Brief Review

Nrf2 as an Endothelial Mechanosensitive Transcription Factor
Going With the Flow

Shane R. McSweeney,* Eiji Warabi,* Richard C.M. Siow

The vascular endothelium is continually exposed to hemodynamic stress induced by the frictional force of blood flow across its surface and pressure changes throughout each cardiac cycle. The interaction between fluid shear stress (FSS) and the endothelium is critical in maintaining vascular homeostasis via the integration of biomechanical forces with signal transduction to maintain redox balance.1 High unidirectional laminar shear (US) forces characteristic of straight regions of the vasculature have been demonstrated to be protected from atherosclerosis, whereas areas of the vasculature exposed to oscillatory disturbed shear (OS) forces, prevalent in curvatures and branches in blood vessels with complex geometry where blood flow forms localized flow-separation zones that include regions of low shear and flow reversal, are prone to atherogenesis.2 Endothelial cells (ECs) respond to the changes in shear stress to modulate redox signaling,3 which leads to alterations of pro- and antioxidant gene expression, inflammatory phenotype, cell alignment, and structural remodelling of vessels.4 The transcription factor nuclear factor (erythroid-derived 2)–like 2 (Nrf2) has been well characterized to play an important role in the antioxidant response element (ARE)–mediated expression of a group of genes encoding phase II detoxification enzymes and antioxidant proteins, such as glutathione-S-transferase, heme oxygenase-1 (HO-1), peroxiredoxin 1 (Prx-1), and nicotinamide adenine dinucleotide phosphate (NADPH) quinone oxidoreductase-1 (NQO1).5–7 The transcriptional regulation of these genes by Nrf2 can be enhanced by phyttonutrients, and it plays a key role for the protection of vascular EC from oxidative stress during the pathogenesis of vascular diseases.8–10 It has been reported that EC exposed to OS but not to US flow patterns in both in vivo and in vitro models exhibits greater nuclear factor-xB (NF-xB) activity and deficiencies in the Nrf2/ARE redox signaling pathway and are thus predisposed to oxidative stress and a proinflammatory phenotype because of the enhanced generation and reduced scavenging of reactive oxygen species (ROS).3,11–14 This review will highlight the role of the Nrf2 pathway in EC as a mechanosensitive transcriptional regulator of redox signaling in maintaining physiological endothelial function and summarize the mechanisms underlying the differential modulation of Nrf2 activity, ARE-dependent antioxidant gene expression and EC redox/inflammatory phenotype by laminar and disturbed FSS. Because therapeutic strategies for modulating Nrf2 activity have become an increasing area of basic and clinical research,15 it is important to consider how these may also alter physiological responses of EC to FSS mediated by redox signaling.

Hemodynamic Shear Forces in Endothelial Pathophysiology

Multiple mechanosensors located at the cell membrane are activated in response to changes in the FSS environment to trigger changes in signal transduction. These have been reviewed in detail elsewhere and include integrins, tyrosine kinase receptors, G-protein–coupled receptors, ion channels, adhesion molecules, intercellular junction proteins, and membrane-associated structures such as caveolae, primary cilia, and the glyocalyx.2,16–18 The triggering of signaling cascades by FSS modulates transcriptional regulation of functional gene expression to maintain the balance between EC proliferation or growth arrest, inflammatory or anti-inflammatory phenotypes, and redox status.4 During ath erosprotective US, the endothelium releases vasoactive molecules such as nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factors that lead to vascular smooth muscle cell (SMC) relaxation.19 The synthesis of NO by endothelial nitric oxide synthase (eNOS) is under the direct regulation by FSS through activation of the transcription factors Krüppel-like factor 2 and 4 (Klf2/4)20 and the phosphorylation status of eNOS21 and availability of tetrahydrobiopterin (BH4).22,23 During disturbed FSS, a reduction in Klf2/4 expression results in reduced eNOS expression and NO bioavailability leading to an inflammatory and oxidative EC phenotype,4 characteristic of atherogenesis, hypertension, and diabetes mellitus.24,25 The altered redox status in EC exposed to OS additionally reduces BH4 levels through its oxidation and diminished synthesis via GTP cyclohydrolase-1,26 leading to the uncoupling of eNOS to generate superoxide instead of NO.27 This in turn enhances EC dysfunction, oxidative stress, and inflammation, characteristic of atheroprone regions of the vasculature that are exposed to disturbed FSS patterns.28

