GRP78 Upregulation by Atheroprone Shear Stress Via p38-, α2β1-Dependent Mechanism in Endothelial Cells

Ryan E. Feaver, Nicole E. Hastings, Andrew Pryor, Brett R. Blackman

Objective—The initiation of atherosclerosis is in part dependent on the hemodynamic shear stress environment promoting a proinflammatory phenotype of the endothelium. Previous studies demonstrated increased expression of ER stress protein and unfolded protein response (UPR) regulator, GRP78, within all vascular cells in atherosclerotic lesions and its regulation in the endothelium by several atherosclerotic stressors; however, regulation of GRP78 by shear stress directly has not been established.

Method and Results—Using an in vitro model to simulate human arterial shear stress waveforms, atheroprone or atheroprotective flow was applied to human endothelial cells. GRP78 was found to be significantly upregulated (3-fold) in a sustained manner under atheroprone, but not atheroprotective flow up to 24 hours. This response was dependent on both sustained activation of p38, as well integrin α2β1. Increased GRP78 correlated with the activation of the ER stress sensing element (ERSE1) promoter by atheroprone flow as a marker of the UPR. Shear stress regulated GRP78 through increased protein stability when compared to other flow regulated proteins, such as connexin-43 and vascular cell adhesion molecule (VCAM)-1. Increased endothelial expression of GRP78 was also observed in atheroprone versus atheroprotective regions of C57BL6 mice.

Conclusions—This study supports a role of the hemodynamic environment in preferentially inducing GRP78 and the UPR in atheroprone regions, before lesion development, and suggests a potential atheroprotective (ie, prosurvival), compensatory effect in response to ER stress within atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2008;28:1534-1541)

Key Words: endothelial ■ GRP78 ■ shear stress ■ atherosclerosis ■ unfolded protein response

Atherosclerosis is a focal inflammatory disease that develops preferentially in areas of disturbed flow, where variations in shear stress have been shown to alter the phenotypes of endothelial cells toward either an atheroprone or atheroprotective state in vitro and in vivo.1,2 Therefore, hemodynamic-induced shear stress provides a major mechanical signal, which causes the overlying endothelium to become at risk for the promotion of atherosclerosis.

Of many proteins of interest, the chaperone protein, glucose regulated protein 78 (GRP78), a common marker for endoplasmic reticulum (ER) stress, is preferentially expressed in advanced atherosclerotic lesions3 and on the fibrous cap surface in ApoE-KO mice.4 Further, cell-surface associated GRP78 has been speculated to serve a protective role in atheroprone environments by inhibiting tissue factor through direct binding to the endothelium overlying the plaque.5

Hyperhomocysteinemia is associated with increased risk of cardiovascular disease possibly by limiting the antioxidant activity and causing ER stress, leading to the activation of GRP78.4 ER stress is further linked to oxidative stress through peroxynitrite-induced GRP78 expression.3 ER stress is present at every stage of atherosclerosis, even preceding free cholesterol accumulation.6 This suggests cardiovascular risk factors induce greater GRP78 expression, and further, there exist localized conditions that cause this stress even before lesion development (eg, hemodynamic shear stress).6

GRP78 is involved in a number of processes but has been well studied as the “master regulator” of the unfolded protein response (UPR). GRP78 binds the 3 ER stress sensors PERK, IRE1, and ATF6 and keeps them in an inactive form.7 On accumulation of unfolded proteins, GRP78 releases the stress sensing elements, allowing ATF6 to translocate to the nucleus and bind to the endoplasmic reticulum stress element (ERSE) and increase the transcription of genes to alleviate the ER stress. The early response to ER stress is the attenuation of protein synthesis by the PERK pathway via ATF4. ATF4 along with XBP1 (downstream of IRE1) have recently been found to regulate many important inflammatory genes, including interleukin (IL)-8, which is regulated by atherogenic levels of shear stress.2,8 Currently, GRP78 is known to be induced by many hallmark atherosclerotic stressors, such as excess cholesterol, oxidized phospholipids, oxidative stress, peroxynitrite, and homocysteine.9–12 GRP78−/− mice are embryonic lethal and show increased apoptosis, and high
levels of unfolded proteins may lead to disassociation of GRP78 from UPR mediators causing JNK and NFκB activation or caspase-mediated apoptosis. Currently little is known about the role of the UPR in ECs or in the context of flow or atherosclerosis.

