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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Running head: Shear stress facilitates statin induction of HO-1

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Summary
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 to 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 siRNA. Moreover, treatment of LSS-exposed EC resulted in a significant fall in intracellular cholesterol. Mechanistically, synergy required Akt phosphorylation, activation of KLF2, 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 signalling contributes significantly to endothelial responsiveness to pharmacological agents. Our findings suggest statin pleiotropy may be sub-optimal at disturbed flow atherosusceptible sites, emphasizing the need for more specific therapeutic agents, such as those targeting KLF2 or Nrf2.

Introduction
The efficacy of 3-hydroxy-3-methylglutaryl coenzyme A reductase antagonists (statins) in reducing low-density lipoprotein (LDL) 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 LDL-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 Fe and carbon monoxide (CO). Biliverdin is subsequently converted to bilirubin by biliverdin reductase, while intracellular Fe 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 (VSMC) proliferation, exert anti-inflammatory, anti-oxidant 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, while a low serum bilirubin constitutes a cardiovascular risk factor (11). Moreover, over-expression of HO-1 inhibited atherogenesis, while Hmox1-/- 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 oxide donors and aspirin being shown to induce HO-1 (reviewed in (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 anti-oxidant 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 anticoagulant, anti-adhesive cell surface. In contrast, endothelium exposed to disturbed blood flow (DF), with low shear reversing or oscillatory flow patterns, such as that located at arterial branch points and curvatures, is atheroprone. Thus endothelial cells exposed to DF exhibit reduced levels of endothelial nitric oxide synthase (eNOS), increased apoptosis, oxidative stress, permeability to LDL 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 atheroprone 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-Aldrich (Poole, UK). Atorvastatin and simvastatin were from Merck Biosciences (Nottingham, UK) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Promega (Southampton, UK). N⁶-nitro-L-arginine methyl ester (L-NAME) from BIOMOL (Plymouth Meeting, PA), leptin from R and D Systems (Abingdon,UK), anti-phospho-Akt (Ser⁴⁷³) antibody from Cell Signaling, (Beverly, MA) and anti-HO-1 antibodies 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 (Rockford, IL). 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 (HAEC; purchased from Promocell, Heidelberg, Germany), were cultured as described (20). The use of human EC was approved by Hammersmith Hospitals Research Ethics Committee (ref no. 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 1Hz (+/- 5 dynes/cm²), for up to 48h using a parallel-plate flow chamber (Cytodyne, La Jolla, CA, USA) 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 24h. After 12h, 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 while 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.

**RNAi design and transfection**

Previously validated siRNA sequences targeting KLF2, HO-1 or scrambled control siRNA were transfected into HUVEC using oligofectamine-based transfection in EBM2 media as described (18).
HO-1:
(Sense: 5’-UGCUGAGUUCAGAGGAACUU-3’)
(Antisense: 5’-GUUCCUCAUGACAGCAAU-3’),
(Sense: 5’-CAUUUCCAGUGCCACCAAGGUU-3’)
(Antisense: 5’-CUUUGGGCACUGCAUGUUU-3’).

KLF2:
(Sense: 5’-GCCCUACCACUGCAACUGGUU-3’)
(Antisense: 5’-CCAGUUGCAGUGGUAGGGCUU-3’),
(Sense: 5’-GUUUGCGCGCUCAGACGAGUU-3’)
(Antisense: 5’-CUCGUCUGAGCGCAAAUU-3’).

EC were cultured for 24h in EBM2 and analysed 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 adenovirus expressing dominant-negative (DN) Akt was a gift from Dr C Wheeler-Jones (Royal Veterinary College, London). The adenovirus expressing DN-Nrf2 which lacks the transactivation domain (Ad-Nrf2-DN) was provided by Dr Jeffrey A. Johnson, University of Wisconsin, Madison (22). Adenoviruses were amplified in HEK-293A cells, purified and titred using BD Adeno-X Purification and Rapid Titer Kits (BD Biosciences). HUVEC were infected by incubation with adenovirus in serum free M199 for 2h at 37°C. The media was then changed to M199/10% FBS and HUVEC incubated overnight prior to experimentation. Infection of HUVEC with a ß-gal control adenovirus demonstrated a transfection efficiency of ≥95%. The optimal multiplicity of infection (MOI) for the DN-Nrf2 and DN-Akt adenoviruses was determined by immunoblotting (not shown). The plasmid pHO-1-Luc was a gift from J Alam (Alton Ochsner Medical Foundation, New Orleans). EC were transfected in triplicate with pGL3-basic or pH0-1-Luc using microporation technology (Digital Bio, Seoul, Korea) as described previously (18).

