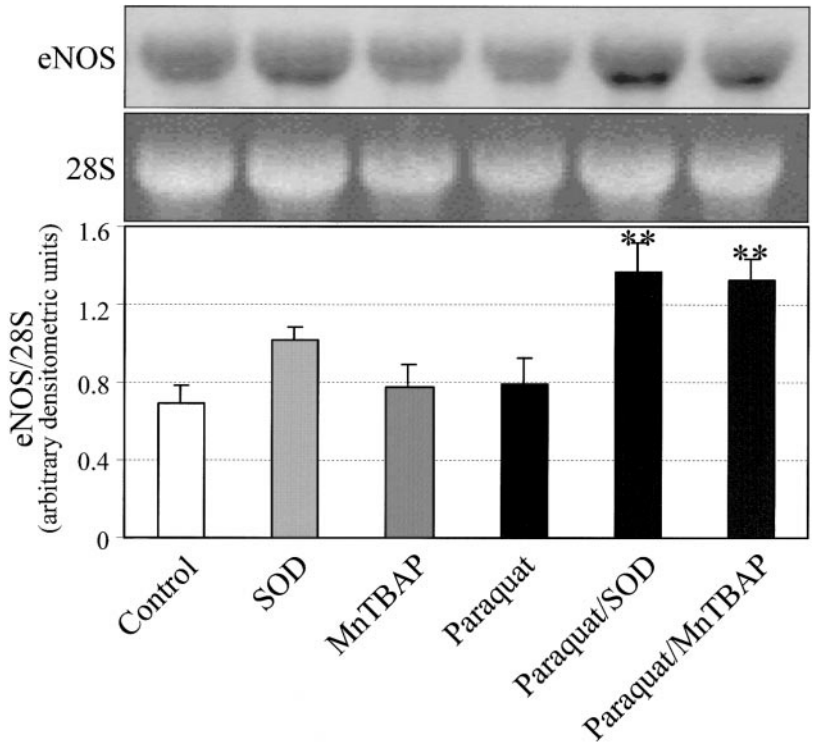


Figure 1. Northern analysis showing effects of paraquat, Cu^{2+}/Zn^{2+} SOD, and MnTBAP on eNOS mRNA expression in BAECs. Cells were treated with control medium or medium containing Cu^{2+}/Zn^{2+} SOD (100 U/mL) or MnTBAP (100 μmol/L) for 1 hour before incubation with paraquat (1 mmol/L) for 24 hours. Top panel is a representative blot, middle panel shows the corresponding 28S bands, and bottom panel contains the grouped densitometric data (mean±SEM) from 5 experiments. Asterisks represent eNOS mRNA levels significantly different from that in control cells (**$P<0.01$, Tukey-Kramer test after 1-way ANOVA).

regions contain putative binding sites for redox-sensitive transcription factors, including activator protein-1 (AP-1), Sp1, and antioxidant-responsive elements.[24–26] Thus, the present study was performed to examine the effects of ROIs on eNOS expression in both BAECs and human aortic endothelial cells (HAECs). Our results indicate that H_2O_2, but not $O_2^{-\cdot}$ or hydroxyl radicals ($OH^{\cdot}$), increases eNOS expression by increasing both the rate of transcription of the eNOS gene and the stability of the eNOS message once it is formed. Finally, because shear stress has been shown to increase both eNOS expression and endothelial cell ROI production, additional studies were performed to determine whether ROIs might mediate increased eNOS expression in response to shear stress.

Materials and Methods

Cell Culture

BAECs (Cell Systems) were cultured in medium 199 (GIBCO Laboratories) containing 10% FCS (Hyclone Laboratories) as described earlier.[5] Postconfluent BAECs between passages 4 and 10 were used for experiments. HAECs (Clonetics) were cultured in endothelial cell growth medium (Clonetics) containing 2% FCS. Postconfluent cells (passages 6 to 10) were used for experiments. In some experiments, endothelial cells were exposed to laminar shear stress (15 dyne/cm^2) using a cone-plate viscometer (cone angle 1°, rotational velocity 4 per second).[27]

Assessment of eNOS Expression

Northern analyses, Western blots, nuclear run-ons, and eNOS enzyme activity assays were performed as previously described.[5]

Drugs and Their Suppliers

Mn(III)tetrakis(4-benzoic acid)porphyrin chloride (MnTBAP), *N-tert-butyl-α*-phenylnitrone (BPN), 5,6-dichloro-1-*β*-D-ribofuranosylbenzimidazole (DRB), and ebselen were obtained from Calbiochem, and bovine liver catalase was from Boehringer Mannheim. All other chemicals were from Sigma. DMSO was used to dissolve BPN (1 mol/L) and DRB (25 mmol/L). MnTBAP (100 mmol/L) was dissolved in $NaHCO_3$ (1 mol/L). All other drugs were dissolved in dH_2O.