Here we tested the hypothesis that hemodynamic shear stress in regions of atherosclerosis, independent of other risk factors, regulates GRP78 expression in vivo and in vitro. It is hypothesized that GRP78 upregulation in the endothelium may provide a protective compensatory effect in response to ER stress within early or developing atherosclerotic lesions.

**Methods**

**Animals**

Tissue sections from wild-type mice (C57BL6, n=4; Jackson Laboratory) between 8 to 10 weeks of age and 8- or 20-week old ApoE<sup>−/−</sup> mice (n=2; Jackson Laboratory, Bar Harbor, Maine) were obtained for immunofluorescence staining for GRP78.

**In Vitro Hemodynamic Flow Model and Analysis**

Passage 2 human umbilical vein endothelial cells were used to investigate the in vitro role of shear stress patterns on the induction of the ER stress via a cone-and-plate flow device. On completion of the flow experiments, the cells were immediately collected for Western blotting or real-time reverse transcriptase polymerase chain reaction (PCR).

**ERSE Luciferase Assay**

Passage 7 to 9 bovine aortic endothelial cells were transfected with GRP78-ERSE1-Luc, GRP78–M–ERSE1-Luc, CHOP-ERSE1-Luc, and CHOP-M-ERSE1-luc (provided by Dr Glembotski, San Diego State University, Calif).

For complete Methods, please see the supplemental materials (available online at http://atvb.ahajournals.org).

**Results**

**Endothelium GRP78 Is Differentially Expressed in Atheroprone and Atheroprotective Regions In Vivo**

GRP78 has been found to be highly expressed in macrophages, smooth muscle, and endothelial cells of atherosclerotic lesions. We found a similar intense GRP78 staining in a 20-week lesion (G) and 8-week cross-section (H) along the descending aorta (intersecting at an intercostal branch) of an ApoE<sup>−/−</sup> mouse.

**Figure 1.** GRP78 is differentially expressed in atheroprone areas and within atherosclerotic lesions of mice aorta. Histological sections of the aorta were stained for GRP78 (red) and matrix (green/autoflourescence) in C57BL/6 (A-F) or ApoE<sup>−/−</sup> (G-H) mice. Representative images of the aortic arch of C57BL/6 mice show the inner arch (atheroprone) and the outer arch (atheroprotective) (A–D). White arrows (100× images) indicate individual ECs. Staining was compared to protected regions of the thoracic aorta (E–F). GRP78 expressed in a 20-week lesion (G) and 8-week cross-section (H) along the descending aorta (intersecting at an intercostal branch) of an ApoE<sup>−/−</sup> mouse.
vasculature, we hypothesized that increased expression of GRP78 might be attributable in part to the local differences in the hemodynamic shear stress environment.

Endothelial GRP78 Regulated by Atheroprone Hemodynamics In Vitro
To test our hypothesis and corroborate the observations found in vivo, the role of hemodynamic shear stress on the regulation on GRP78 in ECs was investigated in vitro using atheroprone and atheroprotective flow patterns derived directly from human carotid circulation (Figure 2A). To assess a more sustained phenotype, ECs were exposed to atheroprone or atheroprotective flow for 24 hours (Figure 2B). GRP78 was increased under atheroprone compared to atheroprotective flow and the time-matched static control. To assess the temporal regulation of GRP78 by arterial hemodynamics, ECs were exposed to both flow paradigms from 4 to 24 hours and subsequently assayed for GRP78 protein expression. Although GRP78 was elevated under both flow paradigms 4 hours after the onset of flow (Prone: \( P=0.03 \); Protective: \( P=0.17 \)), the atheroprone flow resulted in a significant and sustained increase in GRP78 after 6 hours and up to 24 hours when compared to atheroprotective flow (Figure 2C). In contrast, GRP78 expression under atheroprotective flow from 6 to 24 hours was no different than the time-matched static controls (\( P<0.05 \); Figure 2C). The heightened expression levels of GRP78 after atheroprone flow at the onset and longer time points suggests shear stress is initiating and sustaining the activation of the UPR pathway, respectively.