Analysis of oxidative stress and cellular injury
HUVEC were loaded with 5μM 5-(and-6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA) (Invitrogen, Paisley, UK) for 30 min at 37°C. ECs were incubated in serum-free M199 with H2O2 (5μM) for 30 min or leptin 100 ng/ml for 2h and then washed with ice-cold PBS. Intracellular dihydrodichlorofluorescein (H2DCF) is oxidized to 2,7-dichlorofluorescein and quantified by flow-cytometry. H2O2 (50μM) was used to induce cellular injury and this was quantified using either: (i) trypan blue exclusion; or (ii) a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell numbers were not significantly altered by any of the treatment conditions prior to addition of H2O2.

Quantitative real-time PCR
Quantitative real-time PCR was performed using an iCycler (BioRad, Hercules, CA, USA). β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were used as housekeeping genes, with data calculated in relation to the β-ACTIN gene and verified with GAPDH and HPRT. DNase-1-digested total RNA (1 μg) was reverse transcribed using 1 μM oligo(dT) and Superscript reverse transcriptase (Invitrogen, Paisley, UK). 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 H2O. Primer sequences used were: KLF2 forward 5’-CTTTCGCCAGCCCGTGCCGCG-3’; KLF2 reverse 5’- AAGTCCAGCACGCTGTTGAGG-3’, HO-1 forward 5’-CTTTCACCTTCCCCAAC-3’, HO-1 reverse 5’-TTATATACCCTTCTGCCTGA-3’, Nrf2 forward 5’-AAACCAGTGTACTGTCAAC-3’, Nrf2 reverse 5’-GACCGGAATATCACGGAACA-3’ (23), TM forward: 5’-TTGTGAATTGGAGCTTGG-3’, TM reverse 5’-TCTCACTAAGTTGAGATGGTGTG-3’ (24), and eNOS forward 5’-TGGATTTCTTCTGAGGTGAGCT-3’, eNOS reverse 5’-AGAGGCGTTTTCAGCTGTT-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, sodium dodecylsulfate polyacrylamide gel electrophoresis and transfer to polyvinylidene difluoride membranes.
Immunoblots were probed with primary Abs overnight at 4°C, followed by appropriate secondary reagents for 1h at room temperature and developed with a chemiluminescence substrate (Amersham Pharmacia Biotech, Little Chalfont, UK). To ensure equivalent sample loading, protein content was determined using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) and membranes 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 climatic 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 (5mg/kg) or vehicle alone and sacrificed 24h later by CO2 inhalation, followed by perfusion fixation with 2% formalin, 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 Alexafluor568-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 FITC (Invitrogen, Carlsbad, USA). 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 (http://rsbweb.nih.gov/nih-image/). 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 Students t-test. Data are expressed as the mean of individual experiments ± SEM. 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 24h. 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 Figure 1A, treatment with atorvastatin induced a significant increase in HO-1 expression at a site with low probability (LP) 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 (HP) of developing lesions, were relatively refractory to atorvastatin treatment (Figure 1B). Quantification by image analysis confirmed that HO-1 induction at HP sites was significantly less than that at LP sites (Figure 1C).

LSS and statins exhibit synergy
Statins and unidirectional LSS separately induce 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 2.5µM atorvastatin under static conditions, or exposure of HUVEC to LSS for 24h, significantly increased HO-1 mRNA levels (Figure 2A). Pre-conditioning of EC with LSS (12 dynes/cm²) for 12h prior to addition of atorvastatin and continuation of culture under LSS for a further 12h resulted in an additive increase in HO-1 mRNA (Figure 2A). Reduction of the atorvastatin concentration applied to static-cultured EC to 0.6µM led to loss of HO-1 induction. In contrast, a significant increase of
HO-1 mRNA was still seen when this concentration was applied to EC pre-conditioned by LSS, with 0.6µM atorvastatin inducing a 14-fold increase in HO-1 mRNA (Figure 2B). A dose-response study confirmed synergy between LSS (12 dynes/cm²) and atorvastatin (1.25 and 0.6µM), and this was lost with 0.3µM atorvastatin (Figure 2C). Moreover, further studies using an HMOX1 promoter reporter construct confirmed synergy between atorvastatin (0.6µM) and LSS, as indicated by relative luciferase activity (Figure 2D).

A comparable synergistic response was also seen with simvastatin (Supplementary 1A), while immunoblotting confirmed enhanced induction of HO-1 protein by 0.6µM atorvastatin in LSS-conditioned EC (Figure 2E). The importance of the duration of LSS was revealed by loss of the synergistic response when pre-conditioning was reduced to 6h (Supplementary 1B-C). Subsequent experiments performed with HAEC, to represent an endothelial surface affected by atherosclerosis, demonstrated that they responded similarly to HUVEC with synergy observed between LSS and atorvastatin (0.6µM), resulting in a 13-fold increase in HO-1 mRNA (Supplementary 1D).

**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 oscillatory flow (±5 dynes/cm² at 1Hz) (21). A 10-fold increase in HO-1 expression was seen in EC exposed to 24h LSS, while 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 (Figure 2F).

**LSS and atorvastatin induce an enhanced anti-oxidant effect**

To investigate the functional relevance of HO-1 induction, EC were exposed to free radical-induced injury. Atorvastatin (0.6µM) failed to protect static-cultured HUVEC exposed to H₂O₂ (50µM). However, EC exposed to LSS for 24h were protected by 50%, and this was significantly enhanced by atorvastatin (0.6µM) (Figure 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, while exposure of EC to H₂O₂ (5µM) induced a maximal response (Figure 3B). Pre-treatment of static-cultured EC with atorvastatin (0.6µM) 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 (Figure 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 (Figure 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% (Supplementary Figure 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% (Figure 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 oxidant injury in this setting (Figure 3E).

**LSS and atorvastatin in combination reduce intracellular cholesterol**

As shown in Figure 4A, exposure of HUVEC to LSS, or treatment of static-cultured EC with atorvastatin (0.6µM), 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 of up to 60%. These data, combined with that in Figure 2, suggests 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 (2µg/ml) and analysis of HO-1 by qRT-PCR. Treatment with actinomycin D resulted in less than 5% cell death as estimated by trypan blue exclusion studies. The rapid decay of 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 (Figure 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 upregulation of HO-1. KLF2-targeted siRNA reduced expression in HUVEC by 80% (Supplementary Figure 2B) (18). However, interference with the KLF2 transcript did not alter HO-1 induction by LSS (Figure 5A) (29). Likewise, KLF2 depletion did not reduce LSS-mediated cytoprotection against oxidant-induced injury, when compared with control siRNA (Figure 5B).

The Nrf2-Keap1 system regulates cytoprotective gene expression via the antioxidant responsive element (ARE), with several ARE-regulated genes induced by LSS (29). A recombinant adenovirus expressing a DN-Nrf2 construct (MOI 100) demonstrated the importance of Nrf2 in HO-1 induction by LSS (Figure 5C). The functional consequences of HO-1 suppression were evident when HUVEC expressing DN-Nrf2 were exposed to LSS followed by H$_2$O$_2$. DN-Nrf2 significantly reduced LSS-mediated protection against oxidant-induced injury (Figure 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 (0.6µM) in the presence of siRNA or adenoviral vectors had no effect on HO-1 expression (Figure 5E-F). KLF2 siRNA did not affect LSS induction of HO-1, while reducing HO-1 expression in EC exposed to LSS and atorvastatin by 40% (Supplementary Figure 2C). Inhibition of Nrf2 alone had a more marked effect, reducing HO-1 expression in LSS and statin-treated EC by 60% (Figure 5E). Although co-transfection of EC with KLF2 siRNA and DN-Nrf2 significantly attenuated LSS-induced HO-1 when compared to EC transfected with control siRNA and Adv βgal, 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 upregulation, reducing this by 80% from 12.5 to 2.5-fold, supporting the view that both KLF2 and Nrf2 activation is required (Figure 5F).

Akt activation regulates LSS and atorvastatin synergy

We next explored PI-3K/Akt activation, which is known to occur in EC exposed to LSS or statins (30). Atorvastatin (0.6µM) failed to increase Akt phosphorylation (Ser473) in static-cultured cells (Figure 6A). However, prolonged LSS increased Akt phosphorylation and this was further enhanced by atorvastatin. The importance of Akt was confirmed with Adv-DN-Akt (MOI 100) which inhibited LSS-induced HO-1 expression and the synergy between LSS and atorvastatin, a response not seen with the βgal control (Figure 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 upregulation in LSS-conditioned EC exposed to atorvastatin (Figure 6C). Likewise, the induction of Nrf2 mRNA by LSS, and the enhanced response in the presence of atorvastatin, was reversed by DN-Akt (Figure 6D), consistent with a recent study which reported that flow-induced translocation of Nrf2 requires PI-3K/Akt activation (29). Thus, EC pre-conditioned with LSS and treated with atorvastatin exhibit 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 PI-3K 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 (Figure 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 (Figure 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 Figure 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 quantified using a TransAM™ assay. LSS increased Nrf2 activation by 4-fold, while atorvastatin alone (0.6μM) had no effect, nor did it increase activation in EC pre-conditioned with LSS. Finally, pre-treatment with L-NAME did not influence the activation of Nrf2 (Figure 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 upregulation 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 pre-conditioning 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 signalling 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 anti-oxidant (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 anti-thrombotic effects (28,37,38). Nrf2 activity is central to LSS-mediated regulation of anti-oxidant genes including HMOX1, THIOREDOXIN REDUCTASE1 and GLUTATHIONE REDUCTASE (29,39).

