Physiological cyclic strain promotes endothelial cell survival via the induction of heme oxygenase-1

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Heme oxygenase-1 (HO-1) is a highly inducible enzyme that catalyzes the degradation of heme into equimolar amounts of carbon monoxide (CO), biliverdin, and free iron (see Refs. 14, 15, 31). Biliverdin is subsequently metabolized to bilirubin by the enzyme biliverdin reductase, while iron is rapidly sequestered by ferritin. The oxidation of heme by HO-1 is inhibited by various metalloporphyrins, including tin protoporphyrin-IX (SnPP). The induction of HO-1 by various forms of cellular stress provides an important defense mechanism against tissue injury. The cytoprotection afforded by HO-1 is mediated by several distinct mechanisms. In particular, HO-1 catabolizes prooxidant heme to the bile pigments biliverdin and bilirubin, which are effective antioxidants capable of scavenging peroxyl radicals and inhibiting lipid peroxidation (40). Furthermore, the coordinated induction of ferritin with HO-1 exerts an additional antioxidant effect by chelating free iron (44). Finally, HO-1 generates the diatomic gas CO, which possesses potent antiapoptotic properties (4, 28, 48). Interestingly, the application of cyclic strain stimulates HO-1 expression in vascular cells, but the functional significance of this induction remains unclear (9, 35, 47). In the present study, we investigated the mechanism by which a hemodynamically relevant regimen of cyclic strain induces HO-1 expression in human vascular endothelium. In addition, we determined whether the induction of HO-1 by cyclic strain contributes to its ability to modulate endothelial cell survival.

MATERIALS AND METHODS

Materials. M199 medium, streptomycin, penicillin, gelatin, ascorbic acid, uric acid, Trypan blue, sulfanilamide, dithiothreitol, trypsin, naphthyl ethylenediamine dihydrochloride, ceramide, methyl-L-arginine, heparin, formaldehyde, chloroform, sodium dodecyl sulfate (SDS), Nonidet P-40 (NP-40), NaCl, EDTA, glucose-6-phosphate, sucrose, glucose-6-phosphate dehydrogenase, hemin, HEPES, Tris, and N-acetyl-L-cysteine were purchased from Sigma-Aldrich (St. Louis, MO). Phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and peptatin A were from Roche Applied Biosciences (Indianapolis, IN). SnPP was from Frontier Scientific (Logan, UT), and Dowex 50W-X8 was from Bio-Rad Laboratories (Hercules, CA). Tumor necrosis factor-α (TNF-α) was from Genzyme (Boston, MA). An antibody against HO-1 was from Assay Designs (Ann Arbor, MI), antibodies directed against β-actin and nuclear factor erythroid-derived 2-related factor (Nrf2) were from Santa Cruz Biotechnologies (Santa Cruz, CA), antibodies against Nrf1, glutamate-cysteine ligase modifier subunit (GCLM), and NAD(P)H dehydrogenase quinone 1 (NQO1) were from GeneTex (Irvine, CA), an antibody against endothelial nitric oxide synthase (eNOS) was from BD Transduction Laboratories (San...
Jose, CA), and an antibody against platelet-endothelial cell adhesion molecule-1 (PECAM-1) was from BD Biosciences (San Jose, CA). MG-132 was from EMD Millipore (Billerica, MA). 5,6-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) was from Invitrogen (Carlsbad, CA). [32P]dCTP (3,000 Ci/mmol) was from Amersham (Arlington Heights, IL), and L-[3H]arginine (43 Ci/mmol) was from Perkin Elmer (Boston, MA).

**Cell culture.** Human aortic endothelial cells (HAECs) and human umbilical vein endothelial cells (HUVECs) were purchased from Lonza Incorporated (Allendale, NJ) and propagated in M199 medium supplemented with 20% bovine calf serum, 2 mL glutamine, 50 μg/mL penicillin and streptomycin (36). Murine aortic endothelial cells (MAECs) from wild-type (HO-1+/−) and HO-1-deficient (HO-1−/−) mice were initially provided by Dr. Anupam Agarwal (Division of Nephrology, University of Alabama at Birmingham) and subsequently isolated by mice with plating thoracic aortas on Matrigel-coated plates and purifying MAEC outgrowth with a PECAM-1 antibody, as we previously described (23). Cells were characterized as endothelial cells by both positive staining for PECAM-1 and uptake of acetylated low-density lipoprotein by >95% of cells. MAECs were grown in endothelial basal medium supplemented with 10% fetal bovine serum, 50 μg/mL gentamicin, 50 μg/mL amphotericin, 1 μg/mL hydrocortisone, 10 ng/mL human epidermal growth factor, and 6 μg/mL bovine extract (Lonza Incorporated). Endothelial cells were serially cultured on gelatin-coated dishes and propagated in an atmosphere of 95% air-5% CO2.

**Cyclic strain.** Cells were subjected to cyclic strain with the Flexercell 4000T Tension Plus strain unit (Flexercell, McKeesport, PA). This system consists of baseplates equipped with 25-mm loading posts connected to a tension controller and vacuum pump with attached pressure and vacuum reservoir. This system provides uniform radial and circumferential strain across a membrane surface along all radii. Cells were plated onto six-well BioFlex dishes coated with type I collagen, and these flexible-bottom dishes were placed into the baseplate assembly. Vacuum was repetitively applied to the bottom of the dishes via the baseplate, which was placed in a humidified incubator with 5% CO2 at 37°C. Endothelial cells were exposed to a physiologically relevant strain of 6% at a frequency of 1 Hz. Cells grown on BioFlex plates and simultaneously placed in a cell humidified incubator with 5% CO2.

**Western blotting.** Cells were lysed in sample buffer [125 mM Tris (pH 6.8), 12.5% glycerol, 2% SDS, 50 mM sodium fluoride, and trace bromophenol blue], sonicated, boiled at 100°C for 5 min, and centrifuged at 13,000 g for 10 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. Blots were blocked with nonfat milk (5%) in PBS for 1 h and then incubated overnight at 4°C with specific antibodies directed against HO-1 (1:1,500, Nrf2 (1:100), Nrf1 (1: 400), eNOS (1:200), PECAM-1 (1:200), GCLM (1:400), NQO1 (1:800), or β-actin (1:200). Membranes were washed in PBS, incubated with horseradish peroxidase-conjugated secondary antibodies, and developed with commercial chemiluminescence reagents (Amersham). Protein expression was quantified by scanning densitometry and normalized with respect to β-actin.

**Northern blotting.** Total RNA was loaded onto 1.2% agarose gels, fractionated by electrophoresis, and blotted transferred to Gene Screen Plus membranes (Perkin Elmer Life Sciences, Waltham, MA). Membranes were prehybridized for 4 h at 68°C in rapid hybridization buffer (Amersham) and then incubated overnight at 68°C in hybridization buffer containing [32P]DNA probes (1 x 10^6 cpm) for HO-1, Nrf2, GAPDH, or 18S rRNA. DNA probes were generated by reverse transcriptase-polymerase chain reaction (RT-PCR) and labeled with [32P]dCTP with a random priming kit (Amersham) (48). After hybridization, membranes were washed and exposed to X-ray film at -70°C, and HO-1 expression was quantified by scanning densitometry and normalized with respect to GAPDH or 18S rRNA.

**Real-time polymerase chain reaction.** Total RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY) and transcribed to cDNA with a reverse transcription kit (Bio-Rad Laboratories). Real-time PCR was performed with SYBR Green Supermix in a SYBR Green Cycler iQ 5 RT-PCR detection system (Bio-Rad Laboratories) with appropriate primers (1, 38). The primer sequences are as follows: Nox2 forward 5′-AGCTATGAGGTGGTGACCA-3′, reverse 5′-CACAATATTGTGACAGACAGTTGAG-3′; Nox4 forward 5′-tttggcctaggattgt-3′, reverse 5′-AGGCCACCTGTG-TATCCCTG-3′; p2phox forward 5′-GTACCTTGGACCTCTCCTG-3′, reverse 5′-CTGCT- AACCACTTGGGAGCT-3′; and p67phox forward 5′-CAGTTT-AAGCTTTTGGCCTG-3′, reverse 5′-TCTTTGCGCACGCTGACCC. Relative expression of target genes was analyzed with the comparative threshold cycle (2^−ΔΔCt) method, normalized to 18S rRNA, and given as a fraction compared with control, static conditions.

**HO activity.** Cells were sonicated in MgCl2 (2 mM) phosphate (100 mM) buffer (pH 7.4) and centrifuged at 18,000 g for 15 min at 4°C. Supernatants were then added to a reaction mixture containing NADPH (0.8 mM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.2 U), rat liver cytosol (2 mg), and hemin (20 μM). The reaction was conducted for 1 h at 37°C in the dark and terminated by the addition of chloroform. The extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm with an extinction coefficient of 40 mM−1 cm−1.

**HO-1 small interference RNA and HO-1 gene transfer.** HO-1 expression was silenced by transfecting endothelial cells with HO-1 small interference RNA (siRNA) (100 nM) or nontargeting (NT) siRNA (100 nM) that was purchased from Dharmacon (Lafayette, CO) (48). Gene transfer of HO-1 was achieved by infecting endothelial cells with a replication-defective adenovirus expressing HO-1 (AdHO-1), as we previously reported (42). Cells were infected with AdHO-1 or an adenovirus expressing green fluorescent protein (AdGFP) at a multiplicity of infection of 20.

**Reactive oxygen species measurement.** Cell monolayers were incubated with CM-H2DCFDA (5 μM) for 30 min at 37°C prior to collection. The cells were then washed, detached, and centrifuged at 1,000 g for 5 min. Precipitated cells were washed and resuspended in PBS containing 1% fetal bovine serum, and reactive oxygen species (ROS) production was determined by measuring the intracellular oxidation of the dye to 2,7-dichlorofluorescein by flow cytometry using excitation at 488 nm and emission at 530 nm (Dickinson FACSscan, Franklin Lakes, NJ).

**eNOS activity.** eNOS activity was determined by measuring the conversion of L-[3H]arginine to [3H]citrulline, as we previously described (16). Cells were incubated with L-[3H]arginine (2 μCi)-containing culture medium for 4 h, culture medium was then removed, and cells were washed three times with PBS. Cells were then collected in ice-cold Triton lysis buffer (50 mM, pH 7.4) containing Triton X-100 (0.1%) and centrifuged at 13,000 g for 5 min at 4°C. Supernatants were then applied to the cation exchange resin Dowex 50W-X8, and neutrally charged citrulline was eluted and quantified by liquid scintillation counting.

**Nrf2 activation.** Cells were scraped into ice-cold lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, 0.5 mM dithiothreitol, and 0.4% NP-40), incubated for 10 min, and centrifuged at 14,000 g for 5 min at 4°C. The resulting nuclear pellet was resuspended in extraction buffer (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1.0 mM EDTA, 1 mM dithiothreitol, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin A, and 10% glycerol), kept on ice for 15 min, and centrifuged at 14,000 g for 5 min at 4°C. The supernatant containing nuclear protein was collected and stored at -70°C. Nrf2 activity was determined with the TransAM Nrf2 assay (Active Motif, Carlsbad, CA). Nuclear extracts (5 μg)
were incubated with ARE consensus site oligonucleotides (5’-GTCA-CAGTGACTGAGCAT-3’ and 5’-GGGCTAGCA-TGGCAAGTGAG-3’). Bound protein was detected with an antibody specific to DNA-bound Nrf2 and visualized by colorimetric reaction catalyzed by horseradish peroxidase-conjugated secondary antibody, and absorbance was measured at 405 nm.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed with the ChIP assay kit from Upstate Cell Signaling (Charlottesville, VA) as previously described (30). Briefly, cells were fixed with formaldehyde and sonicated, and sheared chromatin was immunoncleolated with protein agarose A. A fraction of the precleared chromatin was stored and labeled as “input DNA.” The remaining chromatin was immunoprecipitated with IgG or Nrf2 antibodies, protein-DNA complexes were eluted with elution buffer (1% SDS, 0.1 NaHCO3), and formaldehyde cross-links were reversed by addition of NaCl (5 M) and heating at 65°C for 4 h. DNA was purified, and PCR was performed with a primer pair that spanned the HO-1 E1 enhancer. The primers used were E1 forward 5’-AAGAGCTCCACCCCCACCCA-3’ and reverse 5’-GGGCTAGCA-TGGCAAGTGAG-3’. A 1.5% agarose gel with ethidium bromide was used to separate and examine the PCR products.

Cell viability and caspase-3 activation. Cell viability was assessed by measuring the uptake of the membrane-impermeant dye Trypan blue, as we previously described (28, 29, 48). Caspase-3 activity was determined by monitoring the cleavage of the p-nitroanilide-conjugated caspase-3 substrate DEVD. Briefly, cells were trypsinized, pooled with detached cells, washed in PBS, and suspended in lysis buffer (50 mM HEPES, pH 7.4, 10% sucrose, 0.1% Triton X-100) on ice for 10 min. After centrifugation at 13,000 g for 5 min at 4°C, supernatants were incubated with 50 μM DEVD and absorbance was measured at 405 nm with a μQuant spectrophotometer (Bio-Tek Instruments, Winooski, VT).

Statistical analysis. Results are expressed as means ± SE. Statistical differences between groups were evaluated with a Student’s two-tailed t-test or by ANOVA with Tukey’s post hoc test when multiple groups were compared. P values <0.05 were considered statistically significant.

RESULTS

Application of a physiological level of cyclic strain (6% at 1 Hz) to HAECs stimulated a time-dependent increase in HO-1 mRNA and protein (Fig. 1, A and B). A significant and progressive elevation in HO-1 mRNA was first detected after 4 h of mechanical strain. The induction of HO-1 protein expression by cyclic strain was delayed, with a significant increase in HO-1 protein detected 8 h after mechanical strain, and levels increased further after 24 h. The induction of HO-1 by cyclic strain was associated with an approximately twofold increase in HO activity (Fig. 1C). Application of cyclic strain also stimulated a time-dependent rise in HO-1 mRNA and protein expression and HO activity in HUVECs (Fig. 2).

In the next series of experiments the molecular mechanism by which cyclic strain induces HO-1 expression was examined. To evaluate whether stabilization of HO-1 mRNA contributes to the induction of HO-1 by cyclic strain, HAECs were treated with actinomycin D and the decay of HO-1 mRNA followed. These actinomycin D chase experiments revealed that HO-1 transcripts have a half-life of ~1.5 h. However, the stability of HO-1 mRNA was unaffected by cyclic strain (Fig. 3A). To determine whether increases in HO-1 expression in response to cyclic strain involve the transcriptional activation of the gene, cells were transiently transfected with a HO-1 reporter construct and promoter activity was monitored. Application of cyclic strain stimulated a nearly fourfold increase in promoter activity (Fig. 3B). Interestingly, mutation of the antioxidant responsive elements (AREs) attenuated basal promoter activity and abolished the response to cyclic strain, suggesting that cyclic strain activates HO-1 gene transcription via the AREs (Fig. 3B). Because the transcription factor Nrf2 plays a predominant role in ARE-mediated gene transcription, we examined whether Nrf2 mediates the activation of HO-1 by cyclic strain. Transfection of endothelial cells with a dominant-negative mutant of Nrf2 that had its transactivation domain deleted inhibited basal promoter activity and the cyclic strain-mediated increase in promoter activity (Fig. 3B). In addition, cyclic strain resulted in a rapid, time-dependent rise in Nrf2 protein expression, independent of any change in Nrf1 protein expression (Fig. 3C). However, cyclic strain failed to elevate Nrf2 mRNA expression (Fig. 3D). Cyclic strain also stimulated the activation of Nrf2, as reflected by the increase in binding of nuclear Nrf2 to the ARE (Fig. 3E). To further assess the involvement of Nrf2 in HO-1 transcription, we determined the binding of Nrf2 to the HO-1 promoter in its native chromatin.
Cyclic strain activates eNOS and releases nitric oxide (NO) (3), which is a well-established inducer of HO-1 (30, 33), we initially determined the role of this enzyme in mediating the induction of HO-1. The application of cyclic strain resulted in a significant increase in eNOS expression and activity (Fig. 4, A). Since oxidative stress has also been implicated in the induction of HO-1 by Nrf2 (2, 22, 51), the involvement of ROS in the induction of HO-1 was investigated. Application of cyclic strain resulted in a time-dependent increase in the level of ROS that peaked 8 h after the onset of strain and returned to near control levels after 24 h (Fig. 5A). The induction of oxidative stress by cyclic strain was not accompanied by any changes in the expression of Nox2 and Nox4 or their associated regulatory proteins (Fig. 5B).

In subsequent experiments, we determined the upstream signaling pathway that stimulated HO-1 expression. Since cyclic strain activates eNOS and releases nitric oxide (NO) (3), which is a well-established inducer of HO-1 (30, 33), we initially determined the role of this enzyme in mediating the induction of HO-1. The application of cyclic strain resulted in a significant increase in eNOS expression and activity (Fig. 4, A and B). Although the eNOS blocker methyl-L-arginine inhibits the activation of eNOS by cyclic strain, it failed to repress the induction of HO-1, indicating that cyclic strain-mediated increases in HO-1 expression are independent of NO (Fig. 4, B and C). Furthermore, the peroxynitrite scavenger uric acid (37) did not affect on the induction of HO-1 by cyclic strain (Fig. 4C). Since oxidative stress has also been implicated in the induction of HO-1 by Nrf2 (2, 22, 51), the involvement of ROS in the induction of HO-1 was investigated. Application of cyclic strain resulted in a time-dependent increase in the level of ROS that peaked 8 h after the onset of strain and returned to near control levels after 24 h (Fig. 5A). The induction of oxidative stress by cyclic strain was not accompanied by any changes in the expression of Nox2 and Nox4 or their associated regulatory proteins (Fig. 5B).

In the following experiments, the functional significance of the induction of HO-1 by cyclic strain was investigated. Treatment of HAECs with the combination of TNF-α (100 ng/mL) and ceramide (25 μM) resulted in a significant decline in the number of viable cells and stimulated a marked increase in the activity of the proapoptotic effector caspase-3 (Fig. 6, A and B, respectively). Interestingly, cyclic strain attenuated the cytokine-mediated loss of cell viability (Fig. 6A). However, the HO inhibitor SnPP increased cytokine-mediated cell death and reversed the cytoprotection afforded by cyclic strain. In the absence of cytokines, the application of cyclic strain or SnPP, either alone or in combination, had no effect on cell viability (Fig. 6A) or caspase-3 activity (data not shown). Cyclic strain also blocked cytokine-induced caspase-3 activation, and this was prevented by SnPP (Fig. 6B).

The cytoprotective role of HO-1 in endothelial cells was further corroborated with two distinct molecular approaches. Infection of HAECs with AdHO-1 resulted in a pronounced increase in HO-1 expression, and this was associated with a decrease in cell death following cytokine exposure (Fig. 7, A and B). The control adenovirus AdGFP had no effect on HO-1 protein expression or cell survival. Alternatively, transfection of HAECs with HO-1 siRNA for 3 days reduced HO-1 protein expression by >80%, whereas the NT siRNA minimally affected protein expression (Fig. 7C). Although HO-1 siRNA had no adverse effect on cell survival under basal conditions, it potentiated cell death in response to the inflammatory mediators (Fig. 7D). In contrast, NT siRNA failed to modulate cell survival. The effect of cyclic strain on endothelial cell HO-1 expression and function was mimicked by the proteasome inhibitor MG-132. Treatment of HAECs with MG-132 caused a time-dependent increase in Nrf2 protein that preceded a rise in HO-1 expression (Fig. 8A). A significant elevation in Nrf2 protein was detected 2 h after MG-132-treatment, and levels peaked between 4 and 8 h. A significant rise in HO-1 protein was first observed 4 h after MG-132 exposure, and levels increased further after 24 h. Interestingly, treatment of HAECs with MG-132 reduced the cytokine-mediated loss in cell viability (Fig. 8B).

To further investigate the role of HO-1 in cell protection, we exposed MAECs isolated from HO-1+/+ and HO-1−/− mice to cyclic strain. Cyclic strain induced a significant increase in HO-1 protein expression in MAECs derived from HO-1+/+ mice (Fig. 9A). In contrast, HO-1 expression was absent in HO-1−/− MAECs and the application of cyclic strain failed to induce the expression of HO-1. Cyclic strain also evoked an increase in oxidative stress in MAECs, and this was enhanced in cells taken from HO-1−/− mice (Fig. 9B). Finally, treatment of HO-1−/− MAECs with TNF-α and ceramide induced cell death that was attenuated by the application of cyclic strain.
Cyclic strain was greater in HO-1-mediated cell death. A strain failed to protect these endothelial cells from cytokine-E1 enhancer following exposure to cyclic strain for 4 and 8 h. To the antioxidant responsive element by ELISA. O.D., optical density. Control, static cells. For various times (0–8 h), and Nrf2 mRNA was quantified by scanning densitometry, normalized with respect to 18S rRNA, and expressed relative to that of

In some experiments, a dominant-negative Nrf2 (dnNrf2) construct was cotransfected into cells. Cyclic strain to induce HO-1 gene expression may provide a

HO-1 underlies this cytoprotective effect. Thus the ability of cyclic strain is dependent on the production of ROS and is

dependent fashion. The stimulation of HO-1 expression by mechanical strain is dependent on the production of ROS and is mediated by Nrf2. Significantly, we found that cyclic strain inhibits cytokine-mediated endothelial cell apoptosis and that HO-1 underlies this cytoprotective effect. Thus the ability of cyclic strain to induce HO-1 gene expression may provide a

novel mechanism by which hemodynamic forces promote endothelial cell survival and vascular homeostasis.

The application of cyclic strain stimulates a time-dependent increase in HO-1 protein and mRNA expression in endothelial cells. A significant elevation in HO-1 protein and activity is detected at 8 h, and this progressively increases during 24 h of cyclic strain exposure. The level of cyclic strain (6%) required to stimulate HO-1 expression is physiologically relevant since the maximum stretch of large systemic vessels during the cardiac cycle has been reported to vary between 5% and 12% of the maximum stretch of large systemic vessels during the cardiac cycle. We found that cyclic strain stimulates HO-1 expression in endothelial cells derived from various blood vessels across multiple species. Cyclic strain also stimulates the expression of

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HO-1 in vascular smooth muscle cells and ventricular myocytes, demonstrating that biomechanical strain induces HO-1 expression in diverse cardiovascular cells (17, 47).

The induction of HO-1 by cyclic strain does not involve alterations in mRNA stabilization and is likely due to the transcriptional activation of the gene since transient luciferase reporter assays demonstrate that cyclic strain directly stimulates HO-1 promoter activity. Interestingly, the induction of HO-1 gene transcription requires the presence of AREs, since mutation of this responsive element abolishes the stimulation of promoter activity by cyclic strain. Although several transcription factors can bind to AREs, Nrf2 plays the predominant role in ARE-dependent HO-1 gene expression (2). Consistent with this, we found that cyclic strain stimulates Nrf2 protein...
HO-1 promotes endothelial cell survival. Furthermore, transfection of endothelial cells with a dominant-negative Nrf2 construct abrogates the activation of HO-1 promoter activity in response to cyclic strain. Thus the mobilization of Nrf2 plays an integral role in mediating HO-1 gene transcription by cyclic strain. In contrast, cyclic strain did not stimulate Nrf1 expression, indicating a selective activation of Cap’n’Collar transcription factors by cyclic strain.

Fig. 6. Cyclic strain inhibits endothelial cell apoptosis in a HO-1-dependent manner. A: cyclic strain blocks the cytokine-mediated loss of cell viability. HAECs were subjected to cyclic strain for 24 h, treated with tumor necrosis factor-α (TNF-α; 100 ng/ml) and ceramide (Cer; 25 μM) in the absence or presence of tin protoporphyrin-IX (SnPP; 10 μM), and then exposed to an additional 24 h of cyclic strain. B: cyclic strain inhibits cytokine-mediated activation of caspase-3. HAECs were subjected to cyclic strain for 24 h, treated with TNF-α (100 ng/ml) and Cer (25 μM) in the absence or presence of SnPP (10 μM), and then exposed to an additional 24 h of cyclic strain. Results are means ± SE (n = 5–7). *Statistically significant effect of TNF-α and Cer; †statistically significant effect of cyclic strain.

expression and nuclear Nrf2 binding to the AREs. However, the increase in Nrf2 protein by cyclic strain is not associated with a rise in Nrf2 mRNA expression, suggesting that cyclic strain stimulates Nrf2 protein expression via a posttranscriptional mechanism that may involve protein stabilization (22, 51). Furthermore, transfection of endothelial cells with a dominant-negative Nrf2 construct abrogates the activation of HO-1 promoter activity in response to cyclic strain. Thus the mobilization of Nrf2 plays an integral role in mediating HO-1 gene transcription by cyclic strain. In contrast, cyclic strain did not stimulate Nrf1 expression, indicating a selective activation of Cap’n’Collar transcription factors by cyclic strain. Our finding

Fig. 7. HO-1 promotes endothelial cell survival. A: infection of cells with adenovirus expressing HO-1 (AdHO-1), but not adenovirus expressing green fluorescent protein (AdGFP), for 2 days stimulates HO-1 protein expression. B: HO-1 overexpression inhibits cytokine-mediated cell death. Cells were infected with AdHO-1 [20 multiplicity of infection (MOI)] and AdGFP (20 MOI) for 2 days and then treated with TNF-α (100 ng/ml) and ceramide (Cer; 25 μM) for 24 h. C: HO-1 small interference RNA (siRNA) inhibits HO-1 protein expression in HAECs. Cells were treated with HO-1 siRNA (100 nM) or nontargeting (NT) siRNA (100 nM) for 3 days. D: HO-1 silencing enhances cytokine-mediated loss of cell viability. HAECs were transfected with HO-1 siRNA (100 nM) or NT siRNA (100 nM) for 3 days and then treated with TNF-α (100 ng/ml) and Cer (25 μM) for 24 h. HO-1 protein was quantified by scanning densitometry, normalized with respect to β-actin, and expressed relative to that of control cells. Results are means ± SE (n = 3–5). *Statistically significant effect of AdHO-1 or HO-1 siRNA; †statistically significant effect of TNF-α and Cer.
that a hemodynamically relevant regimen of cyclic strain activates Nrf2 in HAECs builds on earlier work showing that mechanical pulmonary ventilation-associated stretch and fluid shear stress induce Nrf2 activation in endothelial cells (19, 34), highlighting the mechanosensitive nature of this transcription factor.

The induction of HO-1 by cyclic strain is dependent on the production of ROS. Consistent with earlier reports (18, 20), we found that cyclic strain stimulates a time-dependent increase in oxidative stress that peaks ~8 h after the application of cyclic strain. Interestingly, the generation of ROS returns toward baseline 24 h after the application of cyclic strain. This likely reflects the ability of cyclic strain to induce a battery of Nrf2-responsive antioxidant genes including HO-1, GCLM, NQO1, and others to combat cyclic strain-mediated increases in ROS generation (35, 46). Consistent with this notion, we found that cyclic strain-mediated elevations in ROS are greater in HO-1-deficient endothelial cells. Although NAD(P)H oxidase plays a pivotal role in cyclic strain-induced endothelial ROS production (18, 20), a physiological level of cyclic strain has no effect on the expression of the major endothelial catalytic subunits Nox2 and Nox4 or any of the regulatory subunits, suggesting that cyclic strain stimulates ROS formation by promoting the assembly of a functional NAD(P)H oxidase complex. However, the cyclic strain-mediated increase in oxidative stress is prevented by the glutathione donor N-acetyl-l-cysteine. Moreover, N-acetyl-l-cysteine blocks the activation of Nrf2 and the induction of HO-1 by cyclic strain. In contrast, treatment of endothelial cells with the NOS inhibitor methyl-l-arginine or the peroxynitrite scavenger uric acid fails to inhibit cyclic strain-mediated HO-1 expression. Thus oxidative, but not nitrosative, stress is responsible for the induction of HO-1 by cyclic strain. Although the mechanism by which oxidative stress activates Nrf2 is not fully known, the oxidation of cysteine residues in Kelch-like erythroid cell-derived protein 1 (Keap1) is likely involved, since several cysteine residues in Keap1 are able to undergo redox-dependent changes that result in the release and/or inhibition of Keap1-dependent ubiquitination and degradation of Nrf2 (22, 51).

Recent studies indicate that cyclic strain serves a critical survival function in endothelial cells. Application of physiologically relevant levels of cyclic strain inhibits apoptosis in bovine aortic endothelial cells and in a human endothelial cell line (26, 29, 34). In the present study, we now show that cyclic

![Fig. 8. The proteasome inhibitor MG-132 mimics the actions of cyclic strain. A: MG-132 stimulates Nrf2 and HO-1 protein expression in HAECs in a time-dependent fashion. Cells were treated with MG-132 (2 μM) for various times (0–24 h), and Nrf2 and HO-1 protein were quantified by scanning densitometry, normalized with respect to β-actin, and expressed relative to those of control cells. B: MG-132 blocks the cytokine-mediated loss of endothelial cell viability. HAECs were treated with MG-132 (2 μM) for 8 h and then exposed to TNF-α (100 ng/ml) and Cer (25 μM) for an additional 24 h. Results are means ± SE (n = 3–5). *Statistically significant effect of MG-132; †statistically significant effect of TNF-α and Cer.

![Fig. 9. HO-1 gene deletion abolishes the cytoprotection afforded by cyclic strain in murine aortic endothelial cells (MAECs). A: HO-1 protein expression in MAECs derived from wild-type (HO-1+/+) or HO-1-deficient (HO-1−/−) mice in the absence or presence of cyclic strain. A.U., Arbitrary units. B: cyclic strain for 8 h stimulates ROS production in MAECs. C: cyclic strain-mediated cytoprotection was absent in HO-1−/− MAECs. MAECs were subjected to cyclic strain for 24 h, treated with TNF-α (100 ng/ml) and Cer (25 μM), and then exposed to an additional 24 h of cyclic strain. HO-1 protein was quantified by scanning densitometry, normalized with respect to β-actin, and expressed relative to that of control, static cells. Results are means ± SE (n = 3–8). *Statistically significant effect of cyclic strain; †statistically significant effect of HO-1 gene deletion; ‡statistically significant effect of TNF-α and Cer.
strain also promotes the survival of primary HAECs and MAECs. Moreover, we are the first to demonstrate that HO-1 plays an important role in the cytoprotection mediated by cyclic strain. In particular, the application of a physiological level of cyclic strain attenuates endothelial cell death and caspase-3 activation in response to TNF-α and ceramide. The cytoprotection afforded by cyclic strain is mediated by HO-1, since inhibition of HO-1 activity or genetic ablation of the enzyme abolishes the salutary effect of cyclic strain. Interestingly, cell death in response to inflammatory stress is augmented in endothelial cells derived from HO-1-deficient mice. This later finding is in agreement with an earlier report showing increased susceptibility of HO-1-null endothelial cells to oxidized lipid-induced cell injury (9). We further corroborated the cytoprotective property of the enzyme by showing that adenovirus-mediated gene transfer of HO-1 retards, while HO-1 silencing augments, cytokine-mediated endothelial cell death. Moreover, the induction of HO-1 expression following MG-132 exposure confers endothelial cell protection. The antiapoptotic action of HO-1 is likely mediated by CO since scavenging of this gas with hemoglobin reverses the antiapoptotic action of HO-1 in endothelial cells while the exogenous administration of CO reproduces the protection induced by HO-1 in response to numerous apoptotic stimuli, including TNF-α (4, 32). While HO-1 and CO elicit numerous antiapoptotic actions, their ability to activate the prosurvival kinase Akt (5, 7, 25) may be particularly relevant since inhibition of endothelial cell apoptosis by cyclic strain is dependent on Akt (29, 34).

The ability of cyclic strain to stimulate HO-1 gene expression may provide an important mechanism by which hemodynamic forces preserve vascular homeostasis. Aside from preventing endothelial apoptosis, the induction of HO-1 in endothelial cells may inhibit vascular smooth muscle tone and platelet aggregation by releasing CO (15, 24, 47). In addition, HO-1 exerts potent anti-inflammatory effects by blocking the activation of endothelial cells and the recruitment of immune cells into the vessel wall (27, 39). Furthermore, HO-1 restores endothelial integrity of injured blood vessels by stimulating the proliferation and migration of endothelial cells from adjacent border regions of the injured artery and by recruiting circulating endothelial progenitor cells to the site of damage (11, 12, 49). Thus, the induction of HO-1 by physiological levels of cyclic strain may serve an important homeostatic role to maintain blood flow and fluidity and initiate endothelial repair at sites of arterial injury.

In conclusion, the present study demonstrates that a hemodynamically relevant regime of cyclic strain induces HO-1 gene expression in endothelial cells derived from various vascular beds by activating the ROS-Nrf2 signaling pathway. In addition, it found that cyclic strain inhibits endothelial cell death in a HO-1-dependent fashion. The ability of cyclic strain to stimulate HO-1 expression may provide a critical mechanism by which hemodynamic forces promote endothelial cell survival and vascular homeostasis.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS
Author contributions: X.-m.L., K.J.P., and W.D. conception and design of research; X.-m.L. and K.J.P. performed experiments; X.-m.L., K.J.P., and W.D. analyzed data; X.-m.L., K.J.P., and W.D. interpreted results of experiments; X.-m.L. and K.J.P. edited and revised manuscript; X.-m.L., K.J.P., and W.D. approved final version of manuscript; K.J.P. prepared figures; W.D. drafted manuscript.

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