Mitochondria-Derived Reactive Oxygen Species Mediate Heme Oxygenase-1 Expression in Sheared Endothelial Cells

Zhaosheng Han, Saradhadevi Varadharaj, Randy J. Giedt, Jay L. Zweier, Hazel H. Szeto, and B. Rita Alevriadou

ABSTRACT

Bovine aortic endothelial cells (ECs) respond to nitric oxide (NO) donors by activating the redox-sensitive NF-E2-related factor 2/antioxidant response element pathway and up-regulating heme oxygenase (HO)-1 expression. EC exposure to steady laminar shear stress causes a sustained increase in NO, a transient increase in reactive oxygen species (ROS), and activation of the HO-1 gene. Because steady laminar flow increases the mitochondrial superoxide (O$_2^-$) production, we hypothesized that mitochondria-derived ROS play a role in shear-induced HO-1 expression. Flow (10 dynes/cm$^2$, 6 h)-induced expression of HO-1 protein was abolished when BAECs were preincubated and sheared in the presence of either N$^2$-nitro-$\omega$-arginine methyl ester or N-acetyl-$\omega$-cysteine, suggesting that either NO or ROS up-regulates HO-1. Ebselen and diphenylene iodonium blocked HO-1 expression, and uric acid had no effect. The mitochondrial electron transport chain inhibitors, myxothiazol, rotenone, or antimycin A, and the mitochondrial-targeted antioxidant peptide, Szeto-Schiller (SS)-31, which scavenges O$_2^-$, hydrogen peroxide (H$_2$O$_2$), peroxynitrite, and hydroxyl radicals, markedly inhibited the increase in HO-1 expression. These data collectively suggest that mitochondrial H$_2$O$_2$ mediates the HO-1 induction. MitoSOX and 2',7'-dichlorofluorescein (DCF) fluorescence showed that mitochondrial O$_2^-$ levels and intracellular peroxides, respectively, are higher in sheared ECs compared with static controls and, in part, depend on NO. SS-31 significantly inhibited both the shear-induced MitoSOX and DCF fluorescence signals. Either phosphatidylinositol 3-kinase or mitogen-activated protein kinase cascade inhibitors blocked the HO-1 induction. In conclusion, under shear, EC mitochondria-derived H$_2$O$_2$ diffuses to the cytosol, where it initiates oxidative signaling leading to HO-1 up-regulation and maintenance of the atheroprotective EC status.

The stress proteins heme oxygenases, which consist of constitutive and inducible isozymes (HO-2 and HO-1, respectively), catalyze the rate-limiting step in the degradation of heme to the bile pigments, biliverdin and bilirubin, eventually releasing iron and carbon monoxide. Because the HO reaction products are known to provide cytoprotection from inflammatory diseases, including atherosclerosis, mechanisms of HO-1 induction have been studied extensively (Ryter et al., 2006). HO-1 is induced by chemicals that produce oxidative stress. The signaling pathways leading to the transcriptional regulation of the HO-1 gene are variable in a cell- and inducer-specific fashion, although all of them involve the...
participation of protein phosphorylation cascades (Ryter et al., 2006). NF-E2-related factor 2 (Nrf2) is the transcription factor that, upon activation by oxidative stress, translocates to the nucleus, binds to the antioxidant response element (ARE), and activates transcription of phase II genes, including HO-1 (Nguyen et al., 2004). Under physiological conditions, Kelch-like erythroid-derived Cap-N-Collar-Homology-associated protein 1 (Keap1), a suppressor that binds to Nrf2, retains it in the cytoplasm and promotes its proteasomal degradation. Specific cysteine residues of Keap1 are the sensors that recognize inducers of phase II genes. Under oxidative stress, lipid oxidation products, such as the electrophilic lipid 15-deoxy-A2,14-prostaglandin J2 (15d-PGJ2), may bind to Keap1 and cause alkylation of its critical cysteine residues, rendering Keap1 unable to repress Nrf2. Nrf2 activation may also occur when the inducers or their metabolites stimulate Nrf2 phosphorylation through redox-sensitive protein kinases, which triggers its dissociation from Keap1, its nuclear translocation, and initiation of ARE-dependent transcription (Nguyen et al., 2004).