Results

Effect of Paraquat on eNOS mRNA Expression

To increase intracellular ROIs, endothelial cells were treated with paraquat. This xenobiotic redox cycles with intracellular flavins to produce $O_2^{-\cdot}$. In BAECs, paraquat (1 mmol/L) treatment for 24 hours had no effect on eNOS mRNA levels (Figure 1). In contrast, cotreatment with paraquat and either Cu^{2+}/Zn^{2+} SOD (100 U/mL) or MnTBAP (100 μmol/L) resulted in 2.3- and 2.2-fold increases in eNOS mRNA, respectively (Figure 1). In some experiments, treatment with Cu^{2+}/Zn^{2+} SOD alone appeared to cause a small increase in eNOS mRNA expression, although this failed to reach significance ($P=0.27$; Figure 1). MnTBAP alone had no effect (Figure 1).

H_2O_2 Increases eNOS Expression and Activity

The above results demonstrate that although eNOS expression is not affected by $O_2^{-\cdot}$, dismutation of $O_2^{-\cdot}$ potently stimulates eNOS expression. We therefore examined the effect of directly applied H_2O_2. Treatment of BAECs with 100 and 150 μmol/L H_2O_2 for 24 hours resulted in 3.1- and 5.2-fold increases in eNOS mRNA expression, respectively (Figure 2). The eNOS-inducing effect of H_2O_2 (100 μmol/L) was also time-dependent. After 6 hours of exposure to H_2O_2, eNOS mRNA expression was 1.6 times that in the time-matched controls. eNOS expression continued to increase with time, reaching levels that were 3.1 and 3.6 times those in the time-matched 12- and 24-hour control groups, respectively (Figure 2).

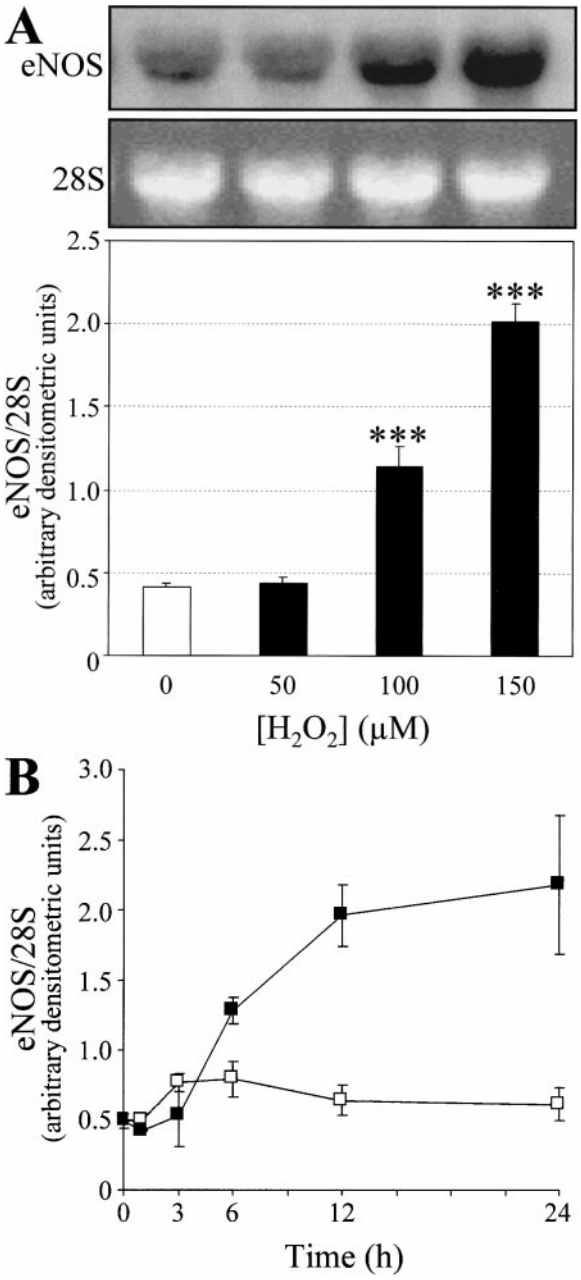


Figure 2. Northern analyses showing concentration-dependent (A) and time-dependent (B) effects of H_2O_2 on eNOS mRNA expression in BAECs. A, Cells were treated with control or H_2O_2 (50 to 150 μmol/L)–containing medium for 24 hours. B, Cells were exposed to control or H_2O_2 (100 μmol/L)–containing medium for 1, 3, 6, 12, and 24 hours. In panel A, top panel is a representative blot, middle panel shows the corresponding 28S bands, and bottom panel contains the grouped densitometric data (mean±SEM) from 8 experiments. Panel B shows the grouped densitometric data (mean±SEM) from 3 experiments. Asterisks represent eNOS mRNA levels significantly different from that in control cells (***$P<0.001$, Tukey-Kramer test after repeated-measures ANOVA).

H_2O_2 also caused a concentration-dependent increase in eNOS protein levels in BAECs as measured by Western analyses. eNOS protein expression in BAECs treated with 100 and 150 μmol/L H_2O_2 was 2.1 and 2.8 times that in control cells, respectively (Figure 3). Likewise, eNOS enzyme activity, as measured by conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline, was increased by H_2O_2 (Figure 3).

