Biomechanical Forces in Atherosclerosis-Resistant Vascular Regions Regulate Endothelial Redox Balance via Phosphoinositol 3-Kinase/Akt-Dependent Activation of Nrf2

Guohao Dai, Saran Vaughn, Yuzhi Zhang, Eric T. Wang, Guillermo Garcia-Cardena, Michael A. Gimbrone Jr

Abstract—Local patterns of biomechanical forces experienced by endothelial cells (ECs) in different vascular geometries appear to play an essential role in regulating EC function and determining the regional susceptibility to atherosclerosis, even in the face of systemic risk factors. To study how biomechanical forces regulate EC redox homeostasis, an important pathogenic factor in atherogenesis, we have cultured human ECs under 2 prototypic arterial shear stress waveforms, “atheroprone” and “atheroprotective,” which were derived from 2 distinct vascular regions in vivo that are typically “susceptible” or “resistant” to atherosclerosis. We demonstrate that atheroprotective flow decreases EC intracellular redox level and protects ECs against oxidative stress–induced injury. To identify the molecular mechanisms that control this cellular response, we examined several major oxidative/antioxidative pathways and found that atheroprotective flow upregulated certain antioxidant genes and strongly activated the transcription factor Nrf2. Using a strategy of small interfering RNA inhibition of Nrf2 expression combined with genome-wide transcriptional profiling, we determined the downstream targets of Nrf2 activation and identified Nrf2 as a critical determinant for the changes in endothelial redox balance exerted by atheroprotective flow. In addition, we showed that atheroprotective flow activates Nrf2 via the phosphoinositol 3-kinase/Akt pathway, and this activation occurs differentially in atherosclerosis-resistant and atherosclerosis-susceptible regions of the mouse aorta. Taken together, our data demonstrate that hemodynamic forces present in atherosclerosis-resistant and -susceptible regions of the vasculature differentially regulate EC redox state and antioxidant potential. These alterations in redox homeostasis are primarily the result of the phosphoinositol 3-kinase/Akt-dependent activation of Nrf2 and its downstream transcriptional targets. (Circ Res. 2007;101:723-733.)

Key Words: biomechanical force • endothelial cells • redox homeostasis • Nrf2

Atherosclerotic lesions frequently develop in areas of the vasculature exposed to disturbed flow, whereas areas that experience pulsatile laminar flow are relatively protected from lesion formation. The magnitude and particular patterns of these biomechanical forces play significant roles in endothelial phenotypic modulation, and increasing evidence suggests that they may also be important determinants of the focal nature of atherosclerotic lesion formation and progression.1–4 On the other hand, many systemic risk factors, such as hypercholesterolemia, hypertension, and hyperhomocysteinemia, generally increase the overall probability of atherosclerosis development. Several studies have indicated that a common denominator of these major systemic pathogenic stimuli for atherogenesis is oxidative stress.5–8 Increased production of reactive oxygen species (ROS) has been shown to initiate several processes involved in atherosclerosis including oxidative modification of LDL, reduction of vascular NO bioavailability, and induction of redox-sensitive genes such as vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1.9,10 Several pathways involved in regulating endothelial cells (ECs) redox balance have been shown to be influenced by biomechanical forces. Laminar shear stress increases the production of NO and intracellular glutathione and upregulates the expression of superoxide dismutase.11 In contrast, oscillatory shear stress decreases intracellular glutathione and increases the production of superoxide.11 These studies demonstrate that different types of biomechanical forces can modulate several important cellular oxidative stress pathways, suggesting that the complex flow patterns actually present in the vasculature may differentially regulate cellular redox balance. Here, we assessed the regulation of EC redox state by well-defined biomechanical forces associated with particular human vascular geometries with known atherosclerosis risk and elucidated the underlying mechanisms using an in vitro cell culture model recently developed in our labora-
In this model, we defined 2 prototypic arterial waveforms: atheroprone and atheroprotective, which were representative of the wall shear stress profiles in the corresponding atherosclerosis-susceptible and atherosclerosis-resistant regions of the human carotid artery bifurcation. These arterial waveforms were then replicated using an in vitro dynamic flow system and applied to cultured human umbilical vein endothelial cells (HUVECs). Using this experimental model, we examined whether the hemodynamic forces could differentially regulate HUVEC redox state and antioxidant potential. We found that atheroprotective flow strongly decreases HUVEC redox level and increases the endothelial resistance to external oxidative stress challenge. We identified Nrf2 (NF-E2–related factor-2) as the major transcription factor responsible for these alterations in HUVEC redox homeostasis and characterized the transcriptional targets of Nrf2 under atheroprotective flow conditioning using genome-wide transcriptional profiling. We have established that the phosphoinositol 3-kinase (PI3K)/Akt pathway is an upstream mediator of atheroprotective flow–induced Nrf2 activation, independent of NO production.

