Mitochondria-Derived Reactive Oxygen Species Mediate Heme Oxygenase-1 Expression in Sheared Endothelial Cells$^{\boxed{S}}$

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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^G-nitro-L-arginine methyl ester or N-acetyl-L-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 mi-tochondria-targeted antioxidant peptide, Szeto-Schiller (SS)-31, which scavenges O_2^-, hydrogen peroxide (H_2O_2), peroxynitrite, and hydroxyl radicals, markedly inhibited the increase in HO-1 expression. These data collectively suggest that mitochondrial H_2O_2 mediates the HO-1 induction. MitoSOX and 2′,7′-dichlorofluorescin (DCF) fluorescence showed that mitochondrial O_2^- levels and intracellular peroxides, respectively, are higher in sheared ECs compared with static controls and, in part, dependent 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_2O_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

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ABBREVIATIONS: HO, heme oxygenase; Nrf2, NF-E2-related factor 2; ARE, antioxidant response element; Keap1, Kelch-like erythroid-derived Cap-N-Collar-Homology-associated protein 1; 15d-PGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; EC, endothelial cell; NO, nitric oxide; O_2^-, superoxide; ROS, reactive oxygen species; HUVEC, human umbilical vein EC; SNO, S-nitrosothiol; ONOO$^-$, peroxynitrite; ETC, electron transport chain; BAEC, bovine aortic EC; NOS, NO synthase; L-NAME, N^G-nitro-L-arginine methyl ester; NAC, N-acetyl-L-cysteine; UA, uric acid; SS, Szeto-Schiller; H$_2$O$_2$, hydrogen peroxide; OH$^\cdot$, hydroxyl radical; GSH, glutathione; DPI, diphenylene iodonium; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; PD98059, 2′-amino-3′-methoxyflavone; ERK, extracellular signal-regulated kinase; SB202190, 4-[4-(4-fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]phenol; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DAPI, 4′,6-diamidino-2-phenylindole; DCF, 2′,7′-dichlorofluorescin.

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-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ$_2$), 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 (O$_2^-$) 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-PGJ$_2$ (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 O$_2^-$, 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-nitroso-thiols (SNO) in the regulatory cysteine residues of Keap1 (Motterlini 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 O$_2^-$ production (Han et al., 2007; Jones et al., 2008). Because steady laminar flow increases O$_2^-$ 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/cm^2 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, N^{G}-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-tar-

geted antioxidant peptide, Szeto-Schiller (SS)-31 (D-Arg-2′,6′-dimethyltyrosine-Lys-Phe-NH$_2$), which exhibits 1000-fold mitochondrial accumulation and scavenges O$_2^-$, hydrogen peroxide (H$_2$O$_2$), ONOO$^-$, and hydroxyl radicals (OH$^{\cdot}$) (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 dimethyltyrosine (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 H$_2$O$_2$, resulting from dismutation of O$_2^-$ 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, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA) in a humidified atmosphere of 95% air-5% CO$_2$. 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 containing 0.05% potassium chromium sulfate (Sigma-Aldrich, St. Louis, MO) and were used within 24 h upon confluence.

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/cm^2 (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% CO$_2$) 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 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, NOS, 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

(PI3K) inhibitor, wortmannin (500 nM for 1 h); the mitogen-activated protein kinase (MAPK) kinase (MEK) 1 inhibitor, PD98059 (25 μM for 1 h), which blocks phosphorylation and activation of extracellular signal-regulated kinase (ERK) 1/2; or the p38 MAPK inhibitor, SB202190 (20 μM for 1 h), and subjected to shear with the same concentration of drug (all from Sigma-Aldrich). Cell viability was determined by trypan blue exclusion at the end of treatments, and it was ≥90%. Concentrations of tested compounds were chosen based on their efficacy in inhibiting intracellular signaling pathways and/or Nrf2 nuclear translocation/HO-1 expression in either ECs or epithelial cells treated with either oxidized lipids or NO donors (Buckley et al., 2003; Iles et al., 2005; Watanabe et al., 2006). Stock solutions of NAC, UA, and SS peptides were made with distilled water and diluted in phosphate-buffered saline (PBS) before mixing with the incubation/perfusion medium. Stock solutions of all other compounds were made with dimethyl sulfoxide (DMSO) and diluted in PBS, but the final DMSO concentration was never higher than 0.03% (v/v). Static controls were treated with DMSO at the same concentration as sheared ECs.