Atheroprone Flow Induces the UPR Through ATF6
GRP78 is known to directly bind to p90 ATF6 (90kDa-form) regulating its activity, and its transcription depends on the induction of ATF6 in a positive feedback manner. \(^{20}\) p38 was found to phosphorylate ATF6 allowing it to translocate to the nucleus (as a 50-kDa form) where it can upregulate genes controlled by the ER stress sensing element (ERSE), including chaperone proteins (GRP78, GRP94) and UPR proteins (ATF6, PERK, IRE1). \(^{21,22}\) Figure 2D shows the early time course of p90 ATF6 expression from 10 minutes to 24 hours after atheroprone flow at the onset and longer time points suggests shear stress is initiating and sustaining the activation of the UPR pathway, respectively.
similar to the exposure to tunicamycin, a potent ER-stress inducer, suggesting activation of the UPR by flow. Longer exposure to atheroprone flow increased ATF6 expression at 4 and 24 hours.

To further assess the activation of the UPR under atheroprone flow, the ERSE1 promoter derived from the GRP78 gene and CHOP gene or a mutated promoter from each gene (ERSE-M) driving a luciferase reporter were transfected into ECs before flow. The ERSE-M reporter does not allow ATF6 to bind. Under atheroprone flow there was a significant increase in both the GRP78 and CHOP ERSE1 promoter activity normalized to the ERSE-M control compared to the initial conditions in static culture or atheroprotective shear stress (Figure 2E, \( P < 0.05 \), 1-tailed t test). This induction of the ERSE promoter by atheroprone flow via ATF6 along with increased expression of ER stress proteins GRP78 and ATF6 indicates the activation of the UPR.

**GRP78 Is Not Regulated by Proinflammatory Cytokines**

Because higher levels of GRP78 were found in atherosclerosis, we wanted to determine whether its upregulation could result from cytokine stimulation in the plaque (ie, released by activated cells in the plaque) rather than solely being regulated by the hemodynamic environment. ECs were exposed to tumor necrosis factor (TNF)-\( \alpha \) or IL-1\( \beta \) from 1 to 24 hours and compared to untreated time-matched controls. Neither IL-1 nor TNF-\( \alpha \) elicited an increase in GRP78 protein expression up to 24 hours of exposure (\( P > 0.3 \); Figure 2F), thus indicating a prominent role for hemodynamic stresses.

**Shear Stress Does Not Affect Cell-Surface GRP78 Function**

GRP78 provides dual functions either in the ER or on the cell surface where it has multiple binding partners, including tissue factor. Bhattacharjee et al found GRP78 to be antithrombogenic by binding cell-surface tissue factor to reduce clotting times. To assess whether the increase in GRP78 expression under atheroprone flow was contributing to an antithrombogenic function, a single-stage clotting assay was performed on ECs pretreated with 24 hours of atheroprone flow. Samples were mixed with anti-GRP78 (Santa Cruz, N terminus), and a single-stage clotting assay was performed and compared to untreated control (for complete methods please see supplemental materials). No significant difference was found between the 2 samples (Prone: 86.19±14.00s, Prone+anti-GRP78: 78.18±17.68s, \( n = 3, P = 0.5 \)). Therefore, atheroprone-induced GRP78 expression appears to participate in sensing unfolded proteins in the ER leading to the release and activation of the UPR-mediators instead of cell-surface reactivity.

**Shear Stress Regulates EC mRNA and Protein Half-Life of GRP78**

To determine whether the increase in protein expression was the result of shear stress differentially regulating GRP78 gene expression, changes in mRNA were assessed after exposure to atheroprone or atheroprotective flow for 1 to 16 hours (Figure 3A). Both flow paradigms stimulated similar transient increases in GRP78 mRNA up to 4 hours of flow that returned to basal levels by 16 hours. This regulation was not consistent with the atheroprone flow-induced protein expression.