### Materials and Methods

HUVECs were isolated and cultured as described previously. HUVECs (passage 1) were plated at an initial density of 70,000 cells/cm² on 0.1% gelatin-coated (Difco Laboratories) polystyrene plastic (Plaskolite Inc, Columbus, Ohio). After 24 hours, the cell culture plate was assembled in the Dynamic Flow System. HUVEC monolayers were then exposed to the atheroprone or atheroprotective waveform stimulation for 24 hours.

An expanded Materials and Methods section is provided in the online data supplement at http://circres.ahajournals.org.

### Results

**Atheroprotective Flow Decreases Intracellular Redox Level and Protects HUVECs From Oxidative Stress–Induced Cell Injury**

Following preconditioning with atheroprotective flow for 24 hours, HUVECs demonstrated a marked decrease in intracellular redox state compared with those cultured under static (no-flow) conditions. Atheroprone flow also reduced the intracellular redox level, compared with static condition, but to a lesser degree than atheroprotective flow (Figure 1A). To examine whether the observed intracellular redox changes over time, additional experiments were performed over a period of 72 hours. It was found that the intracellular redox states at 48 and 72 hours were identical to those at 24 hours (data not shown), suggesting that the decreased intracellular redox potential reflects a relatively stable phenotypic alteration in response to chronic atheroprotective flow exposure.

To assess how these distinct biomechanical stimuli influence HUVEC responses to external oxidative stress challenge, HUVECs, either preconditioned with atheroprone or atheroprotective flow or maintained under static (no-flow) condition, were treated with various doses of the oxidant tert-butyl-hydroperoxide for 4 hours. We found that a lower dose of oxidant (<80 μmol/L tert-butyl-hydroperoxide) did not cause noticeable cellular damage and that the HUVEC monolayer maintained a viability close to 100% under this degree of oxidative stress. When the dose of oxidant was increased beyond 100 μmol/L, considerable damage to the cells cultured under static conditions was observed. However, HUVECs preconditioned with atheroprotective flow showed increased resistance to oxidative stress–induced cell injury (up to 200 μmol/L tert-butyl-hydroperoxide) (Figure 1B). Atheroprotective flow preconditioning, in contrast, only slightly improved the resistance of HUVECs to oxidative stress challenge. Cell viability was further quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay showing that atheroprotective flow preconditioning dramatically enhances the capacity of HUVECs to defend against oxidative stress (Figure 1C).

**Atheroprotective Flow Induces the Expression of Several Cytoprotective Genes**

The homeostasis of ROS could be influenced by either the production of oxidative free radicals or by the enzymatic processes that degrade these free radicals. It is possible that the change in redox level under atheroprotective flow is caused by a decrease in ROS production. We first explored the activity of NAD(P)H oxidase, which has been shown to be a major cellular oxidase in the vasculature. Our results showed that there was no significant change in the NAD(P)H oxidase activity after HUVECs were exposed to atheroprone or atheroprotective flow for 24 hours (data not shown).