Cultured EC exposure to steady laminar shear stress causes a sustained increase in nitric oxide (NO) and a transient one in superoxide (O2-) production, and the shear-induced reactive oxygen species (ROS) act as second messengers in the induction of gene expression (Kuchan and Frangos, 1994; Chiu et al., 1997; Yeh et al., 1999; Han et al., 2007). Human aortic EC exposure to steady laminar shear stress was shown to activate HO-1 at the transcriptional level and to involve the Keap1-Nrf2 pathway and 15d-PGJ2 (Chen et al., 2003; Hosoya et al., 2005). Although it is accepted that induction of ARE-regulated genes is a compensatory response to ROS production (Jones et al., 2007; Warabi et al., 2007), more studies are needed to understand the intracellular mechanisms that regulate this event. HO-1 expression data from sheared human umbilical ECs (HUVECs) in the presence of ROS scavengers suggested that xanthine oxidase, NAD(P)H oxidase, and mitochondria-derived O2- but not NO, mediates HO-1 expression (Warabi et al., 2007). However, agents that release NO induce HO-1 expression in aortic ECs, possibly via formation of S-nitrosothiols (SNO) in the regulatory cysteine residues of Keap1 (Mottolini et al., 2002; Buckley et al., 2003, 2008). We have shown that, in sheared ECs of either aortic or venous origin, endogenous NO by itself and via formation of peroxynitrite (ONOO-) in the mitochondria results in inactivation of the electron transport chain (ETC) and increased O2 production (Han et al., 2007; Jones et al., 2008). Because steady laminar flow increases O2 production by the ETC and oxidative stress mediates HO-1 expression, the purpose of the present study was to better understand the role of mitochondria-derived ROS in the latter event and the signaling pathways that regulate it.

Our study was conducted on bovine aortic ECs (BAECs) exposed to 10 dynes/cm2 for 6 h. To examine whether the shear-induced increase in HO-1 protein expression depends on mitochondrial function, and NO is involved, ECs were preincubated and sheared in the presence of the NO synthase (NOS) inhibitor, Nω-nitro-l-arginine methyl ester (l-NAME); ROS scavengers, such as N-acetyl-l-cysteine (NAC), ebselen, and uric acid (UA); or mitochondrial ETC inhibitors, such as rotenone, myxothiazol, and antimycin A. To delineate the role of mitochondrial ROS, the effect of a mitochondria-targeted antioxidant peptide, Szeto-Schiller (SS)-31 (d-Arg2,6-dimethyltyrosine-Lys-Phe-NHz), which exhibits 1000-fold mitochondrial accumulation and scavenges O2- hydrogen peroxide (H2O2), ONOO-, and hydroxyl radicals (OH-) (Szeto, 2006, 2008), was examined. SS-31 belongs to a family of aromatic cationic tetrapeptides that owe their ROS scavenging activity to the phenolic group on tyrosine or dimethylyrosine (replacement with phenylalanine resulted in a nonscavenging analog, SS-20). Tyrosine-containing SS peptides were shown to reduce mitochondrial ROS levels in epithelial and neuronal cells exposed to ETC inhibitors and to protect from mitochondrial permeability transition and cytotoxicity (Zhao et al., 2004; Szeto, 2004, 2008). Our results confirmed a major role of the mitochondria-derived H2O2, resulting from dissmutation of O2- produced by the ETC, in the shear-induced up-regulation of HO-1 expression.

**Materials and Methods**

**EC Culture.** BAECs were purchased from Cambrex (East Rutherford, NJ) and cultured in Dulbecco’s modified Eagle’s medium (low glucose with l-glutamine and sodium bicarbonate) supplemented with 10% fetal bovine serum, and 100 U/ml streptomycin (Invitrogen, Carlsbad, CA) in a humidified atmosphere of 95% air-5% CO2. ECs (passages 3–9) were seeded onto glass slides (75 × 38 mm; Thermo Fisher Scientific, Waltham, MA) that were sterilized, air-dried, and coated with a 0.5% gelatin-subbing solution. The intracellular mechanisms that regulate this event. HO-1 expression data from sheared human umbilical ECs (HUVECs) in the presence of ROS scavengers suggested that xanthine oxidase, NAD(P)H oxidase, and mitochondria-derived O2- but not NO, mediates HO-1 expression (Warabi et al., 2007). However, agents that release NO induce HO-1 expression in aortic ECs, possibly via formation of S-nitrosothiols (SNO) in the regulatory cysteine residues of Keap1 (Mottolini et al., 2002; Buckley et al., 2003, 2008). We have shown that, in sheared ECs of either aortic or venous origin, endogenous NO by itself and via formation of peroxynitrite (ONOO-) in the mitochondria results in inactivation of the electron transport chain (ETC) and increased O2 production (Han et al., 2007; Jones et al., 2008). Because steady laminar flow increases O2 production by the ETC and oxidative stress mediates HO-1 expression, the purpose of the present study was to better understand the role of mitochondria-derived ROS in the latter event and the signaling pathways that regulate it.