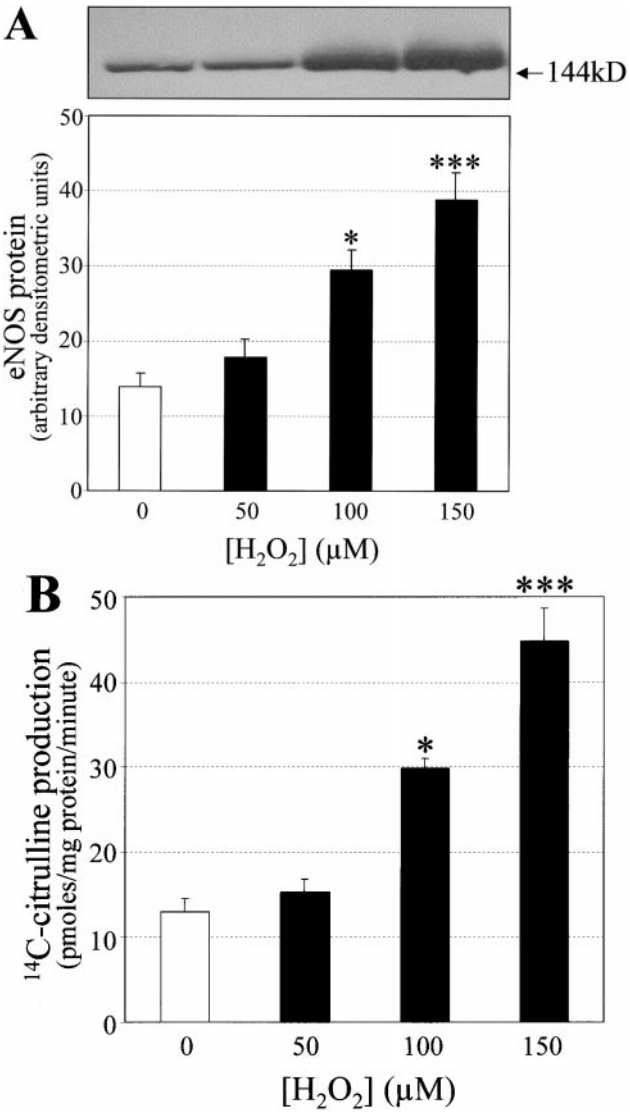


Figure 3. Western analysis (A) and eNOS activity assay (B) showing concentration-dependent increase in expression of functional eNOS protein in BAECs in response to H_2O_2. Cells were exposed to control or H_2O_2 (50 to 100 μmol/L)–containing medium for 24 hours before homogenization. In panel A, each lane was loaded with 15 μg of protein. A monoclonal antibody against human eNOS was used to detect bands. A representative blot is illustrated in the upper panel, whereas lower panel represents grouped densitometric data (mean±SEM) from 5 experiments. In panel B, 250 μg protein was used for the assay. Results (mean±SEM) represent grouped data from 5 experiments. Asterisks represent significantly different levels of eNOS protein expression or activity from that in control cells (*$P<0.05$, ***$P<0.001$, Tukey-Kramer test after 1-way ANOVA).

Similar to its effects on BAECs, H_2O_2 also increased eNOS protein expression in HAECs. In these cells, treatment with 100 and 200 μmol/L H_2O_2 caused 1.6- and 1.7-fold increases in eNOS protein expression, respectively (Figure 4).

Effect of Short-Term Exposure to H_2O_2 on eNOS Expression

Experiments were performed to determine the length of exposure needed for H_2O_2 to stimulate eNOS expression.

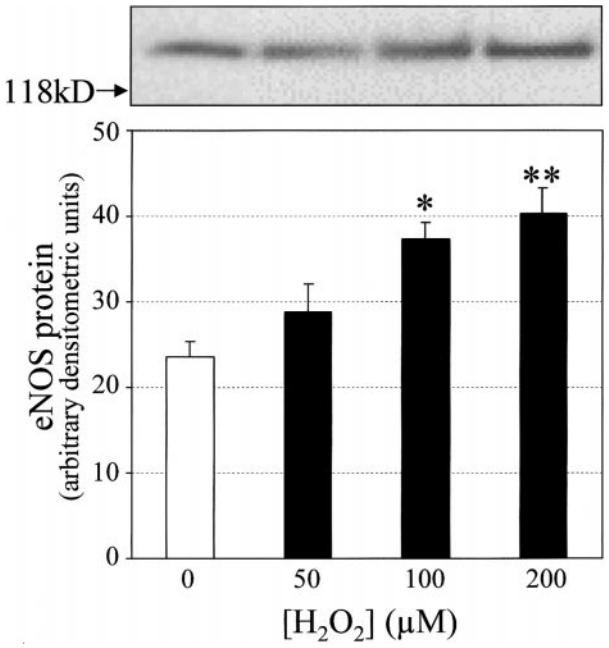


Figure 4. Western analysis showing concentration-dependent increase in eNOS expression in HAECs in response to H_2O_2. Cells were exposed to control or H_2O_2 (50 to 200 μmol/L)–containing medium and were harvested after 48 hours. Each lane was loaded with 15 μg protein. A monoclonal antibody against human eNOS was used to detect bands. Upper panel is a representative blot, whereas lower panel depicts grouped densitometric data (mean±SEM) from 3 experiments. Asterisks represent significantly different levels of eNOS protein expression from that in control cells (*$P<0.05$, **$P<0.01$, Tukey Kramer test after 1-way ANOVA).