Because atheroprotective flow and atheroprone flow are known to differentially regulate the expression of multiple genes in HUVECs, we sought to assess changes in gene expression that might influence redox state and antioxidant potential. Genome-wide microarray analysis of the transcriptional profile revealed that a number of antioxidant genes were significantly regulated by atheroprotective flow, including heme oxygenase-1 (HO-1), ferritin heavy chains (FTH), NAD(P)H:quinone oxidoreductase-1 (NQO1), glutamate-cysteine ligase modifier subunit (GCLM), thioredoxin reductase 1 (TXNRD1), and glutathione reductase (GSR) (Figure 4). Some of these genes were chosen and additional experiments were performed to characterize their expression by Western blot and RT-PCR. As shown in Figure 2A, atheroprotective flow significantly induced the protein expression of HO-1 and FTH. Because ferritin plays an essential role in iron sequestration, and intracellular free iron is indispensable for catalyzing the Fenton reaction to generate highly reactive hydroxyl radical (OH·) that can lead to lipid peroxidation in the face of H₂O₂ challenge, we chose to measure the intracellular catalytic free iron concentration. It was found that HUVECs cultured under atheroprotective flow have a decreased level of catalytic iron (Figure 2B), which is consistent with the elevated ferritin protein in the cells. In addition, atheroprotective flow increased the expression of several other phase II detoxification and antioxidant genes including NQO1, GCLM, TXNRD1, and GSR (Figure 2C). On the contrary, several other major antioxidant genes including manganese superoxide dismutase, Cu/Zn superoxide dismutase (Cu/Zn SOD), and catalase were not regulated by atheroprotective or atheroprone flow at 24 hours (data not shown).
Atheroprotective Flow Activates Nrf2 and Induces Its Nuclear Translocation

Several recent studies have identified Nrf2 as a critical transcription factor that regulates a battery of phase II detoxification and antioxidant genes in the face of xenobiotic and oxidative stress. Recent data also have shown that laminar and oscillatory flow regulates Nrf2 in human endothelial cells. To examine whether Nrf2 is activated under the atheroprotective flow, we investigated the intracellular localization of this transcription factor protein. Western blotting analysis of cytoplasmic and nuclear protein extracts indicated increased nuclear accumulation of Nrf2 protein in cells subjected to atheroprotective flow (Figure 3A). Immunofluorescence microscopy also showed enhanced nuclear staining of Nrf2 in cells under atheroprotective flow in comparison to the cells under atheroprone flow and static conditions (Figure 3B), suggesting that Nrf2 is activated in response to atheroprotective flow exposure.

Nrf2 Is Necessary for Atheroprotective Flow–Induced Antioxidant Gene Expression

To test whether Nrf2 is responsible for atheroprotective flow–induced antioxidant gene expression, we used small interfering (si)RNA to knockdown Nrf2, and the resultant gene expression patterns were analyzed by genome-wide transcriptional profiling. Genes that were induced by atheroprotective flow and the inductions were blocked by Nrf2 siRNA were compiled and color coded by their expression.

Figure 1. Atheroprotective flow decreases intracellular redox level and protects HUVECs from oxidative stress–induced cell injury. A, HUVECs were cultured under no-flow or atheroprone or atheroprotective flow for 24 hours. Intracellular redox level was assessed by 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate/acetyl ester (CM-H2DCFDA) staining, followed by flow cytometric analysis. B, HUVECs were preconditioned by exposure for 24 hours to either no-flow or atheroprone vs atheroprotective flow and then challenged by exposure to tert-butyl hydroperoxide at various doses for 4 hours. Images were taken by phase-contrast microscopy. C, Cell viability was measured by MTT assay and normalized to nontreated cells (100% viability) (n=3).
level relative to static conditions (Figure 4A). It is interesting that Nrf2 activation under atheroprotective flow controls a very small group of genes. Of 29,000 genes that were tested, only 17 genes showed a consistent pattern of significant regulation. Among those genes, most are phase II detoxification and antioxidant genes (HO-1, FTH, FTL, GSR, GCLM, TXNRD1, NQO1). In addition, genes involved in metabolic process, PHGDH (phosphoglycerate dehydrogenase) and inflammation LTB4DH (Leukotriene B4 12-hydroxy dehydrogenase), are also Nrf2 targets under atheroprotective flow. These results demonstrate that Nrf2 is necessary for flow-induced antioxidant gene expression and that this group of genes is also the primary target of Nrf2 activation under atheroprotective flow condition.

Figure 2. Atheroprotective flow induces the expression of several cytoprotective genes. Confluent HUVEC monolayers were exposed to either no-flow or atheroprone vs atheroprotective waveforms for 24 hours. A, Western blot analysis of heme oxygenase 1 (HO-1) and ferritin heavy chain (FTH) protein expression. B, Intracellular catalytic iron was measured and normalized to protein concentration (n=3). *P<0.05. C, mRNA expression of NAD-(P)H:quinone oxidoreductase-1 (NQO1), glutamate-cysteine ligase modifier subunit (GCLM), thioredoxin reductase 1 (TXNRD1), and GSR was measured by TaqMan RT-PCR (n=3). *P<0.05.

Figure 3. Atheroprotective flow increases Nrf2 nuclear translocation. A, Western blot analysis of Nrf2 cytoplasmic and nuclear localization in HUVECs exposed to no flow, atheroprone, or atheroprotective flow for 24 hours. B, Immunofluorescent staining of Nrf2 (×40) in HUVECs.
Activation of Nrf2 by Atheroprotective Flow
Regulates HUVEC Intracellular Redox State and
Protects HUVECs Against Oxidative Stress Challenge

To test whether Nrf2 is the major transcriptional regulator that determines redox homeostasis in HUVECs in response to biomechanical stimuli, we assessed the HUVEC intracellular redox state and antioxidant properties in the face of external oxidative stress challenge after Nrf2 expression was knocked down by siRNA. As shown in Figure 5A and 5B, atheroprotective flow was no longer able to decrease intracellular redox state and protect HUVECs from oxidative stress challenge in cells lacking Nrf2, demonstrating a pivotal role of Nrf2 in cellular redox homeostasis.

Increased Nrf2 Nuclear Localization in Endothelium in Atherosclerosis-Resistant Regions of the Mouse Aorta

To investigate whether the differential activation of Nrf2 by biomechanical forces actually occurs in vivo, we performed experiments to characterize the localization of Nrf2 on the intimal endothelial lining of the mouse aorta by en face confocal immunofluorescence microscopy. The lesser curvature of the aortic arch is typically susceptible to atherosclerosis, whereas the straight portion of the thoracic aorta is a relatively atherosclerosis-resistant region. Figure 6 demonstrates that Nrf2 is predominantly nuclear localized in the atherosclerosis-resistant region, whereas its localization is diffuse in the atherosclerosis-susceptible region, thus illustrating the differential activation status of Nrf2 in vivo.

Atheroprotective Flow Activates Nrf2 Through the PI3K/Akt Pathway Independent of NO

To dissect the molecular mechanisms of atheroprotective flow–induced Nrf2 activation, several protein kinase pathways were explored to determine their roles in the regulation of antioxidant genes. Figure 7A shows that pretreatment with the PI3K inhibitor LY294002 completely blocked the induction of HO-1 protein by atheroprotective flow. In contrast, inhibitors of several other protein kinase pathways (protein kinase C, extracellular signal-regulated kinase 1/2, p38, and c-Jun N-terminal kinase) had no influence. We next infected HUVECs with a dominant-negative form of Akt and found that it blocked the Nrf2 activation and HO-1 expression (Figure 7B). Moreover, a constitutively active form of Akt alone is able to activate Nrf2 (Figure 7C) and increases the expression of several targets (HO-1, Figure 7C; NQO1 and FTH, data not shown), indicating that Akt is sufficient to increase Nrf2 expression and protect HUVECs from oxidative stress challenge.

Figure 4. Nrf2 is necessary for atheroprotective flow–induced antioxidant gene expression. HUVECs transfected with control (ctrl) siRNA or Nrf2 siRNA were cultured under atheroprotective flow for 24 hours. A, Gene expression patterns were analyzed by genome-wide transcriptional profiling. Genes that were induced by atheroprotective flow and the induction blocked by Nrf2 siRNA were compiled and color coded by their relative expression level to static. *Indicates that the results were validated by RT-PCR. B, Western blot analysis of HO-1 and FTH protein. C, mRNA expression measured by TaqMan RT-PCR (n = 3). *P < 0.05.
activate Nrf2. To further explore the mechanisms of Nrf2 activation, we first looked at its cytoplasmic binding partner Keap1 (Kelch-like ECH-associated protein 1). We found that mRNA and protein level of Keap1 did not change in HUVECs exposed to atheroprone or atheroprotective flow for 24 hours (data not shown), suggesting that other regulatory mechanisms of Nrf2 are involved. We then immunoprecipitated the cell lysate using an anti-Keap1 antibody and blotted for Nrf2. The results show that atheroprotective flow dissociates Nrf2 from Keap1, and this dissociation was blocked when the PI3K/Akt pathway was inhibited (Figure 7D). These data suggest that activated PI3K/Akt pathway under the atheroprotective flow stimulation decreases the binding of Nrf2 to Keap1, which may be responsible for the increased Nrf2 nuclear localization.

Akt activation has been shown to affect several important endothelial functions. One of them is NO production via Akt-dependent endothelial NO synthase phosphorylation and activation. Because NO has been implicated in Nrf2 activation from experimental observations made from HUVECs treated with an NO donor, it is possible that flow-induced NO production mediates the Nrf2 activation in our model. To test this hypothesis, we examined the effect of NO synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME) on Nrf2 activation and its mediated gene expression. We found that inhibition of NO production did not affect Nrf2 activation and HO-1 expression in HUVECs exposed to atheroprotective flow (Figure 8A). In addition, the constitutively active Akt upregulated HO-1 expression even in the presence of NO synthase inhibitor L-NAME (Figure 8A), suggesting that NO is not involved in flow- or Akt-mediated Nrf2 activation. Because our data have shown that the PI3K/Akt pathway is involved in Nrf2 activation, and because Nrf2 is implicated in regulating HUVEC redox balance, we tested whether the PI3K/Akt pathway was directly involved in atheroprotective flow–induced HUVEC redox reduction. We examined the HUVEC redox regulation when PI3K/Akt pathway was inhibited by LY294002. As shown in Figure 8B, inhibition of the PI3K/Akt pathway strongly reduced the effect of redox attenuation by atheroprotective flow, to a similar level as when Nrf2 was knocked down by siRNA (Figure 5). In contrast, NO was not involved in regulating HUVEC redox state under the atheroprotective flow condition (Figure 8B). Taken together, these data demonstrate that atheroprotective...
Figure 6. Increased Nrf2 nuclear localization in endothelium in atherosclerosis-resistant regions of the mouse aorta. The aortic arch of C57BL/6J mice was isolated and fixed. The lesser curvature of the arch and the portions of the thoracic aorta were triple stained with CD31, DAPI (4',6-diamidino-2-phenylindole), and Nrf2; en face confocal immunofluorescence microscopy was then performed (×60).

Figure 7. Atheroprotective flow activates Nrf2 through the PI3K/Akt pathway. A, HUVECs were exposed to atheroprotective flow for 24 hours in the presence of various selective protein kinase inhibitors. The protein kinase inhibitors used were as follows: LY294002 (40 μmol/L, PI3K inhibitor), RO-32-0432 (5 μmol/L; protein kinase C inhibitor), PD98059 (20 μmol/L; ERK1/2 inhibitor), SB203580 (10 μmol/L; p38 inhibitor), and SP600125 (10 μmol/L; c-Jun N-terminal kinase inhibitor). B, HUVECs were infected with Ad-DN-Akt or Ad-null for 24 hours and then exposed to atheroprotective flow for 24 hours. B (top), Nuclear protein was blotted for Nrf2 and Lamin B1. B (bottom), Total protein was blotted for α-tubulin and HO-1. C (top), HUVECs were infected with Ad-myr-Akt or Ad-null for 24 hours, and nuclear protein was blotted for Nrf2 and Lamin B1. C (bottom), HUVECs were infected with Ad-myr-Akt at various multiplicities of infection for 24 hours, and total protein was blotted for α-tubulin, HO-1, and phospho-Akt. D, HUVECs were exposed to atheroprotective flow for 24 hours in the presence of dimethyl sulfoxide (DMSO) or LY294002. Total protein lysate was immunoprecipitated with anti-Keap1 antibody and blotted for Nrf2 or Keap1.
flow regulates HUVEC redox homeostasis through PI3K/Akt-dependent, but NO-independent, activation of Nrf2.

**Discussion**

Distinct biomechanical forces in atherosclerosis-resistant and atherosclerosis-susceptible regions of the arterial vasculature are potent mediators of a “vasoprotective phenotype” in endothelium. This biomechanical modulation of endothelial function involves the differential regulation of multiple pathophysiologically important genes involved in inflammation and oxidative stress resistance. For example, oscillatory and steady laminar shear stress differentially regulate NAD(P)H oxidases, xanthine oxidoreductase, and multidrug resistance protein-1, which play important roles in modulating endothelial cell oxidative stress. It has been shown that steady laminar flow inhibits vascular inflammation by decreasing thioredoxin-interacting protein in endothelial cells. Moreover, several studies have identified the mechanosensitive transcription factor Kruppel-like factor 2 (KLF2) as a critical regulator of multiple atheroprotective

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**Figure 8.** Atheroprotective flow regulates EC redox balance through an NO-independent PI3K/Akt pathway. A (left), HUVECs were exposed to atheroprotective flow for 24 hours in the presence of L-NAME or D-NAME. Nuclear protein was blotted for Nrf2 and Lamin B1, and total protein was blotted for α-tubulin and HO-1. A (right), HUVECs were infected with Ad-myr-Akt or Ad-null in the presence of L-NAME or D-NAME for 24 hours, and total protein was blotted for α-tubulin and HO-1. B, HUVECs were exposed to atheroprotective flow for 24 hours in the presence of dimethyl sulfoxide (DMSO) or LY294002 (40 μmol/L) (top) or D-NAME or L-NAME (2 mmol/L) (bottom). Intracellular redox level was assessed by CM-H2DCFDA staining, followed by flow cytometric analysis.
mechanisms including antiinflammatory and antithrombotic genetic programs in endothelial cells exposed to biomechanical forces.14,31

In this study, we explored the regulation of HUVEC redox homeostasis by the shear stress waveforms derived from atherosclerosis-resistant and -susceptible regions of the human carotid bifurcation. Our data revealed that atheroprotective flow dramatically decreases intracellular ROS level and increases EC capacity to defend against exogenous oxidative stresses. These modulations were further demonstrated to be primarily dependent on the activation of Nrf2 and coordinated induction of several antioxidant genes. Atheroprobe flow, conversely, is a much weaker inducer of these changes. Additionally, the differential regulatory status of Nrf2 also was displayed in the atherosclerosis-resistant and -susceptible regions of the mouse aorta in vivo, suggesting that biomechanical forces may play a role in the physiological setting. Interestingly, experiments using siRNA to silence the KLF2 expression in HUVECs exposed to atheroprotective flow conditioning demonstrated that KLF2 did not affect the expression of antioxidant genes (HO-1, FTH, FTL, GSR, GCLM, TXNRD1, NQO1, data not shown), whereas siRNA to Nrf2 affected the expression of those genes, suggesting that Nrf2 is a KLF2-independent regulatory pathway that specifically controls the induction of antioxidant genes and regulates endothelial redox balance.

In this study, we identified the genes that are downstream transcriptional targets of Nrf2 under atheroprotective flow. Importantly, activation of Nrf2 by atheroprotective flow upregulates a small cluster of genes in HUVECs, suggesting a selective role of Nrf2 in response to biomechanical stimulation. Many of these genes are phase II detoxification and antioxidant genes (HO-1, FTH, FTL, GSR, GCLM, TXNRD1, NQO1), which is consistent with previous reports that some of these genes can be induced by laminar or oscillatory shear stress.19–21,32 Additional genes identified included PHGDH (phosphoglycerate dehydrogenase), which is the first and rate-limiting step in the pathway of L-serine biosynthesis, and LTB4DH (leukotriene B4 12-hydroxy dehydrogenase), which constitutes an important inactivator of leukotriene B4 involved in inflammation. Several genes identified in this study have previously been shown to affect atherosclerosis development and progression. For example, HO-1 was shown to inhibit atherosclerotic lesion formation in animal models.33 In humans, polymorphism in GCLM is associated with myocardial infarction and impairment of NO-mediated coronary vasomotor function.35 Additionally, 2 genetic variants in the leukotriene pathway confer risk of myocardial infarction and stroke.36,37 Given the importance of Nrf2 in regulating these genes in endothelial cells exposed to physiologic flow conditions, it is possible that Nrf2 may play a significant role in atherogenesis and this warrants further investigation.

Nrf2 is a recently identified transcription factor that coordinates the induction of genes that contribute to the pivotal cellular defense mechanisms against the toxicity of electrophiles and ROS.16–18 Under basal conditions, Nrf2 resides mainly in the cytoplasm bound to its cysteine-rich, Kelch domain-containing partner, Keap1. On exposure to electrophile or oxidative stresses, Nrf2 is liberated from Keap1-dependent repression and accumulates in the nucleus to regulate the induction of a family of antioxidant genes.16–18 Experimental evidence has suggested that certain classes of electrophilic compounds, such as 15d-PGJ2 (15-deoxy-D12,14-prostaglandin J2), can directly modify cysteine residues of Keap1 and that the resulting conformational changes of the Keap1 protein are essential for releasing Nrf2 in response to electrophile stimulation.20,38,39

The mechanism by which biomechanical forces induce Nrf2 activation is not well understood. In this study, we investigated additional pathways that are important for flow-mediated Nrf2 activation, focusing on the influence of several protein kinase pathways. These signaling pathways, including mitogen-activated protein kinases,40 protein kinase C,41 and the PI3K/Akt pathway,32 have been implicated in Nrf2-mediated transcriptional response to various chemical stresses in different cell types. In addition, previous studies have demonstrated the activation of these signal transduction pathways in endothelial cells exposed to fluid shear stresses.43–46 Our study demonstrates that the PI3K/Akt pathway is necessary and sufficient in atheroprotective flow induced Nrf2 activation and redox attenuation in HUVECs, whereas several other protein kinase pathways appear not to be involved in this process. Shear stress–activated PI3K/Akt has been shown to affect several important endothelial functions such as endothelial NO synthase phosphorylation23 and HUVEC endothelial NO synthase phosphorylation33 and HUVEC survival.47 Our data indicate that the PI3K/Akt pathway also influences the Nrf2 antioxidant pathway in HUVECs. Interestingly, in vivo overexpression of an active form of Akt is protective against oxidative stress challenge, eg, activated Akt protects lung from oxidant-induced injury,48 and protects mouse heart against ischemia/reperfusion injury,49 suggesting that there may be some crosstalk between the Akt survival pathway and the Nrf2 antioxidant pathway.

In summary, this study has revealed that shear stress waveforms representative of those in the atherosclerosis-resistant and -susceptible regions of the human carotid artery in vivo differentially regulate redox homeostasis in cultured human endothelial cells. Further mechanistic studies indicated that this phenotypic modulation occurs principally through the transcription factor Nrf2 and its downstream targets. We identified a well-defined group of genes whose induction is dependent on Nrf2 under the atheroprotective flow condition and demonstrated that the involvement of PI3K/Akt pathway but not NO generation is necessary for this process. Our data suggest that Nrf2-mediated gene expression induced by distinct biomechanical forces may play a role in regional differences in endothelial phenotype in vivo, in health, and in disease.

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Supporting Materials and Methods

Flow Cytometric Analysis of Cellular Redox State

Following exposure to athero-prone or athero-protective flow for 24 hours, HUVEC monolayers were washed with PBS and detached using enzyme-free cell dissociation buffer (Invitrogen). The cells were washed and re-suspended in PBS containing 10µM CM-H$_2$DCFDA (5-(and 6-)chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester), which is oxidized by intracellular reactive oxygen intermediates to generate a highly fluorescent product. After 30 minutes incubation at 37°C, the cells were washed and analyzed in a FACScan Flow Cytometer (Becton Dickinson).

Microarray Hybridization and Statistical Methods for Microarray Data Analysis

For transcriptional profiling studies, whole genome oligo-DNA microarrays (Applied Biosystems) with 32,878 probes representing 29,098 human genes were used according to the manufacturer's instructions. Genes differentially regulated were identified based on three pairs of replicate arrays from three independent experiments. To identify significantly regulated genes, Zpool algorithm$^1$ was used to determine genes differentially regulated at consistent magnitudes across all triplicates, and an iterative standard deviation algorithm$^2$ was used to determine genes differentially regulated at large magnitudes but with a lesser degree of consistency across all triplicates.

RT-PCR Validation of Selected Transcripts

Purified, DNase-treated RNA (1.5µg) was reverse transcribed using a MultiScribe based RT reaction (Applied Biosystems). The cDNA were then subjected to a real-time TaqMan PCR
in 7900HT Fast Real-Time PCR System (Applied Biosystems). All Taqman probes were from
Applied Biosystems and the relative gene expression was normalized to GAPDH.

**Isolation of Nuclear and Cytoplasmic Proteins, Immunoprecipitation and Western Blotting**

Nuclear and cytoplasmic proteins were extracted by using NE-PER nuclear and
cytoplasmic extraction reagents (Pierce Biotechnology). For extraction of total protein, RIPA
buffer (Sigma-Aldrich) supplemented with protease inhibitor cocktails and phosphatase inhibitor
cocktails (Sigma-Aldrich) was used as the protein lysis buffer. The protein concentration was
measured by BCA protein assay (Pierce Biotechnology). For immunoprecipitation of Keap1
associated proteins, anti-Keap1 antibody (1:200, ProteinTech Group, Inc) was used in the total
cell lysate. Proteins were separated on 4-20% polyacrylamide gels (Bio-Rad) and transferred to
PVDF membrane (Millipore). The antibodies used in western blotting were: rabbit anti-Nrf2
(1:1000, Santa Cruz Biotechnology), rabbit anti-ferritin (1:500, Sigma-Aldrich), mouse anti-HO-
1 (1:2000, BD Bioscience), mouse anti-α-tubulin (1:10,000, Zymed) and mouse anti-lamin B1
(1:2000, Zymed). Goat anti-mouse and goat anti-rabbit HRP conjugated secondary antibodies
were from Santa Cruz Biotechnology.

**Measurement of Intracellular Catalytic Iron**

HUVEC were lysed in 20mM Tris/HCl with 0.5% NP-40, PH 7.4. After centrifuging at
16,000g for 10 minutes, the supernatant was collected for analysis of catalytic free iron as
described previously. A standard curve prepared with solutions of ferrous iron sulfate was used
to calculate concentrations of free iron. Intracellular catalytic iron was expressed as nmol/mg
after normalizing to the protein concentration measured from the same sample.
**MTT Cell Viability Assay**

After being challenged by tert-Butyl hydroperoxide at various dose for 4 hours, cells were washed and incubated with MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide, 0.5mg/ml) in culture media without phenol red at 37°C. After 1 hour incubation, cells were washed three times with PBS and lysed in DMSO. The absorbance of the lysate was measured at 570nm and normalized to control cells without exposure to oxidative stress challenge.

**Infection of HUVEC with Adenovirus**

Adenoviruses expressing dominant-negative and constitutively active forms of Akt were kindly provided by Dr. William C. Sessa (Yale University). The dominant-negative Akt mutant (Ad-dn-Akt) expressing a mutant Akt (T308A, S473A) construct cannot be activated by phosphorylation. The constitutively active Akt construct (Ad-myr-Akt) has the c-src myristoylation sequence fused in frame to the NH2-terminus of the HA-Akt coding sequence that targets the fusion protein to the membrane. Experiments were performed 24 hours after HUVEC were infected.

**Transfection of siRNA Duplexes into HUVEC**

siRNA was transfected into HUVEC with Lipofectamine RNAiMAX (Invitrogen) and Opti-MEM (Invitrogen) at a final concentration of 10nM according to manufacturer’s instruction. The sense and antisense strands of human Nrf2 siRNA were: 5’-CCAGAACACUCAGUGGAAUdTdT-3’ (sense) and 5’-AUUCCACUGAGUGUUCUGGdTdT-3’ (antisense). Negative control siRNA were: 5’-UUCUCCGAACGUGUCACG UdTdT-3’ (sense) and 5’-ACGUGACACGUUCGGAGAAdTdT-3’ (antisense). All siRNA were
synthesized by Invitrogen. The efficiency of knockdown was greater than 80% as assessed by both western blot and RT-PCR (data not shown). Flow experiments were performed 24 hours after the siRNA transfection.

**En face Immunofluorescence Microscopy of Mouse Aortic Arch**

*En face* immunofluorescence microscopy of mouse aortic arch was performed as described previously. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained in accordance with Institutional Review Board approved protocols. Aortas were harvested after perfusion with PBS and 2% paraformaldehyde. The aorta was then cut open and suspended in PBS followed by permeabilization in 0.1% Triton X-100 for 1 minute. After blocking with 1% BSA for 1 hour, the aorta was incubated with rabbit anti-Nrf2 (1:500, Santa CruZ Biotechnology) and rat anti-CD31 (1:500, BD Bioscience) in 0.1% BSA at 4°C overnight. Secondary antibodies included Alexa Fluo 488 goat anti-rabbit (1:500, Invitrogen) and Alexa Fluo 546 goat anti-rat (1:500, Invitrogen). To identify nuclei, DAPI (1:10,000, Invitrogen) was added during the incubation of secondary antibodies. The lesser curvature of the aortic arch and a portion of thoracic aorta were cut and mounted with the endothelium facing up. Images of the endothelial cell monolayer were obtained using a Zeiss LSM META 510 confocal microscope system.
References:


