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Mechanisms underlying unidirectional laminar shear stress-mediated Nrf2 activation in endothelial cells: Amplification of low shear stress signaling by primary cilia

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ABSTRACT

Endothelial cells are sensitive to mechanical stress and respond differently to oscillatory flow *versus* unidirectional flow. This review highlights the mechanisms by which a wide range of unidirectional laminar shear stress induces activation of the redox sensitive antioxidant transcription factor nuclear factor-E2-related factor 2 (Nrf2) in cultured endothelial cells. We propose that fibroblast growth factor-2 (FGF-2), brain-derived neurotrophic factor (BDNF) and 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) are potential Nrf2 activators induced by laminar shear stress. Shear stress-dependent secretion of FGF-2 and its receptor-mediated signaling is tightly controlled, requiring neutrophil elastase released by shear stress, αvβ3 integrin and the cell surface glycocalyx. We speculate that primary cilia respond to low laminar shear stress (<10 dyn/cm^2), resulting in secretion of insulin-like growth factor 1 (IGF-1), which facilitates αvβ3 integrin-dependent FGF-2 secretion. Shear stress induces generation of heparan-binding epidermal growth factor-like growth factor (HB-EGF), which contributes to FGF-2 secretion and gene expression. Furthermore, HB-EGF signaling modulates FGF-2-mediated NADPH oxidase 1 activation that favors casein kinase 2 (CK2)-mediated phosphorylation/activation of Nrf2 associated with caveolin 1 in caveolae. Higher shear stress (>15 dyn/cm^2) induces vesicular exocytosis of BDNF from endothelial cells, and we propose that BDNF via the $p75^{NTR}$ receptor could induce CK2-mediated Nrf2 activation. Unidirectional laminar shear stress upregulates gene expression of FGF-2 and BDNF and generation of 15d-PGJ_2, which cooperate in sustaining Nrf2 activation to protect endothelial cells against oxidative damage.

1. Introduction

In the vascular system, endothelial cells lining the inner surface of blood vessels are subjected to a variety of physical stresses including stretch and shear stress. Endothelial cells transduce these mechanical stimuli into sequential biochemical reactions to release vasoactive mediators to control the tone of the underlying vascular smooth muscle and remodeling of vascular structures depending on the flow rate and type (reviewed in Refs. [1–6]).

Although most of the circulation is exposed to pulsatile laminar flow, disturbed or oscillatory flow can occur at bifurcations, especially in large arteries where vessels curve significantly. These regions are exposed to non-uniform shear stresses, which may be associated with vessel wall injury and are prone to the development of early atherosclerotic lesions

Abbreviations: AMPK, AMP-activated protein kinase; BDNF, brain-derived neurotrophic factor; Cav1, caveolin-1; CIC-3, chloride channel 3; CK2, casein kinase 2; Cx43, connexin 43; 15d-PGJ_2, 15-Deoxy-$\Delta^{12\ 14}$-prostaglandin J_2; EGFR, epidermal growth factor receptor; eNOS, endothelial nitric oxide synthase; FGF-2, fibroblast growth factor-2; HB-EGF, heparan-binding epidermal growth factor-like growth factor; HO-1, heme oxygenase 1; HUVEC, human umbilical vein endothelial cells; IGF-1, insulin-like growth factor-1; Keap1, Kelch-like ECH-associated protein 1; KLF2, Krüppel-like factor 2; miR, microRNA; MMP, matrix metalloproteinase; Mrp1, multidrug resistance protein 1; NAD^+, nicotine amido adenine dinucleotide; NF-κB, nuclear factor κ-light chain enhancer of activated B cells; NOXA1, NOX activator 1; NOX1, NADPH oxidase 1; NOXO1, NOX organizer 1; Nrf2, nuclear factor-E2-related factor 2; nSMase2, neutral sphingomyelinase 2; OSS, oscillatory shear stress; PAR1, proteinase-activated receptor-1; PGD_2, prostaglandin D_2; PSA-NCAM, polysialylated neural cell adhesion molecule; RyR, ryanodine receptor; USS, unidirectional shear stress; XD, xanthine dehydrogenase; XO, xanthine oxidase; VEGF, vascular endothelial growth factor.

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(reviewed in Refs. [7–9]). To study the molecular mechanisms of cellular responses to mechanical stress, endothelial cells cultured in flow-controlled devices have been developed. Previous studies have established that cultured endothelial cells respond differentially to oscillatory or turbulent nonunidirectional flow *versus* steady or pulsatile unidirectional flow. Oscillatory shear stress (OSS) induces pathophysiological effects such as activation of transforming growth factor β1 (TGF-β1) and the inflammatory gene regulator NF-κB [10–12].

In contrast, unidirectional shear stress (USS) induces cell alignment and anti-atherogenic responses, such as stable activation of endothelial nitric oxide synthase (eNOS) [13–16] and the transcription factors nuclear factor-E2-related factor 2 (Nrf2) [17–27] (Table 1) and Krüppel--like factor 2 (KLF2) [28,29]. Nrf2 serves as a master regulator in the protection of cells against oxidative stress by upregulating the expression of detoxification enzymes and antioxidant enzymes and proteins [30–33]. Moreover, Nrf2 plays a key role in USS -mediated anti-atherogenic responses in cultured endothelial cells [34]. The transcription factor KLF2 is another important mediator of anti-inflammatory and anti-thrombotic properties in the endothelium (reviewed in Refs. [35,36]). Nrf2 and KLF2 are thus key transcription factors regulating the expression of large number of USS-induced atheroprotective genes [23].

This review aims to provide novel insights into the underlying signaling cascades triggered by USS in cultured endothelial cells, leading to stable activation of Nrf2. Previous studies using different types of flow-controlled devices revealed that wide range of USS from 0.2 to 75 dyn/cm^2 can induce activation of Nrf2 in cultured endothelial cells, suggesting that multiple stress sensors cover this wide range of USS. As summarized in Table 1, USS-mediated Nrf2 activation is influenced by other factors including NADPH oxidase (NOX), xanthine oxidase (XO), protease-activated receptor-1 (PAR-1) and the cell surface glycocalyx. Previous studies have established that USS induces secretion of fibroblast growth factor-2 (FGF-2) [37,38], brain-derived neurotrophic factor (BDNF) [39] and generation of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) [19] in endothelial cells. We postulate that these three factors are directly involved in the activation of Nrf2 by different mechanisms. As FGF-2 receptor signaling can activate NADPH oxidase 1 (NOX1) in smooth muscle cells [40] and Nrf2 via phosphorylation of casein kinase 2 (CK2) in cardiomyocytes [41,42], we postulate that FGF-2-mediated activation of NOX1/CK2 signaling leads to Nrf2 activation/phosphorylation in endothelial cells.

Recent studies indicate that endothelial cells express brain-derived neurotrophic factor (BDNF) [39,43]. In addition to its neuropoietic actions, BDNF promotes endothelial cell survival and induces neoangiogenesis in ischemic tissues [44]. Exposure of endothelial cells to high USS (14 dyn/cm^2), but not low USS (2 dyn/cm^2), stimulates BDNF production and secretion [39]. We previously proposed that BDNF via the pan-neurotrophin receptor $p75^{NTR}$ leads to activation of Nrf2 in astrocytes [45]. In addition to FGF-2 and BDNF, USS also induces generation of the electrophilic prostaglandin 15d-PGJ_2 [19], which can stabilize Nrf2 in a Keap1-dependent manner [46,47]. Under normal conditions, Keap1 facilitates degradation of Nrf2 and electrophiles react with cysteine residues of Keap1, inducing stabilization of Nrf2 [48,49]. Key questions are how USS signals are transduced into cellular biochemical responses such as secretion, receptor signaling and synthesis of FGF-2 and BDNF, and generation of 15d-PGJ_2, respectively. Notably, FGF-2 and BDNF are able to activate both Nrf2 and NF-κB dependent on differential receptor-mediated signaling pathways via as yet unresolved mechanisms. Unveiling the mechanisms of selective activation of Nrf2 by these bioactive protein factors in endothelial cells in relation to USS is a key focus of this review.

Table 1
Unidirectional laminar shear stress dependent activation of Nrf2 in endothelial cells.

Cell type	Shear stress	Key findings	Refs
Human aortic and microvascular EC	Steady, 20 dyn/cm^2 for 48 h	ARE/Nrf2 mediated gene activation	[17]
HUVEC	Steady, 0.2–2 dyn/cm^2 for 4–24 h	XO/XD- and NOX-dependent	[18, 20]
Human aortic EC	Steady, 10 dyn/cm^2 for 6–24 h	Partial contribution of 15d-PGJ_2	[19]
HUVEC	Steady, 10 dyn/cm^2 for 16 h	Superoxide radical dependent	[21]
HUVEC	Steady, 10 dyn/cm^2 for 24 h	PI3-kinase/Akt pathway dependent	[22]
HUVEC	Pulsatile, 12 ± 7 dyn/cm^2 for 24 h	Krüppel-like factor 2 cooperates with Nrf2	[23]
HUVEC	Steady, 15 and 75 dyn/cm^2 for 24 h	Nrf2 activation at 75 dyn/cm^2	[24]
HUVEC	Steady, 12 dyn/cm^2 for 24 h	PAR-1 knockdown diminishes Nrf2 activation	[25]
HUVEC	Steady, 15 dyn/cm^2 for 24–48 h	Neuraminidase treatment diminishes Nrf2 activation	[26]
Mouse glomerular microvascular EC	Steady, 5 dyn/cm^2 for 7 d	Low shear stress upregulates HO-1 and NQO1 expression in glomerular EC	[27]

Abbreviations: HUVEC, human umbilical vein endothelial cells; ARE, antioxidant response element; PAR1, proteinase-activated receptor-1; NQO1, quinone oxidoreductase 1.

2. Overview of shear stress-mediated signal transduction

Endothelial cells sense physical stresses through multiple sensor networks controlling the release of relaxing and contracting factors to adapt to different stresses (reviewed in Refs. [1,50]). Physical stress to the endothelial cell surface also causes structural changes in the cytoskeleton such as intermediate filaments and the actin network (reviewed in Refs. [51,52]). Changes in cytoskeletal structure will activate cell surface mechanosensitive cation channels mediating Ca^{2+} influx into the cytoplasm (reviewed in Refs. [53–56]). Shear stress-dependent Ca^{2+} influx through mechanosensitive transient receptor potential V4 (TRPV4) cation channels mediates rapid activation of eNOS and generates NO in endothelial cells [56,57]. A recent study further shows that shear stress initially activates another mechanosensitive cation channel Piezo1, which in turn causes TRPV4 opening for the sustained phase of calcium elevation [58]. Thus, Ca^{2+} signaling mediated by various ion channels remains a topic of interest in the field of mechanotransduction.

Release of ATP is also known as an important early event associated with flow-induced shear stress [59]. Interestingly, USS rapidly increases the mitochondrial membrane potential [60] and activates mitochondrial oxidative phosphorylation to increase ATP synthesis and subsequent ATP release [61]. Release of ATP from endothelial cells is partly mediated through vesicular exocytosis [62] and connexin 43 (Cx43) hemichannels [63]. Although Cx43 is important for intercellular gap junction formation, it forms uncoupled hemichannels and functions as a mechanosensitive ATP-release channel in a variety of different cell types [64–68]. The gating of Cx43 hemichannels releases not only ATP but also other small signaling molecules such as NAD^+ and prostaglandins in cell type- and metabolism-dependent manners [66–70]. USS-dependent opening of Cx43 hemichannels is an important early step in mechanotransduction in osteocytes and chondrocytes [66–70]. However, the role of Cx43 hemichannels in USS signal transduction in endothelial cells remains to be studied. Released ATP interacts with P2Y and P2X receptors, inducing various cellular responses. One of the ATP/$P2Y_2$ responses is the activation of intercellular junctional platelet endothelial cell adhesion molecule 1 (PECAM-1) to increase cell-cell contact [71–73] and Akt-mediated eNOS phosphorylation/activation [74].

Considering signal transduction induced by fluid shear stress, secreted small signal molecules could easily be diluted and carried away from the sites of secretion. Therefore, secretion of signal transduction molecules most likely occurs at specific cell surface compartments,

where the diffusion of signal mediators is limited and their cell surface receptors and effectors are concentrated. Caveolae are approximately 50–100 nm membrane micro-invaginations associated with plasma membrane and are enriched in glycosphingolipid, cholesterol, sphingomyelin, and lipid-anchored membrane proteins [75,76]. Caveolae contain a marker protein caveolin and a variety of signal transduction molecules forming unique compartments for exporting molecules to the extracellular space and for endocytosis of molecules (reviewed in Refs. [77–79]). Caveolae are particularly abundant in vascular endothelium and are implicated in various types of signal transduction [80–83] (Fig. 1 A). For example, shear stress/TRPV4/Ca^{2+}/calmodulin axis-mediated eNOS activation occurs in caveolae [84,85].

Another cell surface semi-closed compartment important for signal transduction is the ciliary pocket which is an invagination of the peri-ciliary membrane, and a cellular hotspot for endocytosis and exocytosis of vesicles derived from or destined to the ciliary compartment (reviewed in Refs. [86,87] (Fig. 1 A). The ciliary pocket is important for cilia-mediated signal transduction, but its structure and function in endothelial cells remain to be studied. Non-motile primary cilia are microtubule-based organelles that protrude from the cell surface of many mammalian cells including endothelial cells. Primary cilia sense and amplify shear stress with their long rod-like structure and converting these signals into biochemical responses [88–91]. Growth arrested endothelial cells in low serum containing culture media have increased ciliated cells [92]. Ciliogenesis is also enhanced by OSS but downregulated by high USS [93,94].

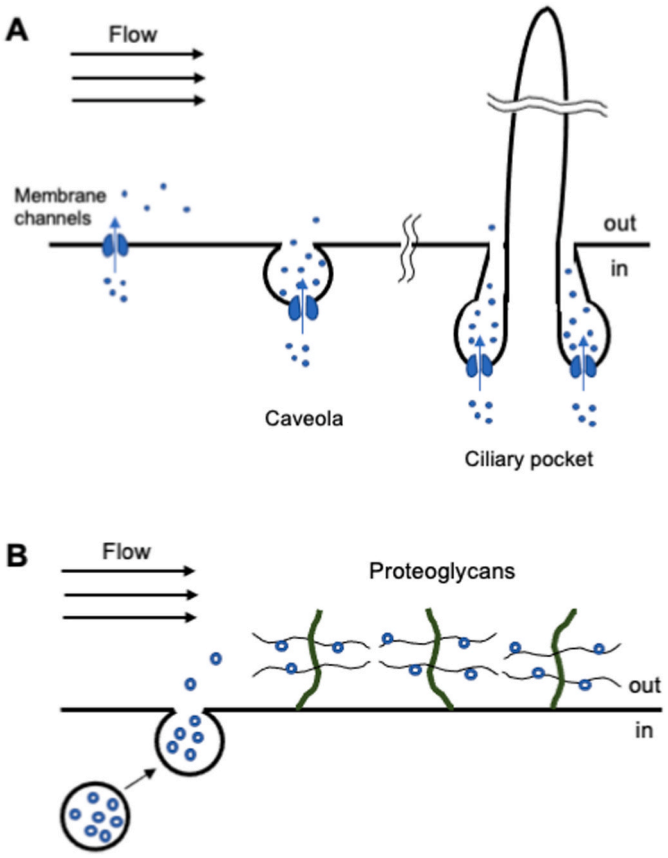

Fig. 1. Important roles of semi-closed compartments and cell surface glycocalyx proteoglycans for signal transduction under fluid shear stress. ***A,*** Small molecules including ATP secreted by endothelial cells through membrane channels are easily diluted by flow, yet cell surface caveolae and ciliary pockets around primary cilia provide semi-closed spaces for signal transduction mediated by these secreted signaling molecules. Importantly, receptors and effectors for signaling molecules are enriched in caveolae and ciliary pockets to facilitate signal transduction. ***B,*** Cell surface proteoglycans provide docking sites for secreted and circulating proteins to facilitate their interaction with their receptors and effectors on cell surface. In particular, heparan sulfate (HS) side chains and polysialylated proteoglycans are important for interaction with FGF-2 and other growth factors.

Previous studies revealed the importance of the endothelial glycocalyx in mechanotransduction of fluid shear stress [95–97]. One of the functions of the cell surface glycocalyx is to trap secreted or circulating proteins and metabolites to facilitate their interaction with their cell surface receptors and targets (reviewed in Ref. [98]) (Fig. 1 B). Cell surface heparan sulfate (HS) side chains of proteoglycans bind FGF-2 and serve as a storage depot for the secreted FGF-2 [99] and furthermore HS side chains interact with FGF-2 receptors modulating receptor-mediated FGF-2 signaling [100–102]. Interestingly, exposure of endothelial cells to USS (15 dyn/cm^2) *in vitro* induces translocation of HS containing glypican-1 to the cell periphery where glypican-1 clusters within the first 30 min [103]. Glypican-1, localized in lipid rafts, controls activation of eNOS by USS [104]. Moreover, treatment of endothelial cells with heparinase III to remove HS chains and/or siRNA to downregulate glypican-1 expression diminishes USS-mediated eNOS activation [105]. Moreover, glypican-1 contributes PECAM-1-mediated eNOS activation [106]. Polysialylated neural cell adhesion molecule (PAS-NCAM) isoforms also retain FGF-2 and facilitate oligomerization of FGF-2 and signaling by interacting with the FGF-2/HS/FGF receptor complex in fibroblasts [107]. PAS-NCAM also binds BDNF and interacts with BDNF receptors in fibroblasts and neuroblastoma cells [107,108], and further studies are required to confirm this signaling cascade in endothelial cells.

Psefteli et al. [26] recently showed that treatment of endothelial cells with neuraminidase to remove sialic acids attenuated USS-dependent Nrf2 activation, suggesting the importance of cell surface sialic acids for Nrf2 activation. Cell surface CD44 also plays an important role in shear stress signal transduction. The CD44 external domain exhibits extracellular matrix adhesion properties by binding hyaluronan (HA), collagen and fibronectin, and the cytoplasmic domain interacts with the cytoskeleton [109,110]. HA is a linear high molecular weight polysaccharide and an important constituent of the endothelial glycocalyx (reviewed in Refs. [111–113]) and HA/CD44 signaling induces angiogenesis and growth and tube formation of endothelial cells [113–116]. Alternatively spliced variants of CD44, that contain exon v3, display both biochemical and functional characteristics of HS proteoglycans [117], as the v3 exon contains Ser-Gly repeats that support covalent attachment of high molecular weight HS side chains [117,118]. CD44 v3 variants (CD44v3) bind growth factors including FGF-2 and BDNF, and are expressed in endothelial cells [119].

3. Potential Nrf2 activators induced by a wide-range of USS

Previous studies suggest multiple factors and signaling pathways interact to support stable activation of Nrf2 in response to a wide range of USS in endothelial cells (Table 1). It is assumed that the average wall shear stress is ~15 dyn/cm^2 for the arterial circulation and 1–6 dyn/cm^2 for the venous circulation [120,121]. We propose three factors, FGF-2, BDNF and 15d-PGJ_2, are potential activators of Nrf2 in endothelial cells exposed to USS (Fig. 2). To elucidate the mechanism of USS-induced signal transduction, we aim to review multiple steps and differential signaling pathways involved in modulating FGF-2 and BDNF secretion and receptor signaling, and generation of 15d-PGJ_2. USS-mediated ATP release from endothelial cells seems to play a key role in the early step of signal transduction, although the precise mechanism of ATP release remains unknown (Fig. 2).

Hennig et al. [38] observed that USS (16 dyn/cm^2) induced secretion of elastase from cultured endothelial cells, with elastase inducing endothelial secretion of FGF-2 [37]. FGF-2 plays an important role in proliferation, differentiation, and survival of many cells and contributes blood vessel growth, tissue vascularization and wound healing (reviewed in Refs. [122–124]). Moreover, USS induces gene expression

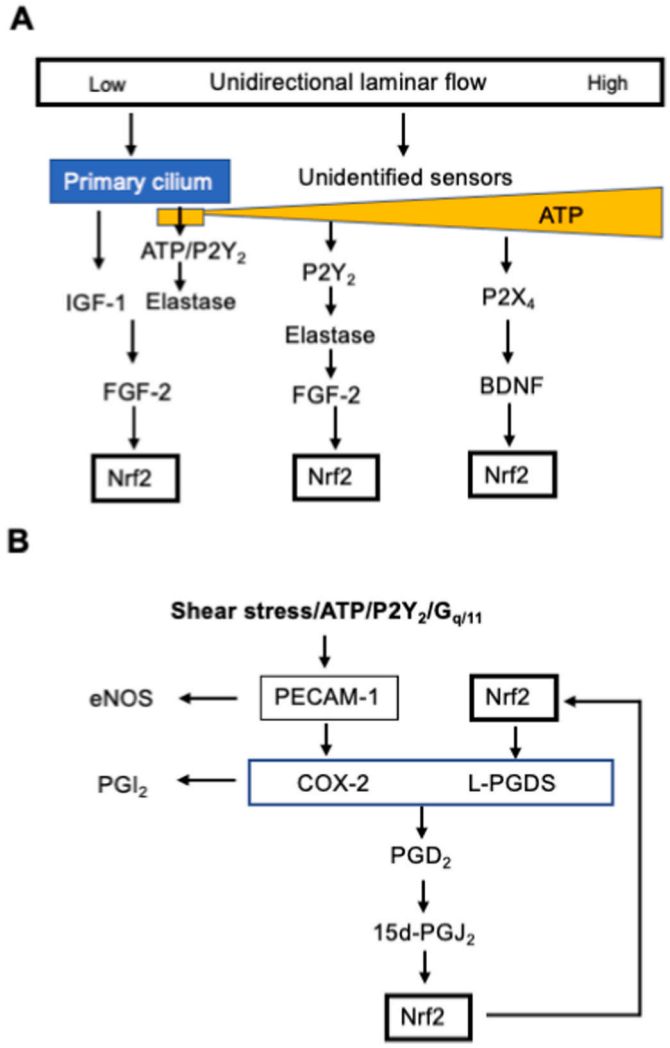

Fig. 2. Proposed scheme of laminar shear stress-dependent activation of Nrf2 in endothelial cells. ***A,*** Unidirectional low laminar shear stress induces cilia-mediated secretion of IGF-1 and elastase, which leads to FGF-2-mediated Nrf2 activation. Higher shear stress is detected by unidentified sensors inducing ATP secretion. ATP induces secretion of elastase, which controls FGF-2 secretion. High shear stress induces ATP-mediated secretion of BDNF, which activates Nrf2 in endothelial cells. ***B***, Shear stress/ATP/$P2Y_2$ signaling induces PECAM-1 activation, which results in COX-2 expression to stimulate prostaglandin synthesis. Nrf2 can induce lipocalin-type PGD synthase (L-PGDS) expression and generation of 15d-PGJ_2. Nrf2/L-PGDS/15d-PGJ_2/Nrf2 signaling composes a positive feedback loop.

of FGF-2 [125]. USS-mediated FGF-2 secretion, gene expression and receptor signaling are tightly controlled processes requiring many factors. Interestingly, the release of elastase containing primary granules from human neutrophils is induced by extracellular ATP via its G-protein-associated $P2Y_2$ receptor [126]. In the activated neutrophils, the EC_{50} for elastase release is 6.7 μM for ATP [126]. Endothelial cells express $P2Y_2$ receptor, and USS-induced $P2Y_2$ receptor activation is important for cell alignment and formation of actin stress fibers [127]. We speculate that USS-mediated ATP/$P2Y_2$ signaling may also control elastase release in endothelial cells (Fig. 2 A). In addition to facilitating secretion of FGF-2, elastase also contributes in FGF-2 gene expression and FGF-2 receptor-mediated signaling as discussed in Sections 7 and 8, with FGF-2-mediated Nrf2 activation discussed in Section 9.

Low USS-dependent activation of Nrf2 is perhaps the most complicated mechanism of signal transduction. Using human umbilical vein endothelial cells (HUVEC), Warabi et al. [18,20] showed that lower rates of USS (0.2–2 dyn/cm^2) induce Nrf2-dependent upregulation of heme oxygenase-1 (HO-1) mRNA over 4–24 h. Mouse glomerular microvascular endothelial cells, which play an important role in glomerular barrier functions [128], also respond to low USS (5 dyn/cm^2) to induce Nrf2 activation [27]. These authors observed Nrf2 mediated upregulation of HO-1 and NAD(P)H:quinone oxidoreductase 1 (NQO1) after 7 days in the cells exposed to steady low USS, suggesting low USS induces long lasting Nrf2 activation in the cells [27]. Interestingly, low USS (5 dyn/cm^2) but not high USS (15 dyn/cm^2) induces secretion of IGF-1 and gene expression of IGF-1 in endothelial cells [129]. Based on these facts, we propose that stress sensor primary cilia amplify low USS enhancing FGF-2 secretion via IGF-1 release and ATP-mediated elastase secretion (Fig. 2 A). This scheme, however, raises an important question, how do primary cilia sense differences between unidirectional and oscillatory flow, and how do they regulate secretion of IGF-1 in endothelial cells? These topics are reviewed in Sections 4 and 5.

Another candidate for USS-mediated Nrf2 activation is BDNF, which can be secreted by endothelial cells in response to high USS [39]. The precise mechanism of BDNF secretion by USS in endothelial cells is not known, but ATP/$P2X_4$ receptor-mediated BDNF release is well known in microglia [130,131]. In endothelial cells, USS induces ATP release and activates Ca^{2+} influx through $P2X_4$ purinoceptors [132,133]. These authors further showed that the highly concentrated ATP release occurs at Cav1-rich regions, presumably caveolae, of the cell membrane. Chemiluminescence imaging methods established that released ATP could reach ~10 and 30 μM in response to USS of 10 and 40 dyn/cm^2, respectively [134]. These results suggest that high USS induces local ATP/$P2X_4$ receptor signaling to induce Ca^{2+} influx, potentially leading to BDNF secretion by endothelial cells. Notably, vascular BDNF protein levels are higher in rat artery than in vein, while BDNF mRNA levels do not differ significantly between vessels [135]. We propose that BDNF contributes to Nrf2 activation in endothelial cells exposed to high USS (>15 dyn/cm^2) through a $p75^{NTR}$-mediated signaling pathway (see Section 10). Notably, cell surface PAS-NCAM associates not only with FGF-2 but also with BDNF, inhibiting degradation of BDNF [136] and enhancing BDNF signaling [108]. Taken together, we propose that endothelial cells sense high USS (>15 dyn/cm^2) via sensor systems (other than cilia) to release elastase via an ATP-dependent manner leading to FGF-2-dependent Nrf2 activation. BDNF released in response to high USS may in turn cooperate with FGF-2 in the activation of Nrf2 (Fig. 2 A).

Interestingly, exposure of endothelial cells to USS in the physiological range (10 dyn/cm^2) induces synthesis of 15d-PGJ_2 [19], a strong activator/stabilizer of Nrf2 [46,47,137]. Synthesis of 15d-PGJ_2 depends on the upregulation of cyclooxygenase-2 (COX-2), which peaks at 4–6 h following USS exposure [138–140]. COX-2 is a key enzyme for prostaglandin synthesis from arachidonic acid. Russell-Puleri et al. [141] showed that the USS/PECAM-1 signaling induces PI3–K/focal adhesion kinase/p38 MAPK-mediated activation of COX-2 gene expression and release of prostaglandin PGI_2, which is an important antiatherogenic prostanoid and vasodilator. USS also induces gene expression of lipocalin-type prostaglandin D_2 synthase (L-PGDS) partly via Nrf2 [142]. PGD_2 is metabolized to 15d-PGJ_2 [143]. Hosoya et al. [19] observed an increase in PGD_2 (6–24 h) and 15d-PGJ_2 (24 h) levels in culture medium following exposure of endothelial cells to constant USS (10 dyn/cm^2), suggesting that once Nrf2 is activated by FGF-2 and/or BDNF, an autocrine Nrf2/L-PGDS/PGD_2/15d-PGJ_2/Nrf2 positive feedback loop may contribute to maintain Nrf2 activation under sustained USS (Fig. 2 B).

4. How do cilia sense differences between OSS and USS?

Primary cilia are shear stress sensors and act as mechano-signal amplifiers [89,90]. Endothelial cells express a nonmotile primary cilia on their surface [89,92]. The common basic structure of the primary

cilium, termed axoneme, is composed of the "9 + 0" microtubule fibers with a diameter of ~200 nm and several μm in length that emerges from the basal body, a structure derived from the mother centriole of the centrosome [144]. A daughter centriole stays aside the mother basal body forming an asymmetrical structure [145] (Fig. 3 A). The basal body of primary cilium is not located just underneath the plasma membrane but is connected to the actin cytoskeleton [146] and is located near the nucleus in the center of the condensed Golgi apparatus, which is important for dynamic control of ciliogenesis [92]. The cilium length is important for the control of cilium-mediated flow sensing and signal transduction, and the actin cytoskeleton around the basal body is involved in the dynamic control of ciliogenesis. Actin polymerization through activation of RhoA induces cilia shortening whereas depolymerization promotes ciliogenesis [147–150]. The axoneme is covered by a lipid bilayer membrane that is continuous with the plasma membrane of the cell body, but enriched in specific lipids, ion channels and receptors that endow the organelle with unique signaling properties [151]. The cilioplasm is separated by a physical diffusion barrier from cytoplasm forming a separate signaling compartment [152] (see Fig. 3 A). As the centriole plays an important role during cell division, cilium formation is restricted to quiescent cells. In the case of confluent HUVEC cultured in endothelial basal medium containing 2% (v/v) fetal bovine serum, approximately 60% of cells extend a primary cilium [92]. In addition to growth stimulation, ciliogenesis is also downregulated under high USS (~15 dyn/cm^2), while OSS enhances primary cilia assembly [93]. For instance, when HUVEC are cultured in a medium containing high serum (24%) supplemented with vascular