Western Blot Analysis. ECs were scraped into ice-cold lysis buffer (0.06 M Tris·HCl, 2% SDS, and 5% glycerol, pH 6.8). Cell extracts were homogenized by sonication, boiled for 5 min, and centrifuged at 10,000 rpm for 10 min at 4°C to remove the insoluble material. Protein concentration in cell lysates was determined using the bicinchoninic acid assay (Pierce Chemical, Rockford, IL). Supernatant containing 30 μg of protein was mixed with 2× sample buffer and applied in duplicate on 4 to 20% Tris·HCl gels (Bio-Rad, Hercules, CA) for protein separation by SDS-polyacrylamide gel electrophoresis at 100-V constant voltage. Proteins were electrophoretically transferred at 100-V constant voltage at room temperature for 1 h onto nitrocellulose membranes (Bio-Rad). After incubation in blocking solution (10 mM Tris·HCl, 0.2% nonfat milk, 100 mM NaCl, and 0.1% Tween 20, pH 7.5), duplicate membranes were hybridized with primary antibodies against HO-1 (Assay Designs, Ann Arbor, MI) and β-actin (Sigma-Aldrich), respectively. Membrane-bound primary antibodies were detected using secondary antibodies conjugated with alkaline phosphatase. Immunoblots were developed on films using the enhanced chemiluminescence method (Bio-Rad) and analyzed by densitometry.

Fluorescence Detection of Mitochondrial O_2^- and Intracellular Peroxides. 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 O_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 DCFH inside the cell, and on exposure to either H_2O_2 or $ONOO^-$, it is oxidized to the green fluorescent product 2′,7′-dichlorofluorescein (DCF) (Tarpey et al., 2004). MitoSOX Red is a mitochondria-targeted form of dihydroethidium that is relatively specific for O_2^- and undergoes oxidation to form the DNA-binding red fluorophore ethidium bromide (Tarpey et al., 2004). Before shear exposure, EC monolayers were incubated with either DCFH-DA (10 μM) or MitoSOX red (5 μM) and DAPI (1 μM) for 10 min in the incubator. At the end of shear (30 min), ECs were washed with PBS, mounted with Fluoromount-G (Southern Biotechnology Associates, Birmingham, AL), and images (62×) 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/β-actin protein expression and fluorescence data were normalized to corresponding static controls and expressed as mean ± 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/cm², 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).

H_2O_2 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 μM), the flavoprotein inhibitor DPI (20 μM), or the $ONOO^-$ scavenger UA (100 μM). Ebselen abolished the HO-1 induction, suggesting that either H_2O_2 (or its metabolite $OH^•$) or $ONOO^-$ is involved (Fig. 2A). DPI also abolished the HO-1 induction, suggesting that the O_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 H_2O_2 (and/or secondary $OH^•$), dismutated from flavoprotein-derived O_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
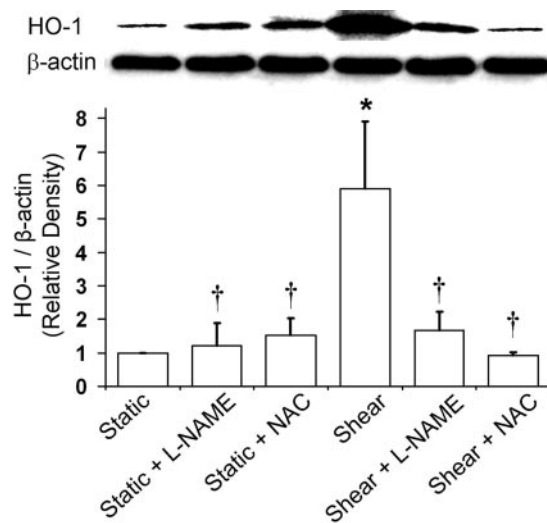