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**EC Exposure to Shear Stress and Antioxidants.** ECs were serum-starved overnight in Dulbecco’s modified Eagle’s medium with 0.5% fetal bovine serum and antibiotics and exposed to steady (gravity-driven) laminar shear stress of 10 dynes/cm2 (in the low range of human arterial shear stresses) for either 30 min or 6 h in the same medium. In brief, three glass slides with EC monolayers were assembled side-by-side into a parallel-plate flow chamber, and the chamber was connected at both ends to a reservoir forming a flow loop, as described previously (Frangos et al., 1985; Han et al., 2007). A schematic diagram of a flow loop is provided in Supplemental Fig. 1. Flow rate through the chamber was monitored by an ultrasonic flow sensor (Transonic Systems Inc., Ithaca, NY). Recirculating medium was constantly exposed to a countercurrent flow of a sterile-filtered gas mixture (95% air-5% CO2) that was warmed and humidified by bubbling through water. The temperature of the entire system was kept at 37°C, and medium temperature was monitored real-time by an inline sensor (World Precision Instruments, Inc., Sarasota, FL). For static controls, ECs were placed in tissue culture incubators for the same period of time as the sheared monolayers. Some monolayers were preincubated with the NOS inhibitor, l-NAME (1 mM for 4 h); the nonspecific antioxidant and glutathione (GSH) precursor, NAC (10 mM for 30 min); the GSH peroxidase mimetic and ONOO- scavenger, ebselen (5 μM for 1 h); the flavoprotein inhibitor, diphenylene iodonium (DPI; 20 μM for 30 min), which inhibits the NAD(P)H oxidase, ROS, xanthine oxidase, cytochrome P450 reductase, and the mitochondrial ETC flavoproteins (including the flavoprotein subcomplex of complex I); the ONOO- scavenger, UA (100 μM for 30 min); the inhibitors of ETC complex III, myxothiazol and antimycin A (each at 10 μM for 30 min); and the inhibitor of ETC complex I, rotenone (2 μM for 1 h), and either subjected to shear or left static in medium containing the same concentration of the respective drug (all from Sigma-Aldrich). Some ECs were preincubated with either the mitochondria-targeted antioxidant peptide SS-31 (1, 10, or 100 nM for 1 h) or the nonscavenging SS-20 and subjected to shear with the same concentration of drug (a gift from Dr. Szeto, Cornell University) (Zhao et al., 2004). Last, some ECs were preincubated with the phosphatidylinositol 3-kinase
and Intracellular O2
488-nm excitation/525-nm emission) were used to detect mitochondrial H2O2-mediated fluorescence. MitoSOX red (514-nm excitation/585-nm emission) and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; 488-nm excitation/525-nm emission) were used to detect mitochondrial O2\(^2\) and intracellular peroxides, respectively, and 4',6-diamidino-2-phenylindole (DAPI; 359-nm excitation/461-nm emission) to label cell nuclei (Invitrogen, Carlsbad, CA). DCFH-DA is converted to DCF inside the cell, and on exposure to either H2O2 or ONOO\(^-\), it is oxidized to the green fluorescent product 2',7'-dichlorodihydrofluorescein (DCF) (Tarpey et al., 2004). MitoSOX Red is a mitochondria-targeted form of dihydroethidium that is relatively specific for O2\(^2\) and undergoes oxidation to form the DNA-binding red fluorescent ethidium bromide (Tarpey et al., 2004). Before shear exposure, EC monolayers were incubated with either DCFH-DA (10 \(\mu\)M) or MitoSOX red (5 \(\mu\)M) and DAPI (1 \(\mu\)M) for 10 min in the incubator. At the end of shear (30 min), ECs were washed with PBS, mounted with Fluormount-G (Southern Biotechnology Associates, Birmingham, AL), and images (62 \(\times\)) were obtained by confocal microscopy (Zeiss LSM 510; Carl Zeiss Inc., Thornwood, NY) and overlaid using LSM software. Digital images from three fields of view were collected per experiment and corrected for autofluorescence, and background fluorescence was excluded from calculations by thresholding. The mean fluorescence intensity per image was calculated and averaged over the three images, using MetaMorph software (Molecular Devices, Sunnyvale, CA).