BAECs were treated with H_2O_2 (100 μmol/L) for 0.25 to 12 hours. In all cases, cells were washed and exposed to control medium and harvested at 12 hours after the beginning of the H_2O_2 exposure. H_2O_2 exposure as short as 0.25 hours caused a 4.4-fold increase in eNOS mRNA expression (Figure 5). Maximal effects (ie, 8.3- and 7.8-fold increases compared with time-matched controls) occurred after 1- and 3-hour exposures (Figure 5).

Additional experiments were performed to determine how long eNOS expression remained elevated after short-term H_2O_2 exposure. Cells were harvested 6, 12, 24, 48, and 72 hours after a 1-hour-pulse exposure to H_2O_2 (100 μmol/L). At all of the time points tested, eNOS mRNA expression was significantly higher in H_2O_2-treated cells than in time-matched controls (Figure 5). Maximum effects of H_2O_2-pulse treatment occurred after 12 and 24 hours (7.6 and 8.4 times control levels, respectively; Figure 5). However, even at 72 hours after the 1-hour H_2O_2 pulse, eNOS mRNA expression remained elevated at 3.9 times that of control levels (Figure 5).

Effect of Scavengers on H_2O_2 Induction of eNOS

Because the effects of H_2O_2 could be mediated either directly or indirectly by OH^-, produced from H_2O_2 via Fenton chemistry, the effects of scavengers of H_2O_2 and OH^- were examined. Induction of eNOS mRNA expression by H_2O_2 was abolished by catalase (1000 U/mL) and was significantly inhibited by ≈50% by the glutathione peroxidase mimetic ebselen (40 μmol/L) (Figure 6). NAC (5 mmol/L), whether

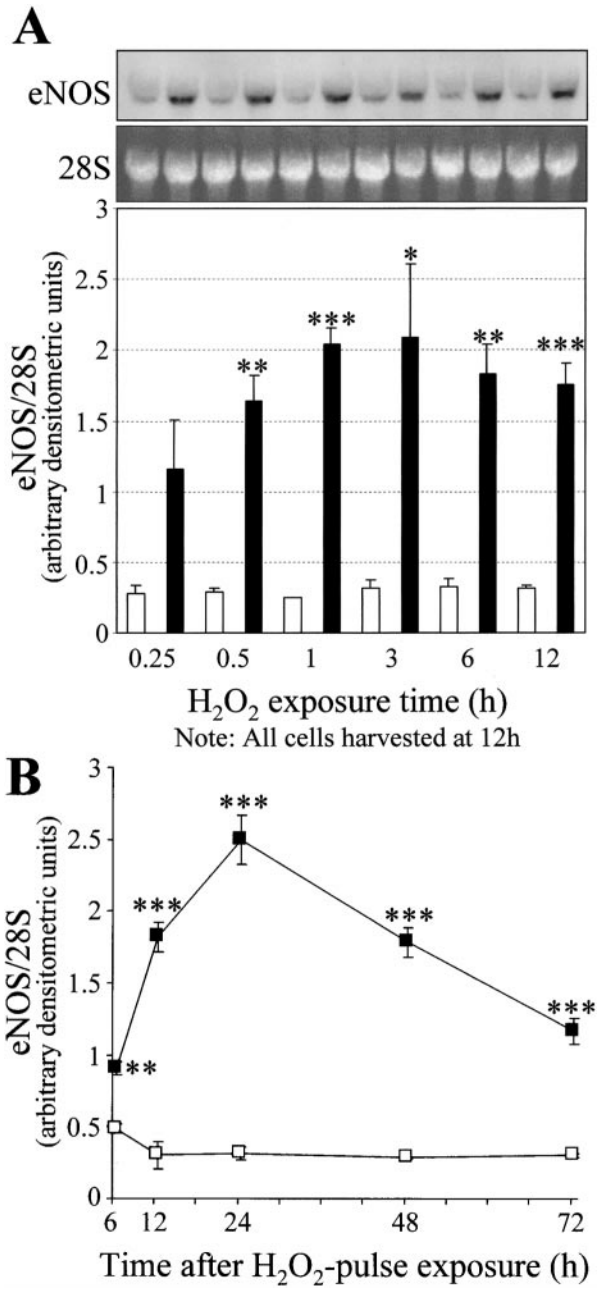
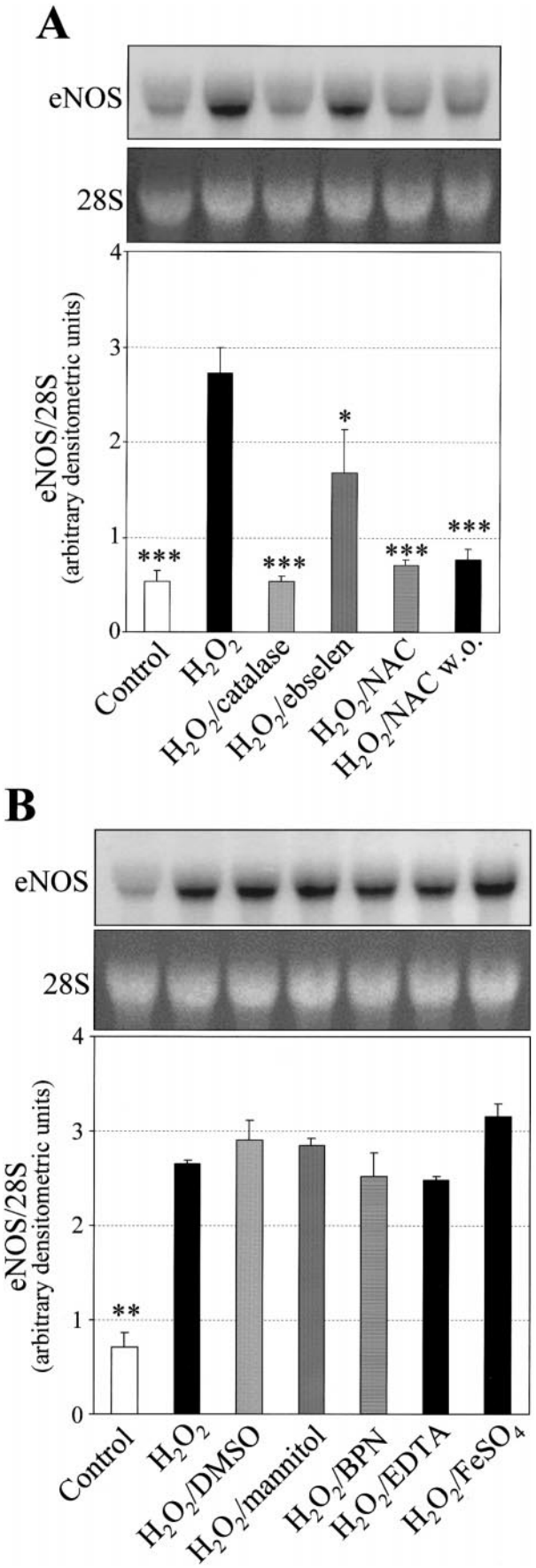