Fig. 1. Effect of L-NAME and NAC on shear-induced HO-1 expression. BAECs were preincubated and sheared (10 dynes/cm², 6 h) in the presence of either L-NAME (1 mM) or NAC (10 mM), and HO-1 protein expression was measured as described under *Materials and Methods*. Representative immunoblot for HO-1 (32 kDa) and loading control β-actin (42 kDa) and densitometric analysis for levels of HO-1 protein relative to β-actin in static or sheared ECs in presence or absence of L-NAME and NAC are shown. Values are mean ± S.E.M. ∗, significantly different from static control group; $P \leq 0.05$; $n = 3$. †, significantly different from shear group; $P \leq 0.05$; $n = 3$.
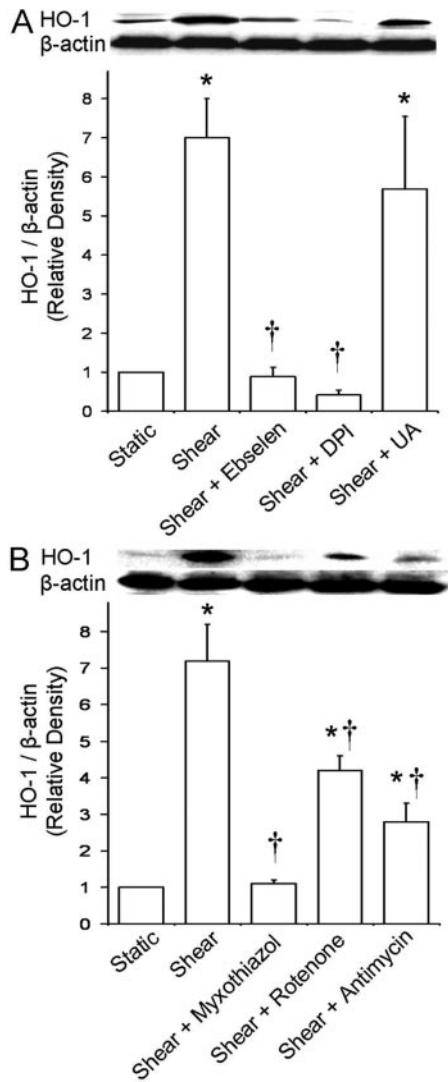
Fig. 2. Effect of selective antioxidants and ETC inhibitors on shear-induced HO-1 expression. ECs were preincubated and sheared in the presence of ebselen (5 μM), DPI (20 μM), UA (100 μM), myxothiazol (10 μM), rotenone (2 μM), or antimycin A (10 μM), and HO-1 protein expression was measured as before. A, representative immunoblot for HO-1 and β-actin and densitometric analysis for HO-1 protein levels relative to β-actin in static or sheared ECs in the presence or absence of antioxidants ebselen, DPI, and UA (because they had no effect on HO-1 expression in static ECs, only their effects on sheared ECs are shown). B, same as in A but in the presence or absence of ETC inhibitors myxothiazol, rotenone, and antimycin A. Values are mean $\pm$ S.E.M. *, significantly different from static control group; $P \leq 0.05$; $n = 3$. †, significantly different from shear group; $P \leq 0.05$; $n = 3$.

A (10 μM), myxothiazol abolished and antimycin A significantly inhibited the shear-induced increase in HO-1 expression (Fig. 2B). The ETC complex I inhibitor rotenone (2 μM) 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_2O_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_2^-, H_2O_2, and $ONOO^-$ and, to a lesser extent, $OH^{\cdot}$ (Szeto, 2008), and H_2O_2 can freely diffuse to the cytosol (Cadenas, 2004), our data suggest that the mitochondrial H_2O_2 plays an important role in shear-induced HO-1 up-regulation.

Mitochondrial H_2O_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_2^- 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 $\sim$80 and $\sim$70%, respectively (Han et al., 2007), and, at least in the case of complex III, decreased complex activity reduces the enzyme-mediated O_2^- 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, suggesting NO involvement in the shear-induced mitochondrial O_2^- production (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
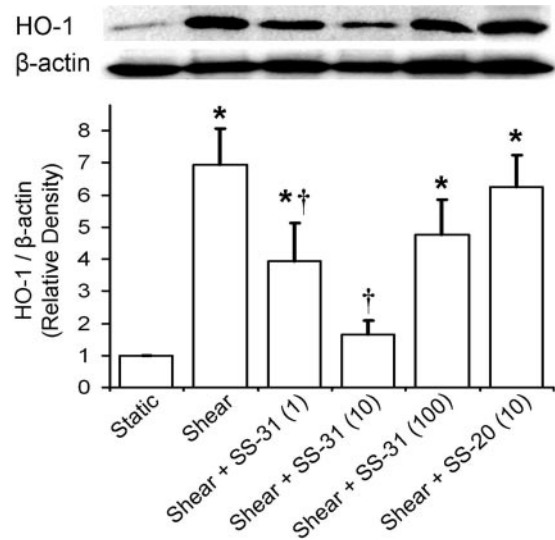