ER stress can lead to increased mRNA and protein stability to UPR effectors, including GRP78. Because the differential expression of GRP78 was not found to be regulated solely by mRNA transcription, the stability of GRP78 was assessed by treating the cells with cycloheximide (CHX) to block synthesis of new protein. ECs were pretreated with 4 hours of atheroprone or atheroprotective flow followed by exposure to DMSO control or cycloheximide (CHX 10 \( \mu \)g/mL) for 2 additional hours under flow. Representative Western blots for GRP78, Cx43, and VCAM1 after the combined 6 hours of flow and normalized to actin from three independent experiments; mean±SE (\( * P < 0.05, ** P < 0.001 \)).

**Figure 3.** Shear stress patterns differentially regulate protein stability. A, GRP78 mRNA levels were measured by real-time RT-PCR after exposure to atheroprone or atheroprotective flow from 1 to 16 hours (\( n = 3 \) to 6). B, ECs were preconditioned with 4 hours of atheroprone or atheroprotective flow followed by exposure to DMSO control or cycloheximide (CHX; 10 \( \mu \)g/mL) for 2 additional hours under flow. Representative Western blots for GRP78, Cx43, and VCAM1 after the combined 6 hours of flow and normalized to actin from three independent experiments; mean±SE (\( * P < 0.05, ** P < 0.001 \)).
both flow conditions, however degradation of VCAM-1 was only observed under atheroprotective flow. After atheroprone flow, GRP78 was found to have a higher protein half-life (ie, stability) compared to flow-regulated proteins that degrade (Cx43, VCAM), as its expression did not change after 2 hours of CHX treatment. Interestingly, CHX prevented the decrease in GRP78 after 6 hours of atheroprotective flow (see Figure 2C), which is observed as increase in GRP78 expression (Figure 3B) relative to the vehicle control. This suggests that the restoration of GRP78 to basal levels under atheroprotective flow is dependent on the new synthesis of an unidentified protein for UPR control.

Both p38 and α2β1 Were Necessary for GRP78 Regulation Under Atheroprotective Flow

p38 is involved in major pathways involved with atherosclerosis, and a previous study had identified p38 as a proximal regulator of GRP78 in human HEp3 cells. We therefore investigated p38 activation as a possible mechanism by which GRP78 was regulated by atheroprotective flow. Figure 4A and 4B shows that flow immediately increased p38 activity significantly after 10 minutes under both flow paradigms, compared to the static condition, in a transient manner up to 60 minutes (P<0.05; Figure 4A), though the relative activity was similar between flow patterns after 1 hour (P=0.17). The activity of p38 remained similar between both flow types after the onset of flow for up to 24 hours (Figure 4B), although significantly increased compared to static time-zero (P<0.03).

Blocking p38 activity with 1 μmol/L SB202190, a potent p38 inhibitor, blocked the atheroprone-induced increase in GRP78 after 24 hours when compared to the nonblocking control, 12F1 as seen in representative Western blots (A) or quantified using densitometry (B). C, Reduction in p38 phosphorylation after 24 hours of atheroprotective flow in the presence of blocking antibody R2-8C8, but not the control 12F1 (P<0.02; NS, not significant n=6 to 9). D, After treatment with R2-8C8 or 12F1, GRP78 expression was measured when challenged with ER stressors DTT, thapsigargin (Tg), or tunicamycin (Tm) for 6 or 24 hours.

Integrin ligation is an important component of EC responsiveness to shear stress and may be a means of adaptive mechanotransduction. Recent work has linked the integrin α2β1 to p38 activation. Here we tested whether α2β1 ligation is upstream of p38 and GRP78 regulation in response to atheroprone flow. Pretreatment for 30 minutes and
throughout the flow experiment with the α2β1 blocking antibody R2-8C8 (1:800; gift from Dr Mark Ginsberg, UCSD) inhibited atheroprone flow-induced increase of GRP78 at 24 hours compared to control nonblocking α2β1 antibody 12F1 (3 μg/mL; gift from Dr Virgil Woods, UCSD) and the time-matched static control (P<0.05; Figure 5A and 5B). Blocking α2β1 was also found to significantly lower (P<0.02) p38 activity by more than 20% after 24 hours of atheroprone flow (Figure 5C). Again, to see whether α2β1 is part of the shear stress-sensing pathway or necessary for ER stress, R2-8C8 and 12F1 were used in conjunction with 3 ER stress inducers (DTT, Tg, Tm) for various amounts of time (Figure 5D). Blocking α2β1 did not reduce ER stress at any of the tested points. Therefore, ligation of α2β1 and activation of p38 are important shear stress signaling mechanisms in the long-term, sustained upregulation of GRP78.