**Statistical Analysis.** Both HO-1/\(\beta\)-actin protein expression and fluorescence data were normalized to corresponding static controls and expressed as mean \(\pm\) S.E.M. of \(n = 3\) independent experiments. Significant differences among treatments were determined by using one-way analysis of variance followed by Bonferroni’s test for pairwise comparisons. \(P\) values < 0.05 were considered significant.

**Results**

**NO and ROS Are Responsible for the HO-1 Induction by Shear Stress.** BAEC exposure to steady laminar shear stress (10 dynes/cm\(^2\), 6 h) caused a 6-fold increase in HO-1 protein expression (Fig. 1). To investigate the role of shear-induced NO and ROS in HO-1 induction, ECs were preincubated and sheared in the presence of the NOS inhibitor L-NAME (1 mM) and the antioxidant and GSH precursor NAC (10 mM), respectively. Although NAC was slightly more potent, either L-NAME or NAC abolished the shear-induced increase in HO-1 expression, suggesting that both NO and ROS play important roles in HO-1 induction (Fig. 1).

**H2O2 Mediates the HO-1 Induction, and Mitochondrial Function Is Required.** To determine the particular ROS that mediates the increase in HO-1 protein expression, ECs were preincubated and sheared in the presence of either the GSH peroxidase mimetic and ONOO\(^-\) scavenger ebselen (5 \(\mu\)M), the flavoprotein inhibitor DPI (20 \(\mu\)M), or the ONOO\(^-\) scavenger UA (100 \(\mu\)M). Ebselen abolished the HO-1 induction, suggesting that either H2O2 (or its metabolite OH\(^-\)) or ONOO\(^-\) is involved (Fig. 2A). DPI also abolished the HO-1 induction, suggesting that the O2\(^2\) produced by NAD(P)H oxidase, NOS, the mitochondrial ETC, and other ROS sources, which contain flavoproteins, is upstream in the redox signaling pathways that regulate HO-1 expression (Fig. 2A). UA did not affect the shear-induced response; hence, ONOO\(^-\) is not involved (Fig. 2A). These data collectively suggest that H2O2 (and/or secondary OH\(^-\)), dismutated from flavoprotein-derived O2\(^2\), plays a role in HO-1 induction.

When ECs were preincubated and sheared in the presence of the ETC complex III inhibitors myxothiazol and antimycin...
A (10 M), myxothiazol abolished and antimycin A significantly inhibited the shear-induced increase in HO-1 expression (Fig. 2B). The ETC complex I inhibitor rotenone (2 M) also significantly blocked the shear-induced response (Fig. 2B). These data suggest that the activities of complexes I and III of the mitochondrial ETC contribute to the pathways leading to HO-1 induction.

**Mitochondrial H\textsubscript{2}O\textsubscript{2} Is Responsible for the Increase in HO-1 Expression.** To investigate the role of mitochondrial ROS, ECs were preincubated and sheared in the presence of either the mitochondria-targeted antioxidant peptide SS-31 (1, 10, or 100 nM) or its nonscavenging analog SS-20. SS-31 displayed a biphasic dose-response relationship, where it abolished HO-1 induction at the middle dose (10 nM) and afforded a significant inhibition at 1 nM and a modest one at 100 nM (Fig. 3). SS-20 had no effect at either dose tested (only 10 nM is shown; Fig. 3). Because SS-31 is known to scavenge mitochondrial O\textsubscript{2}
·H\textsubscript{2}O\textsubscript{2} and ONOO
· and, to a lesser extent, OH\textsuperscript· (Szeto, 2008), and H\textsubscript{2}O\textsubscript{2} can freely diffuse to the cytosol (Cadenas, 2004), our data suggest that the mitochondrial H\textsubscript{2}O\textsubscript{2} plays an important role in shear-induced HO-1 up-regulation.