Figure 5. Northern analyses showing effects of short-term exposure to H_2O_2 on eNOS mRNA expression in BAECs. In panel A, cells were exposed to control (open bars) or H_2O_2 (100 μmol/L)–containing medium (filled bars) for 0.25, 0.5, 1, 3, or 6 hours. Cells were subsequently washed with control medium and further incubated until 12 hours after the initial exposure to H_2O_2, at which time they were harvested. Top panel is a representative blot, middle panel shows the corresponding 28S bands, and bottom panel depicts the grouped densitometric data (mean±SEM) from 3 experiments. In panel B, BAECs were exposed to control (□) or H_2O_2 (100 μmol/L)–containing medium (■) for 1 hour, before being washed with control medium, and harvested after 6, 12, 24, 48, and 72 hours. Values represent grouped densitometric data (mean±SEM) from 3 experiments. Asterisks represent significantly different levels of eNOS mRNA expression from those in time-matched controls (*$P<0.05$, **$P<0.01$, and ***$P<0.001$, Student unpaired t test).



retained in the medium throughout the H_2O_2 incubation or washed out just before the addition of H_2O_2, also abolished the increase in eNOS mRNA expression caused by H_2O_2 (Figure 6). None of these interventions affected basal eNOS mRNA expression (data not shown). In contrast, the $OH^{\cdot}$ scavengers DMSO (0.3%), mannitol (20 mmol/L), and BPN (1 mmol/L) had no effect on H_2O_2-induced eNOS mRNA expression (Figure 6). Moreover, cotreatment with H_2O_2 (100 μmol/L) and either of the Fenton chemistry–enhancing agents, EDTA (100 μmol/L) or $FeSO_4$ (100 μmol/L), was not more effective at inducing eNOS mRNA expression than was H_2O_2 alone (Figure 6). Thus, these data indicate that the effect of H_2O_2 on eNOS expression is unlikely to be mediated by $OH^{\cdot}$.