Pre-conditioning HUVEC with unidirectional LSS increased responsiveness to statins so that atorvastatin (0.6µM), which failed to upregulate HO-1 in static-cultured EC, induced a maximal increase in the HMOX1 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 atorvastatin 0.3µM, 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 the 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 anti-oxidant genes induced by LSS including NAD(P)H-QUININE OXIDOREDUCTASE1, NAD(P)H OXIDASE, SUPEROXIDE DISMUTASE, THIOREDOXIN REDUCTASE1 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 anti-oxidant genes above (29). The mechanisms through which HO-1 products exert anti-oxidant 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, while 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 preconditioned 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 (Lungu, 2004 #5084). 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 24h 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 upregulation 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). We propose that statins, through their ability to increase KLF2 expression, therapeutically manipulate this interaction leading to optimal LSS-induced activation of Nrf2.

LSS-induced PI-3K/Akt signalling, although maximal after 1-2h, may be prolonged and sufficient for Nrf2 activation following 24h of atheroproteective flow (51,52). Thus, PI-3K antagonist LY290042 inhibited the LSS+atorvastatin-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 atheroprone waveform, suggesting that protection from statins may be sub-optimal 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 for novel therapies, such as those targeting KLF2 or Nrf2, to optimize vasculoprotection.
References

Footnotes

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2The abbreviations used are: LDL, low-density lipoprotein; HO-1, heme oxygenase-1; CO, carbon monoxide; VSMC, vascular smooth muscle cells; EC, endothelial cells; LSS, laminar shear stress; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; KLF2, Kruppel-like Factor2; Nrf2, NF-E2-related factor-2; H2O2, hydrogen peroxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI-3K, phosphoinositide-3 kinase; L-NAME, Nω-nitro-L-arginine methyl ester; HUVEC, human umbilical vein endothelial cells; HAEC, human aortic endothelial cells; FBS, fetal bovine serum; OF, oscillatory flow; DN, dominant-negative; H2DCF, dihydrodichlorofluorescein; SEM, standard error of the mean; MOI, multiplicity of infection; NADP, nicotinamide adenine dinucleotide phoshpat; STAT3, signal transducer and activator of transcription 3.

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Figure Legends

Figure 1: Atorvastatin induces endothelial HO-1 expression in murine aortic EC
HO-1 expression in murine aortic endothelium of C57BL/6 mice assessed en face using anti-HO-1 (red). EC were identified by FITC-conjugated anti-CD31 (green) and cell nuclei with Draq5 (purple). An isotype-matched control antibody did not bind (not shown). A, B. Representative images of HO-1 staining from (A) the greater curvature (low probability (LP) area), and (B) the lesser curvature (high probability (HP) area). C. HO-1 expression was quantified (mean ± SD) by image analysis of fluorescence intensity in multiple cells at 3 distinct sites and expressed as EC fluorescence above a threshold intensity defined by background fluorescence. *p<0.05.

Figure 2: LSS and statins synergistically enhance HO-1 expression. HUVEC were exposed to static conditions (grey bars) or unidirectional LSS (12 dynes/cm²) (black bars) for 24h. After 12h, statin or vehicle was added to the culture medium. A. atorvastatin (AT) 2.5µM, B. AT 0.6µM, Dotted lines represent predicted HO-1 mRNA level achieved by an additive response between LSS and atorvastatin. C. Dose response for AT, # = synergistic response, § = additive response, with HO-1 mRNA quantified by real-time PCR. Dotted line represents HO-1 mRNA induced by LSS alone. D. HUVEC were transfected with pHO-1 luciferase reporter construct or pGL3-basic, prior to exposure to static conditions or unidirectional LSS and addition of AT 0.6µM or vehicle as above and analysis of luciferase activity at 24h. E. HUVEC were exposed to static culture or LSS for 24h with atorvastatin added after 12h and HO-1 analyzed by immunoblotting. F. HUVEC were exposed to static conditions (grey bars), LSS (12 dynes/cm²) (black bars) or oscillatory flow (OF) (1Hz, +/-5 dynes/cm²) (hatched bars) for 24h. After 12h, statin or vehicle was added to the culture medium. Data are expressed as mean ± SEM from 3 experiments, *p<0.05, **p<0.01.