Fig. 3. Effect of mitochondria-targeted antioxidant peptides on shear-induced HO-1 expression. ECs were preincubated and sheared in the presence of either SS-31 (1, 10, or 100 nM) or its nonscavenging analog SS-20. Representative immunoblot for HO-1 and β-actin and densitometric analysis for HO-1 protein levels relative to β-actin in static or sheared ECs in the presence or absence of SS-31 and SS-20 are shown. Because both SS peptides had no effect on HO-1 expression in static ECs, only their effects on sheared ECs are included. Because SS-20 had no effect on HO-1 expression in sheared ECs at either concentration tested, only 10 nM is shown. Values are mean $\pm$ S.E.M. *, significantly different from static control group; $P \leq 0.05$; $n = 3$. †, significantly different from shear group; $P \leq 0.05$; $n = 3$.
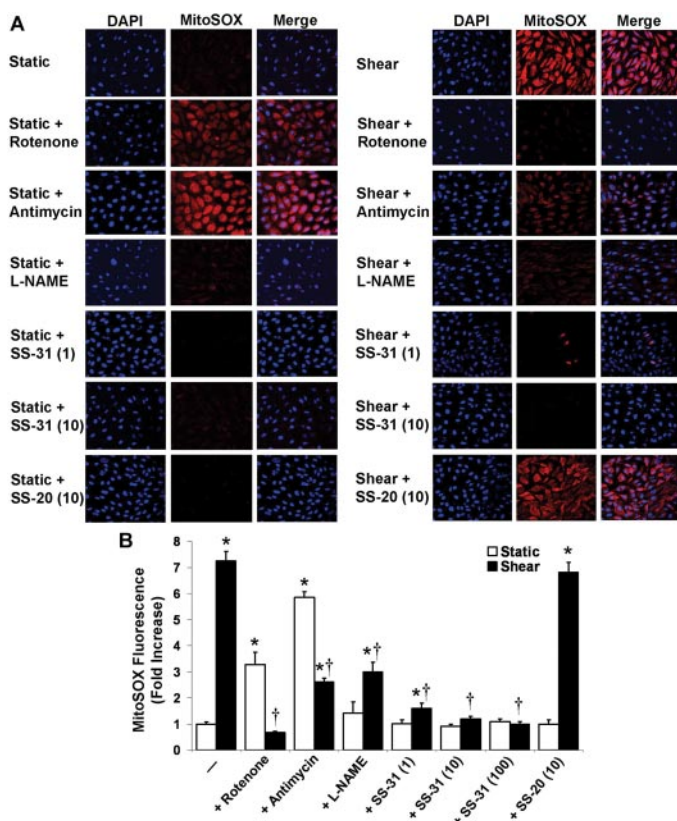
A

DAPI   MitoSOX   Merge   DAPI   MitoSOX   Merge

Static   Shear

Static +   Shear +
Rotenone   Rotenone

Static +   Shear +
Antimycin   Antimycin

Static +   Shear +
L-NAME   L-NAME

Static +   Shear +
SS-31 (1)   SS-31 (1)

Static +   Shear +
SS-31 (10)   SS-31 (10)

Static +   Shear +
SS-20 (10)   SS-20 (10)

B

Fig. 4. Effect of ETC inhibitors and selective antioxidants on mitochondrial O_2^- 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) mitoSOX fluorescence values are mean ± S.E.M. ∗, significantly different from static control group; P ≤ 0.05; n = 3. †, significantly different from shear group; P ≤ 0.05; n = 3.

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 rotenone, L-NAME, or SS-31 (1, 10, or 100 nM), and not affected by SS-20 (only 10 nM is shown; Fig. 5, A and B). Although SS-31 at 1 nM was slightly less potent in blocking both the shear-induced MitoSOX red and DCF signals, the effect was not significantly different from that observed by SS-31 at either 10 or 100 nM (Figs. 4 and 5). Based on the fact that SS-31 scavenges mitochondrial O_2^-, H_2O_2, $ONOO^-$, and $OH^{\cdot}$, the DCF signal reports intracellular H_2O_2 and $ONOO^-$, and mitochondrial H_2O_2 freely diffuses to the cytosol (Cadenas, 2004), the data suggest that mitochondrial H_2O_2 is a major part of the intracellular peroxide pool.