Effect of H_2O_2 on eNOS Gene Transcription and eNOS mRNA Stability

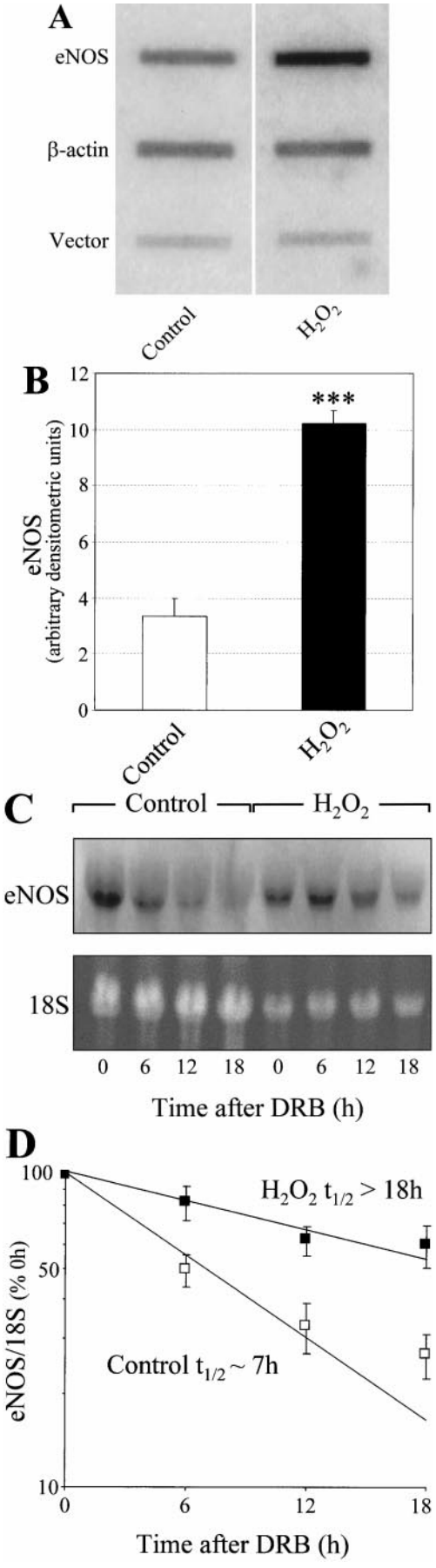
To examine the effect of H_2O_2 on eNOS mRNA transcription rate, nuclear run-on analyses were performed. BAECs were treated with 100 μmol/L H_2O_2 for 6 hours and nuclei harvested. H_2O_2-treated BAECs produced 3.0 times the amount of radiolabeled eNOS mRNA transcripts of those made by control cells (Figure 7). In contrast, H_2O_2 had no effect on transcription rate of the housekeeping gene, β-actin (Figure 7).

To determine the effects of H_2O_2 on eNOS mRNA stability, BAECs were incubated with control or H_2O_2 (100 μmol/L)–containing medium for 6 hours. Next, cells from each group were treated with the RNA polymerase II inhibitor DRB (60 μmol/L). The rate of eNOS mRNA decay was significantly slower in H_2O_2-treated cells than it was in control cells (Figure 7). In H_2O_2-treated cells, the half-life of the eNOS message was $\approx$20 hours compared with $\approx$7 hours in control cells. Thus, H_2O_2 increases eNOS expression both by increasing the rate of transcription and by elevating the half-life of eNOS mRNA.

Effect of H_2O_2 Scavengers on Shear-Induced eNOS Expression

Shear stress has been shown to increase both eNOS expression and production of ROIs by the endothelium. To determine whether H_2O_2 produced in response to shear stress might mediate the increase in eNOS expression, cells were exposed to a laminar shear of 15 dyne/cm^2 for 6 hours. In the

Figure 6. Northern analyses showing effects of H_2O_2 scavengers (A) and $OH^{\cdot}$ scavengers/generators (B) on eNOS mRNA expression in BAECs. Cells were exposed to control medium or medium containing catalase (1000 U/mL), ebselen (40 μmol/L), NAC (5 mmol/L), DMSO (0.3%), mannitol (20 mmol/L), BPN (1 mmol/L), EDTA (100 μmol/L), or $FeSO_4$ (100 μmol/L) for 1 hour before exposure to H_2O_2 (100 μmol/L) for 24 hours. In all groups except the studies in which NAC was washed out before H_2O_2 was added (NAC w.o.), the antioxidant/$OH^{\cdot}$ generator was retained in the medium throughout the H_2O_2 incubation period. In panels A and B, top panels are representative blots, middle panels show the corresponding 28S bands, and bottom panels depict grouped densitometric data (mean$\pm$SEM) from 3 experiments. Asterisks indicate significantly different levels of eNOS mRNA expression from those in groups treated with H_2O_2 (100 μmol/L) alone (*$P<0.05$, **$P<0.01$, and ***$P<0.001$, Dunnett test after 1-way ANOVA).



absence of other interventions, shear caused a 4.0-fold increase in eNOS mRNA expression. Addition of exogenous catalase or NAC had no effect on induction of eNOS by shear (Figure 8). Thus, the increase in eNOS expression produced by shear stress is unlikely to be caused by endogenously produced H_2O_2.

Discussion

The important finding from this study is that expression of eNOS in endothelial cells is upregulated by H_2O_2. We have shown that short-term exposure of BAECs to H_2O_2, but not to $O_2^{-\cdot}$ or $OH^{\cdot}$, causes a sustained increase in eNOS mRNA and functional protein expression. Furthermore, this effect was mediated both by an increase in transcription rate of the eNOS gene and by an increase in stability of the eNOS message after its formation.

