Induction of the Cytoprotective Enzyme Heme Oxygenase-1 by Statins Is Enhanced in Vascular Endothelium Exposed to Laminar Shear Stress and Impaired by Disturbed Flow*§

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In addition to cholesterol-lowering properties, statins exhibit lipid-independent immunomodulatory, anti-inflammatory actions. However, high concentrations are typically required to induce these effects in vitro, raising questions concerning therapeutic relevance. We present evidence that endothelial cell sensitivity to statins depends upon shear stress. Using heme oxygenase-1 expression as a model, we demonstrate differential heme oxygenase-1 induction by atorvastatin in atheroresistant compared with atheroprone sites of the murine aorta. In vitro, exposure of human endothelial cells to laminar shear stress significantly reduced the statin concentration required to induce heme oxygenase-1 and protect against H2O2-mediated injury. Synergy was observed between laminar shear stress and atorvastatin, resulting in optimal expression of heme oxygenase-1 and resistance to oxidative stress, a response inhibited by heme oxygenase-1 small interfering RNA. Moreover, treatment of laminar shear stress-exposed endothelial cells resulted in a significant fall in intracellular cholesterol. Mechanistically, synergy required Akt phosphorylation, activation of Kruppel-like factor 2, NF-E2-related factor-2 (Nrf2), increased nitric-oxide synthase activity, and enhanced HO-1 mRNA stability. In contrast, heme oxygenase-1 induction by atorvastatin in endothelial cells exposed to oscillatory flow was markedly attenuated. We have identified a novel relationship between laminar shear stress and statins, demonstrating that atorvastatin-mediated heme oxygenase-1-dependent antioxidant effects are laminar shear stress-dependent, proving the principle that biomechanical signaling contributes significantly to endothelial responsiveness to pharmacological agents. Our findings suggest statin pleiotropy may be suboptimal at disturbed flow atherosusceptible sites, emphasizing the need for more specific therapeutic agents, such as those targeting Kruppel-like factor 2 or Nrf2.

The efficacy of 3-hydroxy-3-methylglutaryl-coenzyme A reductase antagonists (statins) in reducing low density lipoprotein cholesterol, cardiovascular morbidity, and mortality is widely recognized (1). The observation that beneficial actions of statins on vascular function are detectable prior to any fall in serum cholesterol, extend to normocholesterolemic patients and exceed those of other lipid-lowering drugs despite comparable falls in total cholesterol (2, 3), suggest the existence of low density lipoprotein-cholesterol-independent effects (4, 5). Judging from in vitro studies, these may include immunomodulatory, anti-inflammatory, anti-adhesive, anti-thrombotic, and cytoprotective actions (6). However, the experimental work demonstrating these pleiotropic effects has predominantly used statin concentrations exceeding those achieved by therapeutic dosing, raising questions concerning clinical relevance (4).

Heme oxygenase-1 (HO-1) acts as the rate-limiting factor in the catabolism of heme into biliverdin, releasing free iron and carbon monoxide (CO). Biliverdin is subsequently converted to bilirubin by biliverdin reductase, whereas intracellular iron induces expression of heavy chain-ferritin and the opening of Fe2+ export channels (7). The biologic activity of HO-1 represents an important adaptive response in cellular homeostasis, as revealed by widespread inflammation and persistent endothelial injury in human HO-1 deficiency (8).

Expression of HO-1 in atherosclerotic lesions, and its ability to inhibit vascular smooth muscle cell proliferation, exert anti-inflammatory, antioxidant, and anti-thrombotic effects, suggests a protective role during atherogenesis (9, 10). HMOX1 promoter polymorphisms affecting HO-1 expression may influence susceptibility to intimal hyperplasia and coronary artery disease, whereas a low serum bilirubin constitutes a cardiovascular risk factor (11). Moreover, overexpression of HO-1 inhibited atherogenesis, whereas Hmxox1−/− mice bred onto an ApoE−/− background developed more extensive and complex atherosclerotic plaques (12, 13).

Recent interest has focused on the therapeutic potential of HO-1 and its products, with probucol, statins, rapamycin, nitric

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2 The abbreviations used are: HO-1, heme oxygenase-1; EC, endothelial cells; LSS, laminar shear stress; NO, nitric oxide; eNOS, endothelial nitric-oxide synthase; KLF2, Kruppel-like factor 2; Nrf2, NF-E2-related factor-2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI3K, phosphoinositide 3-kinase; iNOS, inducible nitric oxide synthase; 1,10-phenanthroline; LNAME, L-NAME, Nω-nitro-o-arginine methyl ester; HUVEC, human umbilical vein endothelial cells; OF, oscillatory flow; DN, dominant-negative; H2DCF, dihydrodichlorofluorescein; STAT3, signal transducer and activator of transcription 3; CM-H2DCFDA, 5-(and -6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester; siRNA, small interfering RNA; RT, reverse transcriptase.
oxide donors, and aspirin being shown to induce HO-1 (reviewed in Ref. 10). Indeed, induction of HO-1 may represent an important component of the vasculoprotective profile of statins, with simvastatin, atorvastatin, and rosuvastatin variously shown to increase HMOX1 promoter activity and mRNA levels, to induce enzyme activity and increase antioxidant capacity in human endothelial cells (EC) (14–18). However, induction of HO-1 in vascular EC in vivo has not yet been demonstrated.

Vascular endothelium exposed to unidirectional, pulsatile laminar shear stress (LSS) > 10 dynes/cm² is relatively protected against atherogenesis. LSS increases nitric oxide (NO) biosynthesis, prolongs EC survival, and generates an antioxidant, anti-adhesive cell surface. In contrast, endothelium exposed to disturbed blood flow, with low shear reversing or oscillatory flow patterns, such as that located at arterial branch points and curvatures, is atheroprotective. Thus endothelial cells exposed to disturbed blood flow exhibit reduced levels of endothelial nitric-oxide synthase (eNOS), increased apoptosis, oxidative stress, permeability to low density lipoprotein, and leukocyte adhesion (19).

The atheroprotective influence of unidirectional LSS and the overlap between these actions and those of statins led us to hypothesize that LSS increases endothelial responsiveness to statins. We demonstrate for the first time that treatment of mice with atorvastatin induces HO-1 expression in the aortic endothelium and that this occurs preferentially at sites exposed to LSS. In vitro, pre-conditioning human EC with an atheroprotective, but not an atheroprotective waveform, significantly reduces the concentration of atorvastatin required to enhance HO-1-mediated cytoprotection against oxidant-induced injury. A synergistic relationship between LSS and statins is revealed, resulting in maximal Akt phosphorylation and dependence upon eNOS, Kruppel-like factor 2 (KLF2), and NF-E2-related factor-2 (Nrf2) activation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Actinomycin D, hydrogen peroxide (H₂O₂), paraformaldehyde, Triton X-100, trypan blue, and anti-α-tubulin antibody were from Sigma. Atorvastatin and simvastatin were from Merck Biosciences (Nottingham, UK) and 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Promega (Southampton, UK). N⁶-Nitro-1-arginine methyl ester (L-NAME) from BIOMOL (Plymouth Meeting, PA), leptin from R&D Systems, anti-phospho-Akt (Ser473) antibody from Cell Signaling (Beverly, MA), and anti-HO-1 antibodies were from Cambridge Bioscience (Cambridge, UK) and Stressgen (Victoria, BC). The nuclear extraction kit NE-PER Nuclear and Cytoplasmic Extraction Reagents were from Thermo Fisher Scientific Inc. Nrf2 activation in EC nuclear extracts was analyzed using an Nrf2 TransAM™ assay kit (Active Motif, Carlsbad, CA).

**Endothelial Cell Exposure to Shear Stress**—Human umbilical vein EC (HUVEC) and human aortic EC (purchased from Promocell, Heidelberg, Germany) were cultured as described (20). The use of human EC was approved by Hammersmith Hospitals Research Ethics Committee (number 06/Q0406/21). Confluent EC monolayers (passage 3) on fibronectin-coated glass slides were exposed to control static conditions, high shear unidirectional laminar flow (12 dynes/cm²), or oscillatory flow (OF) with directional changes of flow at 1 Hz (± 5 dynes/cm²), for up to 48 h using a parallel-plate flow chamber (Cytodyne, La Jolla, CA) as described previously (21). To investigate synergy between LSS and statins, EC were exposed to static conditions or unidirectional LSS (12 dynes/cm²) for a total of 24 h. After 12 h, statin or vehicle control was added to the culture medium of static cells or to the medium in the flow apparatus via the injection port, whereas EC remained under conditions of continuous LSS. Cell viability was assessed by examination of EC monolayers using phase-contrast microscopy, cell counting, and estimation of trypan blue exclusion.

**RNA Interference Design and Transfection**—Previously validated siRNA sequences targeting KLF2, HO-1, or scrambled control siRNA were transfected into HUVEC using oligofectamine-based transfection in endothelial basal medium 2 as described (18): HO-1, sense, 5′-UGCUGAGUUAUGAGGAACUU-3′ and antisense, 5′-GUUCUCAUGAUCAGCUAUU-3′; sense, 5′-CAUUGCCAGUGCCACAGU-3′ and antisense, 5′-CUGUGGCAUGCUAUUU-3′; KLF2, sense, 5′-GCCCUACCAUGCAACGUGU-3′ and antisense, 5′-CCAGUUUGCAUGGUGGCUU-3′; sense, 5′-GUUUGCAGACCGAGU-3′ and antisense, 5′-CUGCUUGAGCGCGCAAACU-3′.

EC were cultured for 24 h in endothelial basal medium 2 and analyzed for target gene expression by quantitative RT-PCR or immunoblotting, which demonstrated up to 80% reduction in expression as reported (18). The specificity of siRNA targeting was confirmed using a second set of sequences. Efficacy of siRNA was verified in each experiment.

**Adenoviral Transfection and Luciferase Reporter Assay**—The recombinant adenoavirus expressing dominant-negative (DN) Akt was a gift from Dr. C. Wheeler-Jones (Royal Veterinary College, London). The adenoavirus expressing DN-Nrf2, which lacks the transactivation domain (Ad-Nrf2-DN) was provided by Dr. Jeffrey A. Johnson, University of Wisconsin, Madison, WI (22). Adenoviruses were amplified in HEK-293A cells, purified, and titered using BD Adeno-X Purification and Rapid Titer Kits (BD Biosciences). HUVEC were infected by incubation with adenovirus in serum-free M199 for 2 h at 37 °C. The media was then changed to M199, 10% fetal bovine serum and HUVEC incubated overnight prior to experimentation. Infection of HUVEC with a β-galactosidase control adenovirus demonstrated a transfection efficiency of ≥95%. The optimal multiplicity of infection for the DN-Nrf2 and DN-Akt adenoviruses was determined by immunoblotting (not shown). The plasmid pH01-Luc was a gift from J. Alam (Alton Ochsner Medical Foundation, New Orleans, LA). EC were transfected in triplicate with pGL3-basic or pH01-Luc using microporation technology (Digital Bio, Seoul, Korea) as described previously (18).

**Analysis of Oxidative Stress and Cellular Injury**—HUVEC were loaded with 5 μM 5-(and -6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Invitrogen) for 30 min at 37 °C. ECs were incubated in serum-free M199 with H₂O₂ (5 μM) for 30 min or 100 ng/ml leptin for 2 h and then washed with ice-cold phosphate-buffered saline. Intracellular dihydrodichlorofluorescein (H₂DCF) was ox-
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dized to 2,7-dichlorofluorescein and quantified by flow cytometry. H$_2$O$_2$ (50 μM) was used to induce cellular injury and this was quantified using either: (i) trypan blue exclusion or (ii) a MTT assay. Cell numbers were not significantly altered by any of the treatment conditions prior to addition of H$_2$O$_2$.

Quantitative Real-time PCR—Quantitative real-time PCR was performed using an iCycler (Bio-Rad). β-Actin, glyceraldehyde-3-phosphate dehydrogenase, and hypoxanthine-guanine phosphoribosyltransferase were used as housekeeping genes, with data calculated in relation to the β-Actin gene and verified with gliceraldehyde-3-phosphate dehydrogenase and hypoxanthine-guanine phosphoribosyltransferase. DNase-I-digested total RNA (1 μg) was reverse transcribed using 1 μM oligo(dT) and Superscript reverse transcriptase (Invitrogen). cDNA was amplified in a 25-μl reaction containing 5 μl of cDNA template, 12.5 μl of iSYBR supermix, 0.5 pm sense and antisense gene-specific primers, and double distilled H$_2$O. Primer sequences used were: KLF2 forward, 5’-CTCTGACAGGCCTGGCAT-3’; HO-1 forward, 5’-TTCTTCACCTCCCAACAA-3’; HO-1 reverse, 5’-TTCTATCCCTCTGCTGA-3’; Nrf2 forward, 5’-AAACAGTTGATCTGGCAAC-3’, Nrf2 reverse, 5’-GGCGGGAATATCAGGAAC-3’; TM forward, 5’-TTGTTGAAATTGGAGCTTGG-3’, TM reverse, 5’-TCTC-ATGAACCTGATGGGTT-3’; eNOS forward, 5’-TGGCTCTCCCTCAGTTC-3’, eNOS reverse 5’-AGGGCGT-TTTGCTCTTC-3’ (24). Cycling parameters were 3 min at 95 °C, and 40 cycles of 95 °C for 10 s and 56 °C for 45 s.

Immunoblotting—Immunoblotting was performed as described (25). HUVEC were incubated with atorvastatin for up to 72 h prior to lysis, SDS-PAGE, and transfer to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). Immunoblots were probed with primary antibodies overnight at 4 °C, followed by appropriate secondary reagents for 1 h at room temperature and developed with a chemiluminescence substrate (Amersham Biosciences). To ensure equivalent sample loading, protein content was determined using the Bio-Rad DC protein assay (Bio-Rad) and membranes were stripped and re-probed with a control antibody. Integrated density values were obtained with an Alpha Innotech ChemiImager 5500 (Alpha Innotech, San Leandro, CA).

Cholesterol Analysis—The measurement of intracellular cholesterol was carried out using a procedure previously described in detail by Wang et al. (26).

Animals—C57BL/6 mice were from Harlan Olac (Bicester, Oxford, UK) and housed under controlled climactic conditions in microisolator cages with autoclaved bedding. Irradiated food and drinking water were readily available. All animals were housed and studied according to UK Home Office guidelines. Sentinel mice were housed alongside test animals and regularly screened for a standard panel of murine pathogens.

Confocal Microscopy—En face confocal microscopy was used to assess changes in the expression of HO-1 in the murine aortic vascular endothelium. C57BL/6 mice (n = 6) were injected intraperitoneally with atorvastatin (5 mg/kg) or vehicle alone and sacrificed 24 h later by CO$_2$ inhalation, followed by perfusion fixation with 2% formalin and harvesting of aortae. Fixed aortae were treated with an HO-1 specific primary antibody (Cambridge Biosciences) and an Alexa Fluor 568-conjugated secondary antibody. Stained vessels were mounted prior to visualization of endothelial surfaces en face using confocal laser scanning microscopy (LSM 510 META; Zeiss, Oberkochen, Germany). Changes in the expression of HO-1 in murine aortic EC located in regions of the lesser curvature exposed to disturbed flow and both the greater curvature and descending aorta exposed to laminar flow were quantified as described (27). EC were identified by co-staining with anti-CD31 antibody conjugated to the fluorophore fluorescein isothiocyanate (Invitrogen). Nuclei were identified using a DNA-binding probe with far-red emission (Draq5; Biostatus, Leicester, UK). Isotype-matched monoclonal antibodies against irrelevant antigens were used as experimental controls for specific staining. HO-1 protein expression was quantified by image analysis of fluorescence intensity in 100 cells in at least 3 distinct sites using Image J software. EC fluorescence was measured above a threshold intensity defined by background fluorescence.

Statistics—Data were grouped according to treatment and analyzed using GraphPad Prism software (San Diego, CA) and the analysis of variance with Bonferroni correction or an unpaired Student’s t test. Data are expressed as the mean of individual experiments ± S.E. Differences were considered significant at p values of <0.05.

RESULTS

Atorvastatin Induces Endothelial HO-1 Expression in Murine Aortic EC—To establish whether statins increase endothelial HO-1 expression in vivo, C57Bl/6 mice were treated with atorvastatin for 24 h. Changes in HO-1 expression were quantified by en face confocal microscopy of the aortic endothelium, with endothelial cells identified by CD31 staining. As shown in Fig. 1A, treatment with atorvastatin induced a significant increase in HO-1 expression in murine aortic endothelium at a site with low probability of developing atherosclerotic lesions (27). In contrast, although HO-1 induction was detectable, EC located in the lesser curvature of the aorta, which has a high probability of developing lesions, were relatively refractory to atorvastatin treatment (Fig. 1B). Quantification by image analysis confirmed that HO-1 induction at high probability sites was significantly less than that at low probability sites (Fig. 1C).

LSS and Statins Exhibit Synergy—Statins and unidirectional LSS separately induce EC HO-1 expression in vitro. An established physiologic hemodynamic environment was therefore used to explore the influence of LSS on statin responsiveness. As expected, treatment of HUVEC with either 2.5 μM atorvastatin under static conditions, or exposure of HUVEC to LSS for 24 h, significantly increased HO-1 mRNA levels (Fig. 2A). Pre-conditioning of EC with LSS (12 dynes/cm$^2$) for 12 h prior to addition of atorvastatin and continuation of culture under LSS for a further 12 h resulted in an additive increase in HO-1 mRNA (Fig. 2A). Reduction of the atorvastatin concentration applied to static-cultured EC to 0.6 μM led to loss of HO-1 induction. In contrast, a significant increase of HO-1 mRNA (Fig. 2B). A dose-response study confirmed synergy between LSS (12 dynes/cm$^2$) and atorvastatin.
Differential Regulation of HO-1 in EC Exposed to LSS and OF—To compare the effect of atheroprotective and atheroprone waveforms on responsiveness to statins, EC were exposed to LSS (12 dynes/cm²) or OF (≥5 dynes/cm²) at 1 Hz (21). A 10-fold increase in HO-1 expression was seen in EC exposed to 24 h LSS, whereas HO-1 mRNA induction was reduced to 3-fold in EC exposed to OF. Furthermore, OF-conditioned EC failed to demonstrate a synergistic relationship with atorvastatin (Fig. 2F).

LSS and Atorvastatin Induce an Enhanced Antioxidant Effect—To investigate the functional relevance of HO-1 induction, EC were exposed to free radical-induced injury. Atorvastatin (0.6 μM) failed to protect static-cultured HUVEC exposed to H₂O₂ (50 μM). However, EC exposed to LSS for 24 h were protected by 50%, and this was significantly enhanced by atorvastatin (0.6 μM) (Fig. 3A). An oxygen radical-sensitive fluorescent probe (CM-H₂DCFDA) was used to explore the ability of atorvastatin to modulate oxidative stress. LSS alone led to low-level oxidant generation, whereas exposure of EC to H₂O₂ (5 μM) induced a maximal response (Fig. 3B). Pre-treatment of static-cultured EC with atorvastatin (0.6 μM) failed to protect, whereas LSS alone significantly reduced H₂O₂ generated oxidative stress. However, as predicted, maximal protection was seen in LSS-conditioned EC treated with atorvastatin (Fig. 3B). Treatment with leptin also increased EC oxidant generation by 5-fold. LSS was again protective, with maximal reduction in leptin-induced oxidative stress seen in those cells exposed to both LSS and atorvastatin (Fig. 3C).

To determine the role of HO-1 in the cytoprotective response, HUVEC were transfected with HO-1 specific or control siRNA. HO-1 siRNA reduced mRNA levels by 80% (supplemental Fig. 2A) (18). Interference with HO-1 expression significantly reduced cytoprotection against H₂O₂-induced cell death afforded by atorvastatin in LSS-conditioned EC, from 75 to 40% (Fig. 3D), suggesting HO-1 is an important but not necessarily unique protective mechanism. In line with reduced HO-1 induction, exposure of EC to an atheroprone OF pattern revealed markedly attenuated protection against H₂O₂. Moreover, atorvastatin failed to enhance cytoprotection against oxidative injury in this setting (Fig. 3E).

LSS and Atorvastatin in Combination Reduce Intracellular Cholesterol—As shown in Fig. 4A, exposure of HUVEC to LSS, or treatment of static-cultured EC with atorvastatin (0.6 μM), resulted in a modest reduction in intracellular cholesterol, which did not reach significance. However, consistent with the effect on HO-1 expression, pre-conditioning of EC with LSS prior to addition of atorvastatin resulted in a significant fall in intracellular cholesterol up to 60%. These data, combined with that in Fig. 2, suggest that EC exposure to LSS significantly enhances responsiveness to statins.

LSS and Atorvastatin Stabilize HO-1 mRNA—To determine whether LSS and atorvastatin regulate HO-1 expression post-transcriptionally, EC were exposed to LSS in the presence or absence of atorvastatin, prior to addition of actinomycin D (2 μg/ml) and analysis of HO-1 by quantitative RT-PCR. Treatment with actinomycin D resulted in less than 5% cell death as estimated by trypsin blue exclusion studies. The rapid decay of

![Image](image-url)
HO-1 mRNA in static-cultured EC was not delayed by atorvastatin. In contrast, LSS increased HO-1 mRNA stability and this delay in degradation was further prolonged by atorvastatin (Fig. 4B).

**Inhibition of KLF2 and Nrf2 Prevents LSS and Atorvastatin Synergy**—KLF2 is important for LSS-induced vascular endothelial cytoprotection (28), and in statin-mediated induction of HO-1 in static EC (18). Thus, we explored the role of KLF2 in flow-mediated up-regulation of HO-1. KLF2-targeted siRNA reduced expression in HUVEC by 80% (supplemental Fig. S2B) (18). However, interference with the KLF2 transcript did not alter HO-1 induction by LSS (Fig. 5A) (29). Likewise, KLF2 depletion did not reduce LSS-mediated cytoprotection against oxidant-induced injury, when compared with control siRNA (Fig. 5B).

The Nrf2-Keap1 system regulates cytoprotective gene expression via the antioxidant responsive element, with several antioxidant responsive element-regulated genes induced by LSS (29). A recombinant adenovirus expressing a DN-Nrf2 construct (multiplicity of infection 100) demonstrated the importance of Nrf2 in HO-1 induction by LSS (Fig. 5C). The functional consequences of HO-1 suppression were evident when HUVEC expressing DN-Nrf2 were exposed to LSS followed by H2O2. DN-Nrf2 significantly reduced LSS-mediated protection against oxidant-induced injury (Fig. 5D).

The distinct transcription factors used by atorvastatin and LSS therefore suggested that activation of both was required for maximal synergistic induction of HO-1. To investigate this, HUVEC were transfected with KLF2 siRNA and Adv DN-Nrf2. Atorvastatin (0.6 μM) in the presence of siRNA or adenoviral vectors had no effect on HO-1 expression (Fig. 5, E and F). KLF2 siRNA did not affect LSS induction of HO-1, although reducing HO-1 expression in EC exposed to LSS and atorvastatin by 40% (supplemental Fig. S2C). Inhibition of Nrf2 alone had a more marked effect, reducing HO-1 expression in LSS and statin-treated EC by 60% (supplemental Fig. S2C). Inhibition of Nrf2 alone had a more marked effect, reducing HO-1 expression in LSS and statin-treated EC by 60% (supplemental Fig. S2C). Although co-transfection of EC with KLF2 siRNA and DN-Nrf2 significantly attenuated LSS-induced HO-1 when compared with EC transfected with control siRNA and Adv β-galactosidase, this was equivalent to that seen with Nrf2-DN alone. However, inhibition of both transcription factors led to a maximal reduction in LSS and atorvastatin-induced HO-1 up-regulation, reducing this by 80% from 12.5- to 2.5-fold.
supporting the view that both KLF2 and Nrf2 activation are required (Fig. 5F).

Akt Activation Regulates LSS and Atorvastatin Synergy—We next explored PI3K/Akt activation, which is known to occur in EC exposed to LSS or statins (30). Atorvastatin (0.6 μM) failed to increase Akt phosphorylation (Ser473) in static-cultured cells (Fig. 6A). However, prolonged LSS increased Akt phosphorylation and this was further enhanced by atorvastatin. The importance of Akt was confirmed with Adv-DN-Akt (multiplicity of infection 100), which inhibited LSS-induced HO-1 expression and the synergy between LSS and atorvastatin, a response not seen with the β-galactosidase control (Fig. 6B).

To investigate the relationship between Akt, KLF2, and Nrf2, we analyzed changes in KLF2 and Nrf2 expression following exposure to LSS and atorvastatin. Inhibition of Akt reversed both LSS-induced expression of KLF2 and the synergistic up-regulation in LSS-conditioned EC exposed to atorvastatin (Fig. 6C). Likewise, the induction of Nrf2 mRNA by LSS, and the enhanced response in the presence of atorvastatin, was reversed by DN-Akt (Fig. 6D), consistent with a recent study that reported that flow-induced translocation of Nrf2 requires PI3K/Akt activation (29). Thus, EC pre-conditioned with LSS and treated with atorvastatin exhibit optimal Akt phosphorylation, activation of optimal Akt phosphorylation, activation of KLF2 and Nrf2, resulting in maximal HO-1 expression.

The nitric-oxide synthase (NOS) inhibitor L-NAME was used to investigate eNOS and nitric oxide in HO-1 induction, recognizing that PI3K activity, LSS, and statins increase eNOS expression and phosphorylation. Induction of eNOS mRNA was maximal in EC exposed to LSS and atorvastatin and this was dependent upon activation of Akt (Fig. 6E). Although the presence of L-NAME did not alter basal HO-1 expression, NOS inhibition reduced LSS-mediated HO-1 induction by 50%, and prevented the synergy between atorvastatin and LSS (Fig. 7A). Next we sought to determine the effect of L-NAME on the activation of KLF2 and Nrf2. To assess KLF2 activation, we analyzed expression of its target gene thrombomodulin (31). As seen in Fig. 7B, LSS induced expression of thrombomodulin, a response enhanced by atorvastatin. However, the presence of L-NAME did not significantly inhibit this response. To study Nrf2 activation, EC nuclear extracts were isolated and binding of Nrf2 to the antioxidant responsive element was quantified using a TransAM assay. LSS increased Nrf2 activation by 4-fold, whereas atorvastatin alone (0.6 μM) had no effect, nor did it increase activation in EC pre-conditioned with LSS. Finally, pretreatment with L-NAME did not influence the activation of Nrf2 (Fig. 7C). Together these data
suggest that optimal induction of eNOS in response to LSS and atorvastatin is a consequence of Nrf2 and KLF2 activation and in turn contributes to the increase in HO-1 expression.

**DISCUSSION**

We have explored the hypothesis that LSS conditioning of endothelium enhances the cytoprotective effects of statins, using HO-1 induction as a model. Wide-ranging lipid-independent effects of statins have been reported (6). However, the statin concentration used often exceeds that measured in the plasma during pharmacokinetic studies, raising the question of therapeutic relevance. Alternatively, this may reflect reduced responsiveness of cultured cells to statins and increased hepatic metabolism of these drugs in rodents. Detection of HO-1 protein up-regulation by statins in endothelium has varied, reflecting differences in the EC type studied and the source of statin (14–16, 18, 32). Notwithstanding, we now show that LSS preconditioning of vascular EC significantly reduces the atorvastatin concentration required to induce maximal HO-1 induction *in vitro* with LSS and atorvastatin exhibiting synergy. Moreover, using *en face* confocal microscopy we have demonstrated, to the best of our knowledge for the first time, statin-mediated induction of HO-1 in the aortic endothelium *in vivo*, which was optimal at an atheroprotected site predicted to be exposed to LSS. This study therefore proves the principle that biomechanical signaling makes a significant contribution to endothelial responsiveness to pharmacological agents and specifically suggests that EC at atherosusceptible regions of vessels may fail to be maximally affected by statins.

Atherosclerosis is a geometrically focal disease, predominantly located at arterial branch points and curvatures where the vascular endothelium is exposed to disturbed flow, characterized by a high oscillatory shear index and low time-averaged shear stress amplitude (19, 33). In contrast, unidirectional LSS is an essential component of vascular endothelial homeostasis. The atheroprotective waveform is anti-inflammatory, anti-apoptotic, and antioxidant (34–36). Additional transcriptional responses to biomechanical forces, including those mediated by KLF2 and Nrf2, are important for atheroprotection. KLF2 is induced in endothelium exposed to LSS and is an important regulator of eNOS and thrombomodulin, exerting anti-inflammatory and antithrombotic effects (28, 37, 38). Nrf2 activity is central to LSS-mediated regulation of antioxidant genes including *HMOX1*, thioredoxin reductase 1, and glutathione reductase (29, 39).

Pre-conditioning HUVEC with unidirectional LSS increased responsiveness to statins so that atorvastatin (0.6 μM), which failed to up-regulate HO-1 in static-cultured EC, induced a maximal increase in the *HMXO1* transcript. In contrast, exposure to OF, representing the atheroprone waveform revealed significant attenuation in shear stress-induced HO-1 expression, and no response to atorvastatin. Although exposure to LSS reduced the concentration of statin required to induce HO-1, we failed to demonstrate induction with 0.3 μM atorvastatin, which is thought to be at the upper limit of the plasma concentration achieved therapeutically (40). This may reflect the lack of pulsatility in the LSS model, which is a limitation of our study. Available data suggest that KLF2 expression and Nrf2 translocation are maximal in response to pulsatile unidirectional LSS (29, 41), and we speculate that this would further reduce the statin concentration required to induce optimal HO-1 expression.

The functional importance of LSS and atorvastatin synergy was confirmed by increased resistance of EC to oxidative stress induced by leptin, and protection against H₂O₂-induced EC death. The additional protective effect seen in EC exposed to both LSS and atorvastatin was lost in EC pre-treated with HO-1 siRNA. However, HO-1 depletion did not completely inhibit cytoprotection, suggesting other antioxidant genes induced by LSS including NAD(P)H:quinine oxidoreductase-1, NAD(P)H oxidase, superoxide dismutase, thioredoxin reductase 1, and glutathione reductase may be involved (29). The failure of atorvastatin to increase HO-1 in EC exposed to OF rendered the cells susceptible to oxidant-induced injury, reflecting also...
the failure of OF to induce the antioxidant genes above (29). The mechanisms through which HO-1 products exert antioxidant actions remain to be fully determined. Of note activation of STAT3 is important for the protective effects of HO-1 and CO against hyperoxia-induced murine lung injury (42). However, whereas STAT3 merits further investigation as a downstream mediator of LSS + atorvastatin-induced HO-1, it has been reported that both statins (43, 44) and shear stress (45) may inhibit STAT3 activation.

Analysis of total EC cholesterol emphasized the increased efficacy of atorvastatin in cells pre-conditioned by LSS. A significant fall in cholesterol was only seen in EC exposed to both LSS and atorvastatin. Depletion of membrane cholesterol results in increased aortic EC membrane stiffness via effects on F-actin (46), and may alter LSS-induced intracellular signaling (47). Moreover, inhibition of cholesterol synthesis by statins may reduce caveolin-1 expression through changes in sterol regulatory element activity. This reduces caveolin-1-mediated inhibition of eNOS activity (48, 49), and hence may play a role in the HO-1 induction observed herein. In contrast, cyclosporin A reduced EC cholesterol and inhibited eNOS phosphorylation (54). Thus, further studies are required to investigate the specific effect and consequences of statin-mediated reduction in EC cholesterol, and to identify mechanisms through which EC are rendered more responsive to statins by LSS.

KLF2 and Nrf2 activity is increased in EC in response to LSS, with distinct downstream effects (29, 38, 50). KLF2 expression was induced by 24 h LSS, although as reported (29) siRNA depletion of KLF2 had no effect on HO-1 induction. In contrast, expression of DN-Nrf2 significantly reduced HO-1 up-regulation by LSS. Moreover, combined inhibition of KLF2 and Nrf2 was required to reverse the synergistic induction of HO-1. These data suggest KLF2 and Nrf2 have distinct, complementary actions and together act to maximally enhance vascular cytoprotection against oxidative stress. This concept is supported by a study demonstrating that KLF2 enhances the antioxidant activity of Nrf2 (50).

FIGURE 5. Synergistic HO-1 induction by LSS requires KLF2 and Nrf2. A and B, HUVEC were left untransfected (UT) or transfected with scrambled siRNA (CT) or KLF2 siRNA, prior to exposure to static conditions or LSS (12 dynes/cm²) for 24 h. After 12 h, atorvastatin (0.6 μM) or vehicle were added to the culture medium. A, HO-1 mRNA was quantified by real-time PCR, a representative cDNA gel of PCR products is shown. B, following exposure to atorvastatin and LSS, HUVEC were treated with H₂O₂ (50 μM) or vehicle for 45 min. Live cells were quantified by MTT assay (percent untreated control, n = 4). C and D, HUVEC were left untransfected (UT) or transfected with an adenovirus expressing β-galactosidase (βgal) or DN-Nrf2 prior to exposure to static conditions or LSS (12 dynes/cm²) for 24 h. C, HO-1 mRNA was quantified by real-time PCR. D, HUVEC were treated with H₂O₂ (50 μM) or vehicle for 45 min and live cells were quantified by MTT assay. E and F, HUVEC were left untransfected or transfected with: E, β-galactosidase or DN-Nrf2 adenovirus; or F, control siRNA (CT), KLF2 siRNA, β-galactosidase control, or DN-Nrf2 adenovirus prior to exposure to static conditions (gray bars) or LSS (black bars) for 24 h with addition of vehicle or atorvastatin (AT) (0.6 μM) after 12 h. HO-1 mRNA was quantified by real-time PCR. Data are expressed as mean ± S.E. from three experiments. *, p < 0.05; **, p < 0.01.
tion, therapeutically manipulate this interaction leading to optimal LSS-induced activation of Nrf2.

LSS-induced PI3K/Akt signaling, although maximal after 1–2 h, may be prolonged and sufficient for Nrf2 activation following 24 h of atheroprotective flow (51, 52). Thus, PI3K antagonist LY290042 inhibited the LSS-induced Nrf2-dependent reduction in intracellular redox levels (29). In our model prolonged LSS increased Akt phosphorylation, a response significantly enhanced by atorvastatin. Furthermore, LSS induction of HO-1 and synergy with atorvastatin was inhibited by DN-Akt. Likewise, inhibition of Akt attenuated increases in KLF2, Nrf2, and eNOS mRNA following exposure to LSS and atorvastatin.

Although HO-1 induction by LSS was significantly reduced by l-NAME, expression remained above that in static cells. However, synergistic induction of HO-1 by LSS and atorvastatin was completely inhibited by l-NAME. In contrast, inclusion of l-NAME failed to inhibit LSS + atorvastatin-mediated induction of KLF2 and Nrf2, suggesting NO is acting downstream of these transcription factors. HMOX1 induction is typically transcriptional and independent of changes in mRNA stability (7). Notwithstanding, exposure to LSS delayed HO-1 mRNA degradation, a response enhanced by atorvastatin. Moreover, NO may act to stabilize HO-1 mRNA (53) and is a likely regulator of the post-transcriptional effect observed. Thus, we propose that exposure of LSS-conditioned vascular endothelium to atorvastatin results in sustained Akt phosphorylation, KLF2 and Nrf2 activation, increased eNOS activity, and both transcriptional and post-transcriptional events leading to optimal HO-1 induction and resistance to oxidative stress.

The cytoprotective effects of statins in vascular endothelium are increasingly recognized, although questions remain regarding their clinical relevance. Our data suggest that LSS enhances endothelial responsiveness to statins and that HO-1 induction represents an important component of the vasculoprotective profile of these drugs. Importantly, we also demonstrate an attenuated response in EC exposed to an atheroprotective waveform, suggesting that protection from statins may be suboptimal at sites most susceptible to atherosclerosis. This observation may have important implications for the efficacy of statins in patients with coronary artery disease, and for their increasing use in prevention of accelerated atherosclerosis in patients with systemic inflammatory diseases. The data emphasize the need
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for novel therapies, such as those targeting KLF2 or Nrf2, to optimize vasculoprotection.

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