**Discussion**

We present evidence for the differential control of hemodynamics in influencing EC expression of GRP78, an ER chaperone responsible for alleviating cell toxicity and a key regulator of the UPR. To our knowledge, this is the first instance of GRP78 protein expression to be mechanically responsive, and further is differentially regulated in atheroprone versus atheroprotective regions in vivo and regulated by shear stress in a waveform specific manner in vitro. The mechanisms by which atheroprone flow induces increases in GRP78 were dependent on p38 activity and α2β1. The differential regulation of GRP78 protein and the ERSE activation by the onset of atheroprone flow signify the induction of the UPR and its sustained activity. Inflammatory cytokines found in atheroprone environments had no effect on GRP78 expression in ECs, and its regulation by known chemical agonists was independent of the p38 and α2β1 pathways. This result further accentuates the prominence of shear stress patterns in the atheroprone environment in regulating the basal endothelial phenotype.

GRP78 is shown here and elsewhere in early and late stages of atherosclerotic lesions in ApoE−/− mice. We now show that GRP78 is expressed at greater levels at intercostal branch points and the inner curvature of the aortic arch, arterial regions susceptible to the development atherosclerosis and correlate with disturbed flow patterns. Interestingly, the smooth muscle cells (SMCs) in these areas also showed greater GRP78 staining. Although the immediate consequence of this observation is not known, it is interesting to speculate that hemodynamic regulation of ECs could mediate SMC behavior in atheroprone regions through cell–cell communication. Atheroprone regions in vivo and hemodynamic relevant shear stresses in vitro have been shown to prime the endothelium and smooth muscle cells toward an activated, proinflammatory state. Atheroprone flow in vitro did not affect the clotting time, indicating that the induction of GRP78 by atheroprone flow likely combats ER stress as part of the UPR. Therefore, it is plausible that hemodynamic flow might be the earliest ER stressor in atheroprone environments.

Many survival pathways resulting from atheroprone stimuli converge on MAPKs, including the JNK and p38 cascades. p38 is activated by numerous atherosclerosis stressors such as oscillatory flow, reactive oxygen species, cholesterol, and TNF-α. Despite common convergence on MAPK signaling, cells are capable of controlling specific downstream effects by regulating subcellular localization, protein scaffolds, and extracellular matrix interactions.

Therefore, similar MAPK signaling activity diverge in downstream function as seen in the temporal regulation of the UPR, where inhibitors of p38 block GRP78 induction at 24 hours. This suggests differential regulators downstream of p38 or a protein serving as a CHX-sensitive negative regulator of GRP78, as we observed under atheroprotective flow.

Signals from biomechanical forces and ECM interactions converge and transduce via integrin ligation. α2β1 was found to regulate p38 in agreement with previous studies using purified matrices where p38 localized to focal adhesions, thus possibly providing specificity through subcellular localization. α2β1 can bind multiple ECM components which may be present in the cell derived matrix in cultured ECs or in vivo, which differs from the purified matrices used by Orr et al, possibly accounting for differences in activity levels. Integrins generally provide antiapoptotic/prosurvival signals from the ECM, where lack of integrin signaling, specifically the β1-tail, caused ECs to undergo apoptosis. These effects are often mediated through chaperone heat-shock family proteins to which GRP78 belongs. Collectively, this supports the protective role of α2β1-ECM in using the UPR and GRP78 to counteract apoptosis.