In recent years, it has become apparent that ROIs play an important role in gene regulation in response to several stimuli.[28] These effects likely result from stimulation of intracellular phosphorylation cascades, leading to activation of transcription factors. In the present study, we demonstrated a 3-fold increase in transcription rate of the eNOS gene in response to H_2O_2. The 5′-promoters of the human, bovine, and murine eNOS genes contain putative binding sites for a number of redox-regulated transcription factors, including AP-1, Sp1, and antioxidant-responsive elements.[24–26] In the present study, we did not examine the role of the 5′-promoter in the transcriptional induction of eNOS by H_2O_2. However, a previous study showed through gel-shift assays that the H_2O_2 generating system, glucose oxidase, increased binding of BAEC nuclear proteins to a radiolabeled probe containing an eNOS promoter AP-1 binding site.[29] It is thus tempting to speculate that AP-1 and perhaps other redox-sensitive *cis*-acting elements are involved in the response to H_2O_2.

In addition to increasing eNOS gene transcription, H_2O_2 also enhanced the stability of the eNOS message. Modulation of eNOS mRNA stability is now known to represent an important mechanism for regulation of expression of the enzyme by a number of stimuli. For example, tumor necrosis

Figure 7. Nuclear run-on analysis and DRB-chase experiments demonstrating increase in both rate of eNOS gene transcription and eNOS mRNA stability after treatment with H_2O_2 (100 μmol/L). In panels A and B, nuclei, harvested from BAECs after 6-hour exposure to control or H_2O_2-containing medium, were incubated with [^{32}P]UTP and unlabeled ATP, CTP, and GTP, in reaction buffer for 30 minutes at 30°C. The resulting radiolabeled transcripts from each group were added in equal counts to membranes previously slot-blotted with eNOS, β-actin, and vector DNA. Hybridization was allowed to proceed for 72 hours at 68°C. Panel A is a representative blot, whereas panel B shows the grouped densitometric data (mean±SEM) from 3 experiments. In panels C and D, BAECs were treated with control or H_2O_2-containing medium for 6 hours before the addition of DRB (60 μmol/L). Cells were then harvested at the time points indicated and Northern analyses performed to determine the rate of eNOS mRNA decay in each group. In panel C, top panel is a representative blot, whereas bottom panel shows the corresponding 18S bands. Note: Double the amount of RNA was loaded for the control groups to enhance visualization of eNOS bands at the later time points. Panel D shows the grouped densitometric data (mean±SEM) from 4 experiments. Asterisks indicate significant difference from control (***$P<0.001$; Student unpaired *t* test).

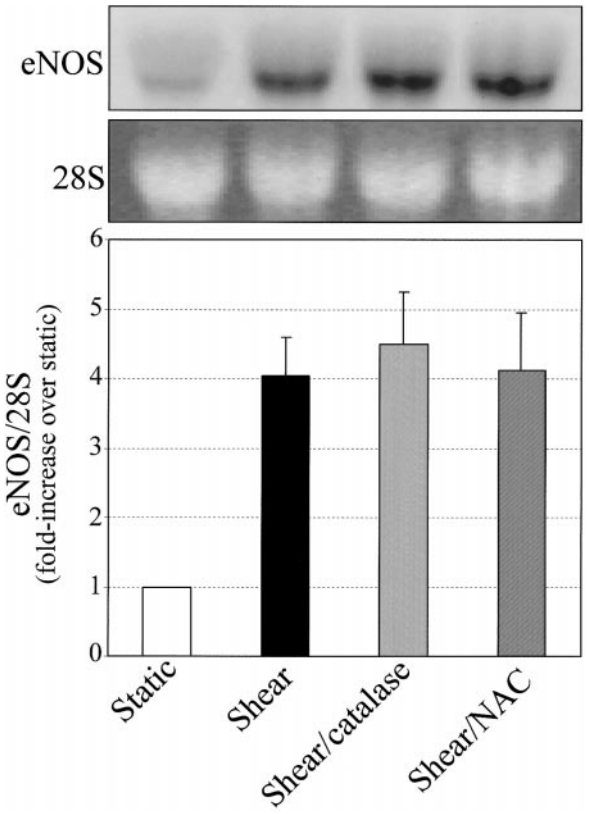


Figure 8. Northern analyses showing effects of H_2O_2 scavengers on eNOS induction by shear stress. BAECs were treated for 1 hour with control or with medium containing catalase (1000 U/mL) or NAC (5 mmol/L), before exposure to laminar shear stress (15 dyne/cm^2), for 6 hours. Top panel is a representative blot, middle panel shows the corresponding 28S bands, and bottom panel depicts the grouped densitometric data (mean±SEM) from 3 experiments.

factor-α decreases eNOS expression by reducing the stability of the eNOS message.[30] In addition, stabilization of the eNOS message augments eNOS expression in response to cell growth[9] and 3-hydroxy-3-methylglutaryl–CoA reductase inhibitors.[10] This effect of cell growth on eNOS mRNA stability has been found to be due to decreased binding of a 51-kDa mRNA-destabilizing protein to a G+C-rich region near the start of the 3′-untranslated region.[9] Interestingly, preliminary UV cross-linking studies in our laboratory showed that H_2O_2 similarly decreased binding of a 51-kDa protein to the eNOS mRNA 3′-untranslated region.

Of note, in preliminary studies, we found that the duration of exposure to H_2O_2 required to elicit an increase in eNOS expression was substantially longer in HAECs than in BAECs. Although the explanation for this remains unclear, it may relate to the longer eNOS mRNA half-life in human[30] as compared with bovine endothelial cells (ie, >24 hours versus 7 to 8 hours). Therefore, although a 24-hour exposure to H_2O_2 in BAECs is equivalent to 3 eNOS mRNA half-lives, it is only equal to 1 half-life in HAECs. Because at least half of the effects of H_2O_2 on eNOS are mediated by enhancing mRNA stability, longer exposures to H_2O_2 may be necessary to impact on steady-state eNOS mRNA levels in human cells.

Recently, we demonstrated that phenolic antioxidants, including NDGA and probucol, increased eNOS mRNA and protein expression.[3] Although these findings may seem to be at odds with those of the present study, it is known that phenolic antioxidants can exert both antioxidant and pro-oxidant effects, depending on the prevailing redox environment. Abstraction of the hydrogen atom from the phenolic group of such antioxidants by $O_2^{-\cdot}$ results in formation of a phenoxyl radical (itself a pro-oxidant species) along with H_2O_2, which could in turn induce eNOS expression. Augmentation of the effects of NDGA by the reducing compounds NAC and ascorbic acid, observed in our previous study,[3] could be explained if these compounds were acting to promote reduction of the phenoxyl radical back to the parent compound,[31] thus allowing further participation in redox cycling.

Diverse stimuli, including cyclic strain, shear stress, and TGF-β_1, have been shown to induce endothelial cell expression of different genes in a manner that is abolished by NAC and catalase,[17–21,23] implying that the upregulation is dependent on generation of endogenous H_2O_2. In these same studies, exogenous H_2O_2 in concentrations of 100 to 150 μmol/L induced a similar magnitude of expression of these genes. Thus, although the concentration of H_2O_2 used in this study and previous studies may seem high, these concentrations likely have physiological and pathophysiological significance. Because of intracellular catalase, glutathione peroxidase, and other antioxidants, the ultimate intracellular concentration of H_2O_2 is likely to be substantially lower than that added exogenously. Importantly, these concentrations of H_2O_2 exerted little or no microscopically visible toxic effects on endothelial cells.

To examine the physiological role of H_2O_2 in regulation of eNOS expression, we studied the effects of antioxidants on eNOS induction by shear stress. Several studies have shown that shear stress increases ROIs within the endothelium,[8,11,18,20] probably via activation of an NADH-dependent oxidase.[17] Furthermore, activation of heme-oxygenase-1,[17] c-fos,[18] and ICAM-1[20] by shear stress was inhibited by catalase and NAC, indicating that ROIs are likely to be important in induction of these genes. Additionally, we have shown that laminar shear stress induces Cu^{2+}/Zn^{2+} SOD expression in BAECs,[32] which should shift the balance of ROIs from $O_2^{-\cdot}$ to H_2O_2. We therefore hypothesized that eNOS induction by shear stress may be due to H_2O_2 production. This hypothesis was proven incorrect, however, as shear stress induction of eNOS was not inhibited by NAC or catalase, both of which abolished eNOS upregulation by H_2O_2. Moreover, preliminary studies in our laboratory demonstrate that the intracellular signaling pathways mediating the effects of H_2O_2 and shear stress on eNOS expression are different. For example, eNOS induction by shear stress seems to be inhibited by the c-Src inhibitor PP1. In contrast, H_2O_2-dependent eNOS induction is not blocked by this compound but can be inhibited by tyrphostin A25 and herbimycin A, implicating a role for a non–Src-related tyrosine kinase in the transduction pathway.

Although H_2O_2 does not mediate the effect of shear stress on eNOS expression, it may be important in modulating eNOS expression in other settings. The production of $O_2^{-\cdot}$ by vascular cells is increased in several pathophysiological

conditions, including hypertension, hypercholesterolemia, and diabetes. This increase in $O_2^{-\cdot}$ production is responsible for decreasing the bioactivity and thus beneficial actions of $NO^{\cdot}$ in these disease states. Because $O_2^{-\cdot}$ is a precursor to other ROIs, including H_2O_2, it is likely that this response of increased eNOS expression represents an important compensatory mechanism. Indeed, in several pathological conditions, including early hypercholesterolemia,[33] hypertension,[34] and diabetes (T. Münzel, personal communication), eNOS expression is increased. It is therefore interesting to speculate that the increase in ROIs (especially H_2O_2) in these diseases underlies the increase in eNOS expression.

Acknowledgments

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