PI3K and MAPK Are Involved in the Increase in HO-1 Expression. To investigate the involvement of protein phosphorylation cascades in the shear-induced HO-1 up-reg-
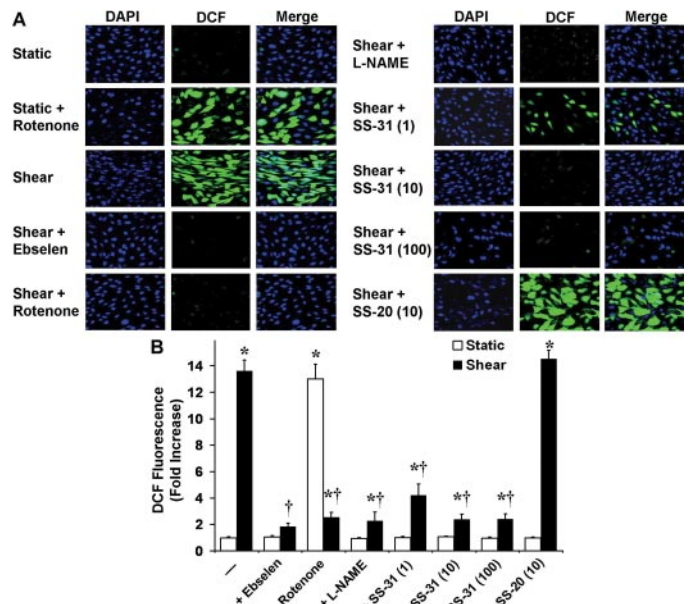
A

DAPI   DCF   Merge   DAPI   DCF   Merge

Static   Shear +
              L-NAME

Static +   Shear +
Rotenone   SS-31 (1)

Shear   Shear +
               SS-31 (10)

Shear +   Shear +
Ebselen   SS-31 (100)

Shear +   Shear +
Rotenone   SS-20 (10)

B

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. ∗, significantly different from static control group; P ≤ 0.05; n = 3. †, significantly different from shear group; P ≤ 0.05; n = 3.

ulation, ECs were preincubated and sheared in the presence of the PI3K inhibitor wortmannin (500 nM), the MEK1 inhibitor PD98059 (25 μM; also an ERK1/2 inhibitor because it inhibits its phosphorylation by MEK1), or the p38 MAPK inhibitor SB202190 (20 μM). Either wortmannin or PD98059 abolished the shear-induced increase in HO-1 expression, and SB202190 had no effect (Fig. 6), suggesting that PI3K and ERK1/2 are signaling molecules in HO-1 induction.

Discussion

The present study provides the first evidence that the mitochondria-derived H_2O_2 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 H_2O_2 (and/or its product $OH^{\cdot}$), dismutated from flavoprotein-derived O_2^-, 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-

oxidant peptide, SS-31, which scavenges O_2^-, H_2O_2, $ONOO^-$, and $OH^\cdot$ (Szeto, 2008), on the HO-1 expression, and because it is known that mitochondrial H_2O_2 freely diffuses to the cytosol (O_2^- is dismutated to H_2O_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_2O_2 is primarily responsible for the shear-induced HO-1 up-regulation. The essential role of mitochondrial H_2O_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_2O_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-κB (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 μM, generating 4 μM 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_2O_2, $ONOO^-$, and $OH^\cdot$, and only H_2O_2 freely diffuses

through membranes, it was concluded that the mitochondria are the major source of H_2O_2 in sheared BAECs. Because NO is responsible for mitochondrial O_2^- generation and mitochondria-derived H_2O_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 (Barbacanne 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_2O_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
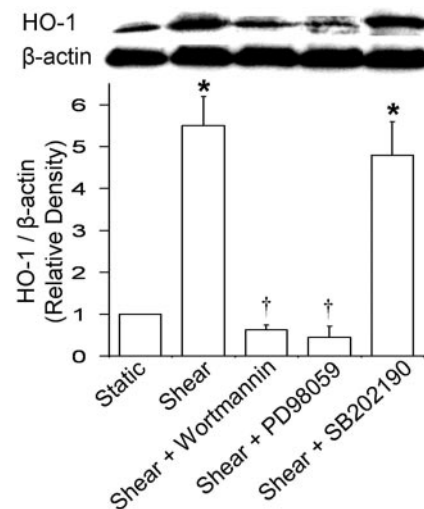