The atheroprone flow-induced increase in GRP78 was linked in part to differential protein stability between the 2 flow paradigms. A previous study demonstrated that the adaptive response to long-term exposure to tunicamycin or thapsigargin increased protein and mRNA half-life of GRP78 and other UPR adaptive proteins, where apoptotic proteins like CHOP were less stable. In ECs, tunicamycin-induced ER stress was found to have no effect on mRNA stability, and in our study GRP78 mRNA levels were transient and showed no difference between the 2 flow conditions. Therefore, we hypothesized the differences may be attributable to GRP78 protein stability. Studies with CHX treatment showed that GRP78 had greater stability under flow when compared to other flow-regulated proteins, Cx43 and VCAM1, also known to be increased by atheroprone flow. CHX had no effect on GRP78 expression, implying that sustained expression at time points greater than 6 hours does not rely on transcription or the synthesis of new protein for its regulation. In contrast, CHX resulted in a ~30% increase in GRP78 expression after atheroprotective flow. One possibility is that atheroprotective flow upregulates an unidentified CHX-sensitive protein that reduces levels of GRP78 through active degradation. The proposed model for hemodynamic regulation of GRP78 is summarized in supplemental Figure 1. Mechanistically, these results suggest that atheroprone flow promotes protein stability (or prevents protein degradation) for important survival pathways including GRP78 to alleviate ER stress and apoptotic signaling.
GRP78 is considered the “master regulator” of the UPR because of its control of the ER stress sensing elements, ATF6, PERK, and IRE1. ATF6 is an important transcription factor that binds to the ERSE1 promoter to increase transcription of important UPR proteins including GRP78 and ATF6 itself. The initial response of ATF6 to the onset of atheroprotease flow was similar to thapsigargin-induced ER stress—a significant decrease in expression. The authors concluded this to be an antiapoptotic/survival mechanism that reduces the expression of CHOP while the cell attempts to alleviate stress. Prolonged exposure of thapsigargin induced an increase in ATF6 expression akin to results reported here under atheroprotease flow. Further, this increase in ATF6 protein corresponds with the activation of the ERSE1 promoter via ATF6 signifying the onset of the UPR under atheroprotease flow.

Atheroprotease flow induces ER stress, which may further aggravate the progression of atherosclerosis in these environments, whereas atheroprotective forces resolve the need for the UPR after 4-hours of flow. GRP78 provides numerous protective effects to minimize stress through UPR signaling, as well as antiapoptotic and antiinflammatory signaling. Therefore, there is a link between hemodynamics, inflammation, apoptosis, and the UPR. There are reports of the ability of atheroprotective flow to reduce inflammatory effects of cytokines; however, this study suggests there may also be compensatory atheroprotective effects when challenged with atheroprotease flow.

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Disclosures

None.

References


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Methods:

Animals

Wild type mice (C57BL6, n=4; Jackson Laboratory) between 8-10 weeks of age and 8 or 20 week old ApoE-/- mice (Jackson Laboratory, n=2) were used in accordance with a protocol approved by the animal care and use committee at our institution to harvest vascular tissue from mice. Immediately following euthanizing the mice, the vasculature was perfusion fixed with 4% paraformaldehyde, and the entire aortic tree was processed for paraffin embedding. Tissue sections were obtained for immunofluorescence staining. Anti-mouse GRP78 antibody (Becton Dickinson Biosciences, 1:200) was applied followed by Alexa Fluor 546 anti-mouse secondary (Molecular Probes, 1:300). Images were obtained using Nikon Eclipse C1 confocal microscope with 20x, 60x, and 100x lenses.

Cell Culture

Passage two human umbilical vein endothelial cells (HUVEC), isolated as previously described\(^1\), were plated in M199 growth media (Biowhitaker) supplemented with 10% fetal bovine serum (FBS; Gibco), 5ug/ml endothelial cell growth supplement (ECGS; Biomedical Technologies), 10ug/ml heparin (Sigma), 2mM L-glutamine (Gibco), and 100U penicillin/streptomycin (Invitrogen) at 80,000 cells/cm\(^2\) on surface treated plastic coated with 1% gelatin and allowed to grow to confluence over 18-24 hours.

Prior to flow, cells were washed in DPBS and the media was exchanged with reduced serum media (M199 supplemented with 2% FBS, 5ug/ml ECGS, 10ug/ml
heparin, 2mM L-glutamine, 100U penicillin/streptomycin, and 2% dextran by weight to increase the viscosity).