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The production of NO by EC exposed to FSS is characterized by a rapid initial Ca$^{2+}$-dependent synthesis followed by a sustained plateau phase that occurs independent of changes in intracellular Ca$^{2+}$ levels, but is primarily regulated by shear-induced post-translational modifications of eNOS protein by phosphorylation and dephosphorylation of eNOS serine and threonine residues, events mediated by the phosphatidylinositoll 3-kinase (PI3K)/Akt pathway and protein phosphatases. Phosphorylation of eNOS at Ser-1177 by FSS has been shown to be more efficacious than phosphorylation at Ser-1177.21 eNOS is basally phosphorylated at Thr-495, acting as a negative regulator, and has been shown to be dephosphorylated after exposure of EC to FSS, which is also coupled to the phosphorylation of Ser-1177, acting as a positive regulator.29 However, dephosphorylation of eNOS Thr-495 does not always lead to enhanced eNOS activity because its dephosphorylation under inflammatory and oxidative conditions can also lead to the uncoupling of eNOS to produce superoxide.30 Phosphorylation of eNOS at Ser-1177 by FSS has been shown to be activated first, followed by the subsequent activation of a second phosphorylation site at Ser-633, which acts as the main site after exposure of EC to long-term FSS and reported to be more efficacious than phosphorylation at Ser-1177.31,32

In addition to the regulation of eNOS phosphorylation by FSS, the enzyme can also undergo covalent modifications with the fatty acids myristate and palmitate, which enhances its localization with caveolae and binding to caveolin-1 (Cav-1), which attenuates eNOS activation by interfering with the CaM-binding domain.33 Phosphorylation of eNOS at Ser-116, which attenuates eNOS activation by interfering with the CaM-binding domain,33 has been shown to increase the association of eNOS with Cav-1 resulting in decreased NO activity.34 It is of interest that Cav-1 can act as a FSS mechanosensor5,36 and has been shown to be negatively regulated by Klf2, an FSS-induced transcription factor.37 Moreover, Cav-1 has been demonstrated in lung epithelial cells to directly interact with Nrf2 to suppress its nuclear translocation, thereby diminishing the inducement of ARE-linked antioxidant defense genes38; in addition, it can also interact with HO-1, an Nrf2/ARE-regulated antioxidant enzyme,39 to attenuate its activity as shown in lung fibroblasts.40 Taken together, the enhanced Cav-1 expression observed in EC exposed to disturbed FSS41 is likely to contribute to enhanced impaired vascular function, oxidative stress and inflammation, not only via the reduction in NO synthesis but also possibly through diminished cytoprotective activities of both Nrf2 and HO-1.

During exposure of EC to sustained OS, enhanced levels of ROS such as superoxide and hydrogen peroxide are derived from uncoupled eNOS, NADPH oxidases, mitochondria, and xanthine oxidase.42 An increase in the generation of ROS during OS can directly decrease the bioavailability of NO and form reactive nitrogen species such as peroxynitrite,43 which can further increase ROS production and oxidative stress by oxidizing BH$_4$, resulting in uncoupling of eNOS to synthesize more superoxide.23 The onset of both OS and US in cultured human umbilical vein EC (HUVEC) has been demonstrated to enhance NADPH oxidase activity. However, sustained OS leads to prolonged superoxide generation, whereas US decreases this and increases both HO-1 and Cu/Zn SOD expression to maintain redox balance.44 OS-induced superoxide generation is attenuated in EC derived from mice lacking the catalytic p47phox subunit of NADPH oxidase, highlighting its role during OS.25 mRNA levels of both NADPH oxidase subunit isoforms Nox2 and Nox4 have been demonstrated to be upregulated in bovine aortic EC after exposure to OS but not to US, leading to enhanced superoxide generation and low-density lipoprotein oxidation.46 Interestingly, the Nox4 promoter has also been shown to have ARE-binding sites such that Nrf2 can negatively regulate the expression of Nox4, thus providing a further transcriptional role of Nrf2 to diminish generation of ROS in EC exposed to US.47 It is of note that OS has been shown to induce bone morphogenic protein-4-mediated induction of Nox1 in mouse aortic EC leading to enhanced H$_2$O$_2$ and superoxide generation and monocyte adhesion.45 Inhibition of NADPH oxidase but not of mitochondrial activity in an ex vivo porcine common femoral artery model of disturbed FSS has been shown to enhance NO generation and vasodilation, suggesting that superoxide generation resulting from directional changes of flow during OS are derived from NADPH oxidase (Nox) activity.44 It has been shown in bovine aortic EC that after exposure to OS, the main producer of superoxide was xanthine oxidase; however, this was dependent on maintenance of xanthine oxidase levels by endothelial NADPH oxidase activity.45 Apart from the enhanced ROS generation characteristic of EC exposed to disturbed shear patterns, perturbations in redox status can also arise from reduced levels of glutathione, the major intracellular thiol antioxidant. It has been demonstrated in human aortic EC that exposure to OS but not to US, significantly diminishes intracellular levels of reduced glutathione, because of enhanced export of oxidized glutathione via increased activity of multidrug resistance protein-1.46 Moreover, the major enzymes mediating the synthesis and recycling of glutathione are under the transcriptional regulation of Nrf2, and thus their expression may be decreased in EC exposed to OS.3,11,12 It has been recently demonstrated that 2 highly conserved cysteine residues on eNOS are sites for potential S-glutathionylation and critical for the redox-regulation of eNOS uncoupling;47 therefore alterations in glutathione and BH$_4$ status in EC exposed to OS further highlight the role of eNOS as the link between ROS generation and redox-dependent targets of NO,2,2 such as the Nrf2 pathway.48 Additional studies are necessary to fully elucidate the mechanosensitive signaling mechanisms by which enhanced endothelial ROS and decreased NO synthesis are coupled with dysregulation of Nrf2-mediated transcription of antioxidant genes in regions prone to atherogenesis.3,11

**Nrf2 as a Shear-Responsive Transcription Factor in the Endothelium**

After exposure of a wide variety of cell types to electrophiles, ROS, oxidative stress agents, and proinflammatory factors, Nrf2 has been demonstrated to regulate expression of a battery of phase II detoxification and antioxidant genes such as NQO1, HO-1, Prx-1, glutathione-S-transferase and sequestosome-1, and genes involved in the synthesis of glutathione such as γ-glutamyl-cysteine synthetase and glutamate-cysteine ligase.5,53 Nrf2 has been regarded as a master transcriptional regulator of cellular redox homeostasis over the past decade and the mechanisms by which Nrf2 activity is closely regulated by the redox environment and inflammatory status...
of cells have been reviewed extensively elsewhere.\textsuperscript{6-8,54-57} However, the involvement of Nrf2 as a shear-responsive transcription factor in EC has only been demonstrated in relatively few in vitro FSS culture models (Table) using primarily HUVEC which do not represent a cell type of relevance to the study of atherosclerosis.\textsuperscript{3,11,12,47,58-60} and in vivo mouse models of atherosclerosis.\textsuperscript{3,13,46} As disturbed oscillatory FSS patterns have been established as modulators of EC redox status and inflammatory phenotype in regions of the vasculature prone to atherogenesis,\textsuperscript{2,4} it is likely that augmented Nrf2 activity and ARE-linked gene expression in EC exposed to laminar FSS confers protection against vascular dysfunction and atherosclerosis.\textsuperscript{3,28}

Studies in cultured human EC have shown that the Nrf2 pathway is highly sensitive to laminar FSS and leads to induction of ARE-related genes such as HO-1, NQO1, sequestosome-1, glutamate-cysteine ligase modifier subunit, and ferritin heavy chain, an effect that is attenuated by siRNA knockdown of Nrf2.\textsuperscript{11,12,60} Because the Nrf2 pathway can be activated to a greater extent by US in EC, the ARE has been regarded as a novel shear stress response element\textsuperscript{11} because mutations in the ARE sequence directly diminish Nrf2 binding to the promoter regions of target genes such as NQO1 and HO-1, thereby attenuating their induction by FSS.\textsuperscript{61} FSS has been shown to enhance Nrf2 protein stability in EC without affecting its mRNA levels, an effect mediated by the production of lipid hydroperoxides derived from NOX, xanthine oxidase, and mitochondrial ROS.\textsuperscript{13} Nuclear translocation of Nrf2 after exposure of EC to US has been shown to be dependent on activation of PI3K, protein kinase C, and enhanced ROS generation (Figure 1).\textsuperscript{58} PI3K signaling has been implicated in Nrf2 nuclear translocation via depolymerization of the actin cytoskeleton resulting in translocation of Nrf2 with bound actin.\textsuperscript{63} Moreover, protein kinase C–mediated phosphorylation of serine residues (Ser40) on Nrf2 has shown to prevent its association with kelch-like ECH-associated protein 1 (Keap-1) and provides a possible molecular mechanism for shear-stress–induced Nrf2 activation.\textsuperscript{64} Cultured HUVEC exposed to US have enhanced PI3K/AKT activity, which results in dissociation of Nrf2 from Keap-1 in a NO-independent manner.\textsuperscript{13} It is likely that cysteine residues on Keap-1 are also subject to oxidative modification after exposure of EC to FSS, possibly mediated by 15-deoxy-prostaglandin J2.\textsuperscript{11} The onset of either OS or US can result in the generation of ROS by EC, which is likely to be a major mechanism for the activation of Nrf2 signaling in response to shear as treatment with the antioxidant N-acetylcysteine attenuates this.\textsuperscript{11,42,58} It is also possible that NO can both activate protein kinase C and modify cysteine residues on Keap-1 acting as an additional mechanism to augment Nrf2 activity.

Although nuclear translocation and binding of Nrf2 to the ARE in the promoter regions of target genes are suppressed in EC exposed long term to OS,\textsuperscript{3,11} the exact mechanisms by which this occurs remain to be fully elucidated. Interestingly, Lee et al\textsuperscript{65} have elegantly demonstrated that exposure of HUVEC to OS induces the expression of both class I and II histone deacetylases (HDACs) and their nuclear accumulation in a PI3K/Akt-dependent manner, whereas pulsatile US induced phosphorylation-dependent nuclear export of class II HDACs. Moreover, OS induced the association of HDAC-1/2/3 with Nrf2 and the association of HDAC-3/5/7 with myocyte enhancer factor-2, leading to diminished expression of NQO1 and the transcription factor Klf2. The study provides mechanistic evidence that different classes of HDACs modulate EC responses to different flow patterns and shear stresses and implicate HDAC-3 as an epigenetic factor that may modulate endothelial oxidative and inflammatory responses to disturbed OS through deacetylation of Nrf2, thereby leading to decreases in Nrf2 ARE-binding activity and antioxidant gene induction.\textsuperscript{65,66} Nuclear localization of Nrf2 has been shown to prevent its association with kelch-like ECH-associated protein 1 (Keap-1) and provides a possible molecular mechanism for shear-stress–induced Nrf2 activation.\textsuperscript{64} Cultured HUVEC exposed to US have enhanced PI3K/AKT activity, which results in dissociation of Nrf2 from Keap-1 in a NO-independent manner.\textsuperscript{13}

### Table. Endothelial Nrf2 Activation by Fluid Shear Stress

<table>
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<tr>
<th>Cell Type</th>
<th>FSS Culture Model</th>
<th>Shear Stress Pattern</th>
<th>Time</th>
<th>Key Findings</th>
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<tr>
<td>HUVEC</td>
<td>Cone-and-plate viscometer</td>
<td>US: 1–30 dyn/cm(^2)</td>
<td>24 h</td>
<td>↓ ROS and N0X4/↑ Nrf2 nuclear translocation</td>
<td>47</td>
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<tr>
<td>HUVEC</td>
<td>Dynamic flow system</td>
<td>Atheroprotective and atheroprone flow</td>
<td>24 h</td>
<td>Atheroprotective flow activates Nrf2 via PI3K/Akt leading to ↓ Nrf2/Keap-1 association</td>
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<tr>
<td>HAEC</td>
<td>Parallel plate</td>
<td>US: 10 dyn/cm(^2), OS: ±10 dyn/cm(^2), 1 Hz</td>
<td>6–24 h</td>
<td>Both US and OS cause Nrf2 nuclear translocation, only US ↑ N0Q1 and Nrf2 binding DNA</td>
<td>11</td>
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<tr>
<td>HAEC</td>
<td>Parallel plate</td>
<td>US: 5–20 dyn/cm(^2), OS: ±5 dyn/cm(^2), 1 Hz</td>
<td>48 h</td>
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<td>HUVEC</td>
<td>Diaphragm pump</td>
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<td>US: 12 dyn/cm(^2)</td>
<td>48 h</td>
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<tr>
<td>HUVEC</td>
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<td>US: 12 dyn/cm(^2)</td>
<td>0–2 h</td>
<td>↑ Nuclear Nrf2 and HO-1 and NQO1 after ↑ Klf2</td>
<td>58</td>
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</tbody>
</table>

\textsuperscript{ARE indicates antioxidant response element; GST, glutathione-S-transferase; HAEC, human aortic endothelial cells; HO-1, heme oxygenase-1; HUVEC, human umbilical vein endothelial cells; Keap-1, kelch-like ECH-associated protein 1; Klf2, Krüppel-like factor 2; N0X4, NADPH oxidase 4; NQO1, NADPH quinine oxidoreductase-1; Nrf2, NF-E2-related factor 2; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; OS, oscillatory shear; ROS, reactive oxygen species; and US, unidirectional shear.}
of Nrf2 in the endothelium of mouse aorta has been shown by enface immunofluorescence in atheroprotected regions of the descending aorta exposed to laminar FSS; however, its expression remains predominantly cytoplasmic in the inner curvature of the aortic arch where FSS patterns are oscillatory and disturbed, indicative of diminished ARE-mediated antioxidant gene induction in these atheroprotected regions. In the atheroprotected regions of the vasculature, sustained activation of the mitogen-activated protein kinase (MAPK), p38, and c-Jun N-terminal kinase-1/2 (JNK-1/2) results in enhanced NF-κB and activator protein-1 transcriptional activity, which subsequently promotes a proinflammatory EC phenotype through induction of adhesion molecules such as vascular cell adhesion molecule-1. Activation of the Nrf2 pathway has been shown to negatively regulate the activity of MAPK signaling by enhancing the catalytic activity of MAPK kinase phosphatases by promoting its reduced form and suppressing the activation of the MAPK kinase 3/6, resulting in the reduced activation of p38 MAPK-mediated signaling. Interestingly, treatment of mice with sulforaphane, a dietary isothiocyanate from cruciferous vegetables that interacts with Keap-1 to augment Nrf2 activity, reduced proinflammatory p38 and vascular cell adhesion molecule-1 signaling at atherosusceptible sites in wild-type but not in Nrf2 deficient mice, suggesting that dietary and pharmacological intervention to activate Nrf2 activity may suppress inflammation and atherogenesis in regions of disturbed FSS.

Another well-characterized effect of prolonged exposure of EC to atheroprotective US, but not disturbed atheroprotective OS, is the induction of the transcription factors Klf2 and Klf4. In vivo, atheroprotective regions of the aorta have been shown to be deficient in Klf2 and Klf4, whereas their basal expression is augmented in atheroprotected regions. Klf2 can be considered a master integrator of EC function as out of 74 of the most highly regulated genes when EC are exposed to FSS, 46% of these depended on Klf2 for their upregulation. Induction of Klf2 results in the regulation of endothelial transcriptional control of inflammation, thrombosis/hemostasis, vascular tone, and blood vessel development and is associated with regions of the vasculature that are resistant to atherogenesis. Overexpression of Klf2 in cultured ECs leads to the upregulation of eNOS and the inhibition of NF-κB activity. The induction of Klf2 by US leading to a downregulation of Cav-1 expression is not only likely to augment NO generation by eNOS but also increase the activity of both Nrf2 and HO-1.

The activation of Klf2 by FSS has been shown to involve MAPK kinase/MEK5, leading to the phosphorylation of ERK5 and subsequent activation of myocyte enhancer factor-2, which binds to the Klf2 promoter. Pulsatile US induces Klf2 via the activation of AMP kinase upstream of ERK5 and myocyte enhancer factor-2. ERK5 can be negatively regulated by protein kinase C-ζ, and its activity has been associated with atheroprotective regions of the porcine aortic arch. In a similar pattern to Klf2, the signaling mechanisms regulating Klf4 activity have been shown to be upregulated by FSS in HUVEC and dependent on MEK5/myocyte enhancer factor-2 but independent of ERK5. However, induction of Klf4 by FSS is dependent on ERK5 and MEK5 in human dermal microvascular ECs, possibly because of differences in the shear rates and vascular beds from which the EC were sourced. Knockdown of Klf2 expression by siRNA during long-term exposure of HUVEC to US has been shown to reduce NQO1 but not Nrf2 mRNA levels, suggesting that Klf2 can augment Nrf2/ARE activity, whereas in static cultures, adenosivir overexpression of Klf2 enhances the nuclear translocation of Nrf2 after stimulation with tert-butyl hydroquinone, together suggesting that Klf2 primes Nrf2 nuclear translocation for ARE activation. Klf2 is, thus, an effecter of shear-induced inhibition of the transcriptional activity of proinflammatory factors such as NF-κB, ATF2, c-Jun, and Smad3/4, while it is required for shear stress–mediated Nrf2 activation and concomitant ARE-dependent target gene expression. A summary of the atheroprotic and atheroprotective effects of oscillatory and unidirectional FSS on EC redox signaling is depicted in Figure 1.

We have previously demonstrated that decreased DJ-1 levels and increased phosphorylation of active glycogen synthase kinase-3β expression in HUVEC derived from gestational...
diabetic pregnancies may contribute to deficits in Nrf2 nuclear accumulation and signaling. To date, these Keap-1-independent Nrf2-regulatory pathways have not been fully characterized in EC exposed to FSS. DJ-1 is a cancer and Parkinson disease–associated protein that protects cells from toxic stresses by stabilizing Nrf2 to prevent its association with Keap-1 and subsequent ubiquitination, thereby enhancing ARE gene transcription. Although the effects on FSS on DJ-1 levels in EC remain to be elucidated, the enhanced oxidative stress associated with OS is likely to lead to DJ-1 oxidation and thus reduce its interactions with Nrf2. Moreover, atherosclerotic lesions in low-density lipoprotein receptor–deficient mice exhibit diminished DJ-1 levels, and this may account for reduced Nrf2 activity and ARE-gene induction in atheroprome regions of the aorta exposed to OS. Activated glycogen synthase kinase-3β has been reported to phosphorylate Fyn tyrosine kinase, leading to enhanced nuclear export of Nrf2 and proteasomal degradation via the adaptor protein β-TrCP (β-transducin repeat-containing protein) independent of Keap-1. It is, therefore, possible that ECs exposed to OS have increased glycogen synthase kinase-3β activity, thus contributing to the diminished Nrf2 nuclear accumulation and associated ARE-gene transcription. In this context, platelet EC adhesion molecule-1, a mechanosensitive membrane protein that is upregulated during inflammation, may mediate not only NOX2-derived superoxide generation in response to disturbed FSS but also glycogen synthase kinase-3β activation leading to enhanced EC oxidative stress because of diminished Nrf2 activity. BTB and CNC homology 1 (Bach1) is a Nrf2-regulated transcriptional repressor of a subset of ARE-regulated genes such as HO-1 and thus antagonizes the activator function of Nrf2. Although to date there are no reports directly demonstrating that Bach-1 activity is altered in EC cultured under FSS, it is of note that disruption of Bach-1 in apoE knockout mice has been reported to inhibit oxidative stress and atherogenesis through the upregulation of HO-1 in the endothelium. However, it remains to be established whether increased Bach-1 levels or activity in atheroprome regions of disturbed FSS lead to diminished Nrf2/ARE activity and antioxidant gene expression.

**Shear Stress Regulated Nrf2-Antioxidant Gene Expression**

Because Nrf2 regulates the induction of a battery of antioxidant defense genes in vitro and in vivo have been reported to exhibit enhanced expression of these enzymes when exposed to laminar FSS. HO-1, one of the most widely reported atheroprotective enzymes that is predominantly under the regulation of Nrf2, catalyzes heme degradation forming biliverdin, iron, and the vasodilator carbon monoxide (CO). Biliverdin can be converted to the potent antioxidant bilirubin via biliverdin reductase and together they form an important redox couple and the Fe3+ formed after the cleavage of the heme porphyrin ring can be directly sequestered by ferritin. In addition to scavenging reactive oxygen and nitrogen species in EC, bilirubin and biliverdin can also directly inhibit NADPH oxidase activity, thereby contributing to the actions of HO-1 to sustain vascular homeostasis. Vascular expression of HO-1 in vivo has been shown in atheroprotected regions of the wild-type mouse aortic arch, but is attenuated in these areas in Nrf2-deficient mice. Moreover, induction of HO-1 has been shown to suppress NADPH oxidase activity in ApoE-deficient mice prone to atherosclerosis, mediated partially by enhanced bilirubin production by HO-1. After exposure of EC to FSS in vitro, Nrf2 is known to directly bind the ARE consensus sequence in the HO-1 promoter, thereby mediating its upregulation; however, induction of HO-1 by US in bovine aortic endothelial cells has also been shown to be mediated by generation of mitochondria-derived H2O2. NQO1 is a constitutively expressed phase II detoxifying enzyme involved in the reduction of quinones to hydroquinones using the pyridine nucleotides NADPH or NADH as an electron donor. It is a flavoprotein induced by oxidative stress and prevents redox cycling of quinones to semiquinones by quinone reductases, thereby reducing ROS generation. Apart from being able to reduce a host of pharmacological and physiological quinones, NQO1 can also directly scavenge superoxide, although its interaction with superoxide is an order of magnitude less than the rate of superoxide dismutase, it may function as an important antioxidant enzyme during enhanced oxidative stress. Expression of NQO1 has been shown to be differentially regulated, as EC exposed to US but not to OS, exhibit direct binding of Nrf2 to the NQO1 promoter ARE as demonstrated by chromatin immunoprecipitation.

**Figure 2.** Unifying the shear stress concepts: micro-RNA and redox regulation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Fluid shear stress (FSS) is a potent activator of endothelial nitric oxide synthase (eNOS) resulting in enhanced NO production. Oscillatory FSS enhances reactive oxygen species (ROS) generation by nicotinamide adenine dinucleotide phosphate oxidase (NOX), and xanthine oxidase (XO). Nucleophilic attack of sulfhydryl groups on kelch-like ECH-associated protein 1 (Keap-1) by ROS results in a conformational change to enhance Nrf2 binding. Phosphorylation of Nrf2 by phosphatidylinositol 3-kinase (PI3K)/Akt enhances its nuclear translocation, which enhances expression of protective antioxidant enzymes. NO can also enhance mitochondrial ROS generation resulting in subsequent activation of PI3K/AKT kinases leading to the phosphorylation of Nrf2 and eNOS. Unidirectional FSS enhances Krüppel-like factor (KLF2) activity, which augments Nrf2 induction and eNOS expression while reducing caveolin-1 (Cav-1)-expression. Heat-shock protein 90 (HSP90) and tetrahydrobiopterin (BH4) maintain eNOS in a coupled state to reduce ROS generation. Redoxi-miRs and mechanosensitive micro-RNAs can attenuate expression of antioxidant enzymes and also regulatory mediators of the Nrf2 pathway, eg, BTB and CNC homology 1 (Bach1) by miR-155 and KLF2 by miR-92a. PKA indicates protein kinase A.
The induction of NQO1 mRNA is known to be upregulated in vitro by long-term exposure of EC to unidirectional pulsatile shear for 7 days.\textsuperscript{69} Enhanced oxidative stress-mediated Nrf2/ARE activation, as opposed to nitrosative stress, has been implicated in the upregulation of NQO1 protein by exposure of HUVEC to laminar shear stress.\textsuperscript{60,62} It is also likely that increased HDAC1/2/3 association with Nrf2 and EC redox responses mediated by PI3K/Akt is responsible for diminished NQO1 expression in HUVEC exposed to disturbed FSS patterns because of deacetylation of Nrf2.\textsuperscript{65}

Pxr are an important class of highly conserved thiol antioxidants containing redox active cysteine residues that play essential roles in the reduction of $\text{H}_2\text{O}_2$, peroxynitrite, and hydroperoxides.\textsuperscript{101} The human Pxr-1 promoter contains ARE sequences at the proximal and distal end of its promoter region,\textsuperscript{102} and its FSS-dependent induction in bovine aortic EC has been shown to be upregulated by US but not by OS, thereby reducing oxidative stress and ROS generation in cells exposed to laminar shear\textsuperscript{103} and conferring protection against EC activation and atherosclerosis.\textsuperscript{104} After oxidation of Pxr-1, it can be reduced by thioredoxin-1 (Trx-1) as a part of a redox couple because Trx is a thioldioxidoreductase and functions as major disulfide reductase which can itself be reduced by thioredoxin reductase.\textsuperscript{105} Trx-1 along with glutaredoxins are the main enzymes responsible for maintaining thiois in a reduced state and preventing the formation of inter- or intramolecular disulfides.\textsuperscript{106} Both Trx-1 and thioredoxin reductase are transcriptionally regulated by Nrf2 and have ARE sequences in their promoter regions.\textsuperscript{107} Expression of Trx-1 has been shown to be FSS sensitive in HUVEC, where high US enhances Trx-1 activity via the downregulation of the Trx interacting protein, resulting in resistance to EC inflammation in response to tumor necrosis factor-$\alpha$.\textsuperscript{108} It is likely that augmented Nrf2 activity induced by FSS contributes to Trx-mediated EC redox homeostasis and its atheroprotective properties.\textsuperscript{109,110}

Regulation of Nrf2 by Micro-RNAs in Endothelial Responses to Shear Stress

Micro-RNAs regulate gene expression by modulating the translation and stability of target mRNAs and they have now become an integral component in our understanding of post-transcriptional mechanisms regulating gene expression.\textsuperscript{111} The expression of micro-RNAs can be altered by cellular redox environment to provide a complex additional layer of micromanagement of cellular redox status.\textsuperscript{112} It has been reported in a variety of cell types that redox-sensitive micro-RNAs (redox-miRs) can modulate levels of Nrf2 and the regulators of Nrf2 signaling, ARE-associated antioxidant gene expression, and the enzymes involved in ROS generation.\textsuperscript{56,113} Given that FSS patterns are major physiological and pathophysiological stimuli that induce or suppress an array of pro- and anti-inflammatory genes in EC, it is likely that mechanosensitive micro-RNAs are involved in the regulation of endothelial redox and inflammatory phenotype.\textsuperscript{114,115} Micro-RNA expression profiles have been shown to be differentially regulated in EC exposed to laminar and oscillatory FSS patterns,\textsuperscript{116-118} leading to alterations in both pro- and anti-inflammatory gene expression, and thus may constitute an additional factor that contributes to atherogenesis.\textsuperscript{119} For example, miR-92a has been shown to be downregulated in HUVEC exposed to US leading to enhanced Klf2 levels and hence expression of eNOS.\textsuperscript{120} Enhanced levels of miR-92a have been demonstrated in atheroprotected areas of the porcine aortic arch where Klf2 and Klf4 expressions were lower than in atheroprotected areas.\textsuperscript{121} It is of interest that suppression of miR-92a levels in HUVEC with anti-miRs led to diminished EC inflammation in response to low FSS and oxidized low-density lipoprotein stimulation and prevented monocyte adhesion, whereas blockade of miR-92a using an antago-mir stabilizes atherosclerotic lesions and reverses EC dysfunction in low-density lipoprotein receptor deficient mice.\textsuperscript{122} Although Nrf2 signaling was not directly assessed in these studies, it is likely that the induction of Klf2 and augmented eNOS activity reported would, in turn, enhance ARE-linked gene transcription.\textsuperscript{59} Moreover, miR-155 has been demonstrated in HUVEC to be both mechanosensitive to FSS\textsuperscript{123} and also a modulator of Nrf2/ARE activity through the suppression of Bach-1 levels,\textsuperscript{124} leading to enhanced HO-1 expression in response to tumor necrosis factor-$\alpha$. In mouse aortas, miR-155 expression has been shown to be increased in the intima of thoracic aorta exposed to US patterns when compared with the intima of the lower curvature of the aortic arch associated with oscillatory and low FSS.\textsuperscript{125} In this study, the effects of miR-155 in vitro were shown to be mediated through suppression of RhoA and myosin light chain kinase, regulators of EC cytoskeleton organization.

Interestingly, miR-143 and miR-145 secreted by HUVEC exposed to US have been shown to target gene expression and regulate the phenotype in cocultured SMCs.\textsuperscript{124} In this study, extracellular vesicles containing miR-143 and miR-145 derived from Klf2 overexpressing EC reduced atherosclerotic lesion formation in the aorta of apoE-deficient mice. However, it remains to be elucidated whether mechanosensitive redoxi-miRs can be transferred from EC to SMC within exosomes or microvesicles\textsuperscript{125} to modulate redox status by either Nrf2 activation or antagonism. In this context, it is of note that miR-143 has been shown to promote downregulation of miR-21, which has been implicated in promoting a synthetic SMC phenotype.\textsuperscript{126} ROS generated by NADPH oxidase has been recently reported to be essential for the expression and function of miR-21 in prostate cancer cells\textsuperscript{127}; therefore, miR-143 expression is likely to affect SMC phenotype through increased miR-21 expression, resulting in reduced contractile gene expression in SMC that may involve altered redox signaling. In addition, expression of miR-145 in a model of murine myocardial ischemia/reperfusion or $\text{H}_2\text{O}_2$-treated neonatal rat cardiac myocytes in culture was markedly downregulated and overexpression of miR-145 significantly inhibited $\text{H}_2\text{O}_2$-induced cellular apoptosis and ROS production.\textsuperscript{128} Therefore, micro-RNAs such as miR-21, miR-92a, miR-143, miR-145, and miR-155 are likely to provide an additional level of biomechanical regulation by which vascular Nrf2 activity may be augmented by FSS to confer protection against atherosclerosis in regions of laminar shear. Additional studies are necessary for the identification of mechanosensitive redoxi-miRs and elucidation of the mechanisms by which they regulate Nrf2/ARE signaling and antioxidant gene expression in EC exposed to FSS to fully assess the potential of micro-RNAs as therapeutic targets to
prevent or treat atherosclerosis. The evidence linking Nrf2 signaling to FSS patterns is unequivocal and mediated by a change in EC redox status, resulting in enhanced antioxidant gene induction thereby maintaining redox homeostasis. The likely regulation of key mediators of the Nrf2 pathway by redoxi-miRs and mechanosensitive micro-RNAs, as well as potentially long noncoding RNAs, add an additional dimension to provide fine tuning of EC redox signaling (Figure 2).

Summary and Future Perspectives
The effects of FSS on endothelial gene expression and the functional consequences on atherogenesis have been previously reviewed in depth. The aim of this review is to provide a brief summary of the literature with a focus on the potential of shear stress as a modulator of vascular Nrf2/ARE signaling and its subsequent regulation of antioxidant gene expression and changes in EC redox phenotype, leading either to protection from or susceptibility to atherosclerosis as summarized in Figure 1. Although the Nrf2 pathway is considered to be a master regulator of redox signaling, it is of importance to consider other major transcriptional networks commonly associated with mechanotransduction in atherogenesis to more accurately determine the integrated intercellular and intracellular responses to changes in FSS. These pathways include Klf2, Klf4, NF-xB and activator protein-1, which together with Nrf2, are likely to account for regulation of >85% of the shear stress-modulated EC genes. A major obstacle in this field is the integration of data on EC responses to shear stress with different flow patterns at the molecular level and the global scale to identify FSS-specific endothelial redox gene expression and phenotype. Most in vitro studies use different FSS models, often involving EC cultured in static conditions that have not been sufficiently preadapted to relevant shear forces, before assessment of changes in gene expression. To date few studies have investigated pulsatile unidirectional FSS patterns to fully elucidate the mechanisms by which physiological FSS maintains redox balance. Moreover, the predominant cell type used in FSS studies is often HUVEC which do not experience high pressure and shear or oscillatory flow patterns in vivo, and hence the interpretation of data from these studies should be treated with caution unless correlated with observations in a more relevant cell type to assess mechanobiology (eg, human aortic, carotid or coronary artery EC), as well as in vivo data. Further investigation of genomic, epigenomic, translational, and post-translational regulation of Nrf2 in atheroprotected and atheroprone regions of arteries will enable the identification of key regulatory networks that have a direct relevance to endothelial function in health and disease to develop pharmacological and dietary strategies to reduce the incidence and progression of atherosclerosis. Endothelial redox and inflammatory phenotypes are likely to be heterogeneous over different vascular regions; therefore, further assessment of both spatial and temporal changes in FSS activity is also required. Moreover, the interaction between mechanical (eg, FSS and vessel wall stretch), circulating (eg, stem cells, leukocytes, hormones, and cytokines), biochemical (eg, lipids, cholesterol, glucose, antioxidants, and proteins), and molecular (eg, micro-RNAs and long noncoding RNAs) factors in mediating atherogenesis remain to be determined.

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Nrf2 as an Endothelial Mechano-sensitive Transcription Factor: Going With the Flow
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