**Mitochondrial H\textsubscript{2}O\textsubscript{2} Is a Major Source of Intracellular ROS in Sheared ECs.** MitoSOX red fluorescence was significantly higher in sheared (30 min) ECs compared with static controls, suggesting a shear-induced increase in O\textsubscript{2}.\textsuperscript· production by the ETC (Fig. 4, A and B). Rotenone and antimycin A enhanced the MitoSOX red fluorescence in static ECs, as expected (Turrens, 2003), but significantly inhibited the increase in fluorescence in sheared ECs (Fig. 4, A and B). This is probably because shear exposure inhibits complex I and III activities by ~80 and ~70%, respectively (Han et al., 2007), and, at least in the case of complex III, decreased complex activity reduces the enzyme-mediated O\textsubscript{2}.\textsuperscript· production (Chen et al., 2006). L-NAME did not affect the fluorescence signal in static ECs but significantly inhibited the increase in fluorescence in sheared ECs (Fig. 4, A and B). At each concentration tested, SS-31 significantly inhibited the MitoSOX red fluorescence in sheared ECs (10 and 100 nM totally abolished the signal; only 1 and 10 nM are shown), whereas SS-20 had no effect (only 10 nM is shown; Fig. 4, A and B).

When ECs were pretreated with ebselen, rotenone, L-NAME, SS-31, or SS-20 and with DCFH-DA and then left static (30 min) in the presence of the respective drug, DCF
fluorescence was not affected in any case, except in the case of rotenone, which caused a significant increase (only rotenone is shown; Fig. 5, A and B). When ECs were pretreated as before and then sheared (30 min) in the presence of the respective drug, the shear-induced increase in DCF fluorescence was abolished by ebselen, significantly inhibited by L-NAME (1 mM), or SS-31 or SS-20 (1, 10, or 100 nM) and sheared (30 min) in the presence of the respective drug. Digital images (62×) of mitoSOX and DAPI fluorescence were obtained by confocal microscopy and overlaid. A, representative digital images from ECs exposed to different treatments. Because SS-31 at either 10 or 100 nM totally abolished the shear-induced fluorescence signal, only 1 and 10 nM are shown. Because SS-20 had no effect at either concentration tested, only 10 nM is shown. B, using digital image processing, the mean mitoSOX fluorescence intensity per image was calculated, averaged over three fields of view per experiment, and then averaged over three independent experiments. Normalized (to static controls) mitoSOX fluorescence values are mean ± S.E.M. *p < 0.05, significantly different from static control group; †p < 0.05, significantly different from shear group; ‡p < 0.05; n = 3.

Discussion
The present study provides the first evidence that the mitochondria-derived H₂O₂ plays an important role in the intracellular signaling pathways, leading to shear-induced up-regulation of the cytoprotective HO-1 in cultured BAECs. The shear-induced increase in HO-1 protein expression was abolished by saturating concentrations of either L-NAME or NAC, suggesting that both NO and ROS mediate the HO-1 up-regulation. Based on the effects of the antioxidants ebselen, DPI, and UA on HO-1 expression, it was concluded that H₂O₂ (and/or its product OH⁻), dismutated from flavoprotein-derived O₂⁻, is essential for HO-1 induction. The ETC inhibitors myxothiazol, rotenone, and antimycin A at the concentrations tested either abolished or significantly inhibited the shear-induced increase in HO-1 expression, suggesting that mitochondrial function is required for this event. Based on the effect of the mitochondria-targeted anti-

Fig. 4. Effect of ETC inhibitors and selective antioxidants on mitochondrial O₂⁻ production. ECs were preincubated with mitoSOX red (5 μM) and DAPI (1 μM) and with rotenone (2 μM), antimycin A (10 μM), L-NAME (1 mM), or SS-31 or SS-20 (1, 10, or 100 nM) and sheared (30 min) in the presence of the respective drug. Digital images (62×) of mitoSOX and DAPI fluorescence were obtained by confocal microscopy and overlaid. A, representative digital images from ECs exposed to different treatments. Because SS-31 at either 10 or 100 nM totally abolished the shear-induced fluorescence signal, only 1 and 10 nM are shown. Because SS-20 had no effect at either concentration tested, only 10 nM is shown. B, using digital image processing, the mean mitoSOX fluorescence intensity per image was calculated, averaged over three fields of view per experiment, and then averaged over three independent experiments. Normalized (to static controls) DCF fluorescence values are mean ± S.E.M. *p < 0.05, significantly different from static control group; †p < 0.05, significantly different from shear group; P ≤ 0.05; n = 3.

Fig. 5. Effect of ETC inhibitors and selective antioxidants on intracellular peroxide levels. ECs were preincubated with DCFH-DA (10 μM) and DAPI (1 μM) and with ebselen (5 μM), rotenone (2 μM), L-NAME (1 mM), or SS-31 or SS-20 (1, 10, or 100 nM) and sheared (30 min) in the presence of the respective drug. Digital images (62×) of DCF and DAPI fluorescence were obtained by confocal microscopy and overlaid. A, representative digital images from ECs exposed to different treatments. From all drugs tested on static ECs, only rotenone changed the DCF fluorescence signal (only rotenone is included). Because SS-20 had no effect on the fluorescence in sheared ECs at either concentration tested, only 10 nM is shown. B, using digital image processing, the mean DCF fluorescence intensity per image was calculated, averaged over three fields of view per experiment, and then averaged over three independent experiments. Normalized (to static controls) DCF fluorescence values are mean ± S.E.M. *p < 0.05, significantly different from static control group; †p < 0.05, significantly different from shear group; ‡p < 0.05; n = 3.
oxidant peptide, SS-31, which scavenges O$_2^-$, H$_2$O$_2$, ONOO$^-$, and OH$^\cdot$ (Szteo, 2008), on the HO-1 expression, and because it is known that mitochondrial H$_2$O$_2$ freely diffuses to the cytosol (O$_2^-$ is dismutated to H$_2$O$_2$ by superoxide dismutase within the matrix and intermembrane space; ONOO$^-$ and OH$^\cdot$ react with intramitochondrial targets) (Cadenas, 2004), it was concluded that the mitochondria-derived H$_2$O$_2$ is primarily responsible for the shear-induced HO-1 up-regulation. The essential role of mitochondrial H$_2$O$_2$ in HO-1 expression was demonstrated previously in human hepatoma cells exposed to metabolic stress by glucose deprivation (Chang et al., 2003). In vascular ECs, the functional significance of mitochondrial ROS has started receiving attention only in recent years (Zhang and Gutterman, 2007). Mitochondria-derived H$_2$O$_2$ was found responsible for the flow-induced vasodilation in human coronary arterioles (Liu et al., 2003). Furthermore, it was shown that mitochondria-derived ROS are required for the cyclic strain-induced increases in vascular cell adhesion molecule-1 expression via nuclear factor-$\kappa$B (Ali et al., 2004). To our knowledge, before this study, none has demonstrated a specific role for mitochondrial ROS in sheared ECs.

Our finding that NO is an important mediator in shear-induced HO-1 up-regulation in BAECs agrees with studies where diverse NO donors were shown to induce HO-1 expression and activity in aortic ECs (Motterlini et al., 2002). To be specific, BAEC exposure to the NO donor spermine NONOate (500 $\mu$M, generating 4 $\mu$M NO/min) led to increases in Nrf2 nuclear translocation and HO-1 protein expression that were blocked by EC pretreatment with either NAC or MAPK pathway inhibitors (Buckley et al., 2003). Furthermore, NO was recently shown to cause thiol-dependent modulation of Keap1, possibly via $S$-nitrosylation, leading to loss of its ability to negatively regulate Nrf2 (Buckley et al., 2008). NO can modify thiol residues to produce SNO via interaction with: 1) oxidants, such as O$_2^-$ and formation of higher oxides of nitrogen, such as nitrogen dioxide, dinitrogen trioxide, and ONOO$^-$ (probably not the main pathway because UA did not block the shear-induced HO-1 up-regulation); 2) reduced thiol followed by electron abstraction; or 3) metal centers, such as iron, either operating as catalysts or through the generation of nitrosonium cation (Gow et al., 2004). Shear stress increases the amount of $S$-nitrosylated proteins in ECs, but both the basal and the shear-induced levels of SNO differ in HUVECs compared with human aortic ECs (BAECs were not examined) (Hoffmann et al., 2003), which may account for the fact that NO does not play a role in HO-1 induction in sheared HUVECs (Dai et al., 2007; Warabi et al., 2007).

MitoSOX fluorescence responded to ETC inhibitors, and, based on the effect of $L$-NAME on the shear-induced increase in fluorescence, it was concluded that NO is, at least in part, responsible for mitochondrial O$_2^-$ production in sheared BAECs. Utilization of excess NO by mitochondria is known to involve ubiquinol oxidation that increases the O$_2^-$ production rate and formation of ONOO$^-$ that, via inhibition of the ETC at multiple sites, amplifies the O$_2^-$ generation (Boveris and Cadenas, 2000). SS-31, at each dose tested, markedly inhibited the shear-induced increase in both DCF and MitoSOX fluorescence. Because it is known that DCF responds to intracellular peroxides, SS-31 scavenges mitochondrial O$_2^-$, H$_2$O$_2$, ONOO$^-$, and OH$^\cdot$, and only H$_2$O$_2$ freely diffuses through membranes, it was concluded that the mitochondria are the major source of H$_2$O$_2$ in sheared BAECs. Because NO is responsible for mitochondrial O$_2^-$ generation and mitochondria-derived H$_2$O$_2$ is a major part of intracellular ROS, inhibition of the endothelial NOS also significantly decreased the shear-induced DCF signal. However, several groups, including ours, have shown that EC exposure to steady laminar flow causes a transient increase in O$_2^-$ levels that originates from the plasma membrane-bound NAD(P)H oxidase (De Keulenaer et al., 1998; Yeh et al., 1999; Duerrschmidt et al., 2006). Because a significant portion of the O$_2^-$ produced by NAD(P)H oxidase diffuses to the extracellular space (Barbacane et al., 2000), and shear-induced O$_2^-$ generation was measured using the cytochrome c assay in media samples and was blocked by the NAD(P)H oxidase inhibitor gp91ds-tat (Duerrschmidt et al., 2006), it may be that, depending on the method for ROS detection, the contribution of mitochondrial ROS is less apparent. It may also be that the relative contribution of the two ROS sources, NAD(P)H oxidase and mitochondria, changes with time during shear. Last, it is possible that $L$-NAME inhibits the fluorescence signals because shear-induced NOS activation results in O$_2^-$ production because of NOS uncoupling, as was shown in sheared pulmonary artery ECs (Mata-Greenwood et al., 2006). It is worth noting that SS-31 demonstrated a biphasic dose-response relationship in the inhibition of HO-1 induction, but this was not the case with its effect on the MitoSOX and DCF signals, suggesting that there may be H$_2$O$_2$-independent pathways leading to shear-induced HO-1 up-regulation. These may depend exclusively on NO because theoretically, NO can modify thiols (in Keap1) independently of its interaction with oxidants.

Studies have implicated a major role for protein phosphorylation-dependent signaling cascades in HO-1 induction by different agents (Ryter et al., 2006). Under our experimental conditions, however, none of the common HO-1 activators or inhibitors tested (Fig. 6) modified the shear-induced HO-1 expression. The reasons for these differences are not clear, and they may be related to the fact that BAECs do not respond to a variety of shear stressors in the same way as other EC types. For example, the shear-induced MitoSOX fluorescence response to ETC inhibitors, and, based on the effect of $L$-NAME on the shear-induced increase in fluorescence, it was concluded that NO is, at least in part, responsible for mitochondrial O$_2^-$ production in sheared BAECs. Utilization of excess NO by mitochondria is known to involve ubiquinol oxidation that increases the O$_2^-$ production rate and formation of ONOO$^-$ that, via inhibition of the ETC at multiple sites, amplifies the O$_2^-$ generation (Boveris and Cadenas, 2000). SS-31, at each dose tested, markedly inhibited the shear-induced increase in both DCF and MitoSOX fluorescence. Because it is known that DCF responds to intracellular peroxides, SS-31 scavenges mitochondrial O$_2^-$, H$_2$O$_2$, ONOO$^-$, and OH$^\cdot$, and only H$_2$O$_2$ freely diffuses through membranes, it was concluded that the mitochondria are the major source of H$_2$O$_2$ in sheared BAECs. Because NO is responsible for mitochondrial O$_2^-$ generation and mitochondria-derived H$_2$O$_2$ is a major part of intracellular ROS, inhibition of the endothelial NOS also significantly decreased the shear-induced DCF signal. However, several groups, including ours, have shown that EC exposure to steady laminar flow causes a transient increase in O$_2^-$ levels that originates from the plasma membrane-bound NAD(P)H oxidase (De Keulenaer et al., 1998; Yeh et al., 1999; Duerrschmidt et al., 2006). Because a significant portion of the O$_2^-$ produced by NAD(P)H oxidase diffuses to the extracellular space (Barbacane et al., 2000), and shear-induced O$_2^-$ generation was measured using the cytochrome c assay in media samples and was blocked by the NAD(P)H oxidase inhibitor gp91ds-tat (Duerrschmidt et al., 2006), it may be that, depending on the method for ROS detection, the contribution of mitochondrial ROS is less apparent. It may also be that the relative contribution of the two ROS sources, NAD(P)H oxidase and mitochondria, changes with time during shear. Last, it is possible that $L$-NAME inhibits the fluorescence signals because shear-induced NOS activation results in O$_2^-$ production because of NOS uncoupling, as was shown in sheared pulmonary artery ECs (Mata-Greenwood et al., 2006). It is worth noting that SS-31 demonstrated a biphasic dose-response relationship in the inhibition of HO-1 induction, but this was not the case with its effect on the MitoSOX and DCF signals, suggesting that there may be H$_2$O$_2$-independent pathways leading to shear-induced HO-1 up-regulation. These may depend exclusively on NO because theoretically, NO can modify thiols (in Keap1) independently of its interaction with oxidants.

Studies have implicated a major role for protein phosphorylation-dependent signaling cascades in HO-1 induction by different agents (Ryter et al., 2006). Under our experimental
conditions, PI3K and ERK1/2, but not p38 MAPK, were involved in HO-1 up-regulation. Although we did not examine the role of lipid oxidation products, 15d-PGJ2 is known to be essential for steady laminar flow to activate the Keap1-Nrf2 pathway (Hosoya et al., 2005). 15d-PGJ2 can bind directly to and modify thiols in Keap1 leading to its dissociation from Nrf2 and transactivation of ARE-regulated genes (Levonen et al., 2004; Hosoya et al., 2005). In ECs exposed to lipid oxidation products, it was shown that they primarily localize to the mitochondria (Landar et al., 2006), where they form protein adducts and increase the mitochondrial Ca2+ uptake, leading to O2− generation by the ETC (Watanabe et al., 2006). In these ECs, either inhibitors of oxidative phosphorylation (rotenone, antimycin A) or mitochondria-targeted vitamin E inhibited ERK1/2 activation and HO-1 induction, providing important evidence that the mitochondrial ROS mediate HO-1 expression via ERK1/2 (Watanabe et al., 2006).

In summary, the present study, in combination with work by others, allows us to delineate the pathways that lead to Keap1-Nrf2 dissociation, Nrf2 nuclear translocation, and Nrf2-mediated HO-1 expression in BAECs exposed to steady laminar flow (Fig. 7). Shear-induced NO increases the mitochondrial O2− production, which is dismutated to H2O2, and the excess H2O2 escapes to the cytosol (Cadenas, 2004). H2O2 forms lipid oxidation products (Zmijewski et al., 2005) that can activate protein kinases, such as PI3K (associated with the serine/threonine kinase Akt) and ERK1/2 (Watanabe et al., 2006), resulting in Nrf2 phosphorylation and its dissociation from Keap1 (Ryter et al., 2006; Salazar et al., 2006). Lipid oxidation products also cause alkylation of critical cysteine residues of Keap1, leading to its dissociation from Nrf2 (Levonen et al., 2004), and can localize to the mitochondria, where they further increase O2− production (Landar et al., 2006). Last, NO can directly contribute to Nrf2 activation by modifying the critical cysteine residues of Keap1, possibly via S-nitrosylation (Buckley et al., 2008). However, flow in the arterial circulation, although still laminar, is unsteady. Flow is pulsatile in the straight portions of the arteries and oscillatory in curvatures and bifurcations, which are the areas prone to atherosclerotic lesion formation (Chatzizisis et al., 2007). Steady laminar and pulsatile laminar flows (considered “atheroprotective”) induce EC HO-1 up-regulation, whereas oscillatory laminar flow (considered “atheroprone”) does not (Hosoya et al., 2005; Dai et al., 2007). In oscillatory flow, there is less bioavailable NO because of down-regulation of endothelial NOS expression and increased O2− generation by the NADPH oxidase (Hsiai et al., 2007). According to the present study, less NO may be, at least in part, responsible for decreased Nrf2 nuclear translocation and suppressed HO-1 expression in areas of the vasculature that are exposed to oscillatory flow. Suppressed expression of phase II genes contributes to the proinflammatory phenotype observed in those areas and leads to atherogenesis.

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References