In Vitro Hemodynamic Flow Model

To investigate the in vitro role of shear stress patterns on the induction of the ER stress in EC, a cone and plate flow device imposed shear stress waveforms derived from the human internal carotid sinus, atheroprone region or the common carotid artery, atheroprotective region as defined previously by our lab\textsuperscript{1,2}. The flow device provides exchange of fresh media with all time points greater than 4 hours, as well as a continuously controlled environment to maintain humidity, gas concentration, and temperature at 37°C\textsuperscript{1}.

Western Blotting

Upon completion of the flow experiments, the cells were immediately collected directly into SDS-MAPK sample buffer (Cell Signaling). Western blotting was used to measure relative protein expression, and the total protein expression was normalized to α-actin to control for loading. Antibodies were used to detect expression for GRP78 (BD, 1:2000), α-actin (Sigma-Aldrich, 1:1000), phospho- and total p38 (Cell Signaling, 1:500), ATF6α (Santa Cruz, 1:500), connexin 43 (Sigma, 1:1000), and VCAM-1 (R&D, 1:1000).

RNA Isolation and Real-Time RT-PCR Analysis
Total RNA was extracted using TRIzol reagent (Invitrogen), as described by the manufacturer, and reverse transcribed using the iScript cDNA Synthesis Kit (BioRad). Primers were designed using Beacon Designer 2.0 for GRP78 and β-2-microglobulin³. Primers for GRP78 are forward primer, 5’-GCGATGCTGCTGCTGCTCAG-3’ and reverse 5’-ACGCCGACGCAGGAGTAGGT-3’. The expression of mRNA was analyzed via real-time reverse transcriptase polymerase chain reaction (RT-PCR) using AmpliTaq Gold (Applied Biosystems), SYBR Green (Invitrogen) and an iCycler (Bio-Rad).

**GRP78- and CHOP-ERSE Luciferase Assay**

For transfection experiments, passage 7-9 bovine aortic endothelial cells (BAEC) were isolated and grown in DMEM supplemented with 10%FBS (Gibco), 2mM L-glutamine (Gibco), and 100U penicillin/streptomycin (Invitrogen) at 80,000 cells/cm² on surface treated plastic. After preplating, BAECs were placed in Optimem I media (Gibco). 10µg of plasmids were mixed with 8µl Lipofectamine2000 (Invitrogen) in 1.2ml of Optimem I for 15 minutes before added to approximately 2 million cells in total volume of 3ml for 2 hours. The following plasmids were generously provided by Dr. Christopher Glembotski, San Diego State University: GRP78-ERSE1-Luc, GRP78-M-ERSE1-Luc, CHOP-ERSE1-Luc, and CHOP-M-ERSE1-luc. Each construct drives the SV-40/luciferase from the active ERSE1 promoter or a mutated version denoted by the – M. ERSE-M constructs are not inducible by ATF6 or during ER stress response⁴.

After cell lysis using passive lysis buffer (Promega), 5µl of sample were combined with 50µl Luciferase Assay Reagent (Promega) and measured using a Fluostar
plate reader. Duplicate averages were normalized to total protein and presented as fold averages to the ERSE1-mutant controls.

*Single-Stage Clotting Assay*

ECs were preconditioned with 24 hours of atheroprone or atheroprotective flow, after which the cells were collected and pelleted in ice cold PBS without calcium. The pellet (~2 million cells) was resuspended in 400µl PBS without calcium, 25mM Heps, and 0.25% TritonX. Half of the suspension was incubated for 30 minutes with anti-GRP78 (Santa Cruz, 1:100). The suspensions (50µl) were then mixed during an activated partial thromboplastine time (APTT) assay in an ST4 Coagulation Analyzer with 50µl of APTT reagent (Diagnostico Stago) in duplicate. After adding 50µl of 25mM CaCl$_2$ (Diagnostico Stago) the clot time was recorded.

*Data Analysis and Statistics*

A student’s 2-tailed t-test was performed to evaluate significant differences in data sets of static controls or between different flow patterns (p<0.05). All values are reported as the mean±standard error.

**Supplemental Figures:**

**Figure I. Hypothesized GRP78 regulation by hemodynamic shear stress.** Flow was found to differentially regulate GRP78 expression. Solid lines show pathways directly investigated here using specific inhibitors, while dashed arrows are hypothesized or have been shown in response to other ER stressors.