Fig. 6. Effect of protein phosphorylation cascade inhibitors on shear-induced HO-1 expression. ECs were preincubated and sheared in the presence of wortmannin (500 nM), PD98059 (25 μM), or SB202190 (20 μM). Representative immunoblot for HO-1 and β-actin and densitometric analysis for HO-1 protein levels relative to β-actin in static or sheared ECs in the presence or absence of wortmannin, PD98059, and SB202190 are shown. Because they all had no effect on HO-1 expression in static ECs, only their effects on sheared ECs are included. Values are mean ± S.E.M. *, significantly different from static control group; $P \leq 0.05$; $n = 3$. †, significantly different from shear group; $P \leq 0.05$; $n = 3$.
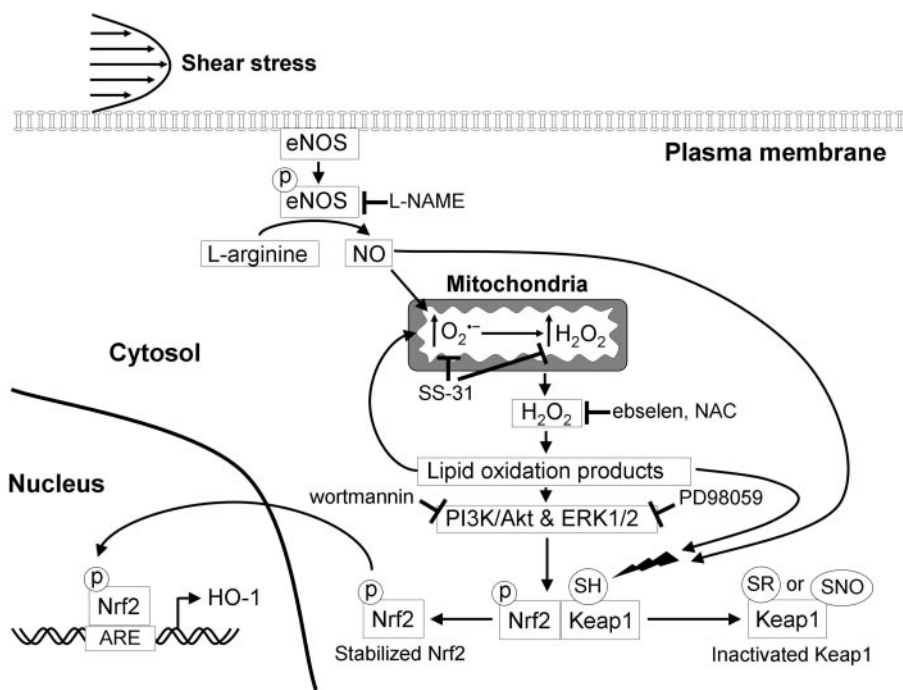
Fig. 7. A proposed scheme for HO-1 induction in BAECs exposed to steady laminar flow. Shear-induced NO, produced by the endothelial NOS, diffuses to the mitochondria where, by inactivating the ETC, increases O_2^- production. Excess H_2O_2 diffuses to the cytosol, where it forms reactive lipid products, which can activate protein kinases, such as PI3K/Akt and ERK1/2, leading to Nrf2 phosphorylation and its release from Keap1. Lipid oxidation products can directly modify Keap1 thiols by alkylation of critical cysteine residues, inhibiting its activity and contributing to the stabilization of Nrf2. Lipid oxidations products may preferentially localize to the mitochondria resulting in further increases in O_2^- production. NO can directly modify Keap1 thiols by, for example, forming SNO. Stabilized Nrf2 translocates to the nucleus, where it binds to the ARE and initiates gene transcription of phase II genes, including HO-1. Sites of inhibition of the pathway by L-NAME, SS-31, ebselen, NAC, wortmannin, and PD98059 are shown.

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-PGJ$_2$ is known to be essential for steady laminar flow to activate the Keap1-Nrf2 pathway (Hosoya et al., 2005). 15d-PGJ$_2$ 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 Ca^{2+} uptake, leading to O_2^- 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 O_2^- production, which is dismutated to H_2O_2, and the excess H_2O_2 escapes to the cytosol (Cadenas, 2004). H_2O_2 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 O_2^- 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 O_2^- generation by the NAD(P)H 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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