Figure 3: LSS and statins synergistically enhance resistance to oxidative stress. HUVEC were exposed to static conditions (grey bars) or LSS (12 dynes/cm²) (black bars) for 24h. After 12h, statin or vehicle was added to the culture medium. A. HUVEC were treated with H₂O₂ (50 µM) or vehicle for 45 min. Live cells were quantified by trypan blue exclusion, expressed as a percentage of untreated control (n=3). B and C. Cells were treated as in A, followed by loading with CM-H₂DCFDA (5µM) and exposure to B. H₂O₂ (5µM) for 30 min or C. leptin (100 ng/ml) for 120 mins. Oxidative stress was quantified by flow-cytometric analysis (n=3). D. HUVEC were left untreated or transfected with scrambled siRNA (CT) or HO-1 siRNA for 24h, prior to exposure to LSS and atorvastatin (0.6µM) for 24h as in A. EC were treated with H₂O₂ (50 µM) as above and live cells quantified by MTT assay (percent untreated control, n=4). E. HUVEC were exposed to static conditions (grey bars) or oscillatory flow (OF) (1Hz, +/-5 dynes/cm²) (hatched bars) for 24h. After 12h, atorvastatin (0.6µM) (AT) or vehicle were added to the culture medium. After exposure to H₂O₂ live cells were quantified by MTT assay. Data expressed as mean ± SEM from 3 experiments. *p<0.05, **p<0.01.

Figure 4: Statin and LSS reduce cholesterol and enhance HO-1 mRNA stability. HUVEC were exposed to static conditions or LSS (12 dynes/cm²) for 24h. After 12h, atorvastatin (0.6µM) or vehicle was added to the culture medium. A. EC were homogenized and cholesterol content was analyzed by mass spectrometry and expressed as µg/10⁶ cells. B. HUVEC monolayers were divided into sections using a water-resistant pen and actinomycin D (2 µg/ml) or vehicle added. EC were harvested after 0-6h for RNA extraction. HO-1 mRNA was quantified by qRT-PCR, plotted as a percentage of mRNA expression prior to the addition of actinomycin D. Data are presented as mean ± SEM (n=3), *p<0.05.

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 24h. After 12h, atorvastatin (0.6µM) or vehicle was 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 24h. 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 quantified by MTT assay. E and F. HUVEC were
left untransfected (UT) or transfected with E. βgal or DN-Nrf2 adenovirus or F. control siRNA (CT), KLF2 siRNA, βgal control or DN-Nrf2 adenovirus prior to exposure to static conditions (grey bars) or LSS (black bars) for 24h with addition of vehicle or atorvastatin (0.6μM) after 12h. HO-1 mRNA was quantified by real-time PCR. Data are expressed as mean ± SEM from 3 experiments *p<0.05, **p<0.01.

**Figure 6: Synergistic induction of HO-1 is dependent upon Akt.** HUVEC were exposed to static conditions (grey bars) or unidirectional LSS (12 dynes/cm²) (black bars) for 24h. After 12h, atorvastatin (0.6μM) (AT) or vehicle control was added. A. EC were immunoblotted with Abs against phosphorylated-Akt (Ser⁴⁷³) and α-tubulin. The histogram shows phospho-Akt expression quantified by densitometry relative to the α-tubulin bands. B-E. HUVEC were left untransfected (UT) or transfected with a βgal control adenovirus (βgal) or DN-Akt adenovirus prior to exposure to static conditions or LSS (12 dynes/cm²) for 24h as above. HO-1 (B), KLF2 (C), Nrf2 (D) and eNOS (E) mRNA levels were quantified by real-time PCR. Data are expressed as mean ± SEM from 3 experiments *p<0.05, **p<0.01.

**Figure 7: Synergistic induction of HO-1 is dependent upon activity of eNOS.** HUVEC were pretreated with L-NAME (L-N) (1mM) or vehicle prior to exposure to static conditions or unidirectional LSS (12 dynes/cm²) for 24h with atorvastatin (0.6μM) (AT) or vehicle control added after 12h. A. HO-1 and B. thrombomodulin (TM) mRNA levels were analyzed by real-time PCR, or C. Nrf2 activation was assessed in EC nuclear extracts by analysis of Nrf2 binding to the antioxidant responsive element, quantified using a TransAM™ assay. Data are expressed as mean ± SEM from 2-3 experiments *p<0.05, **p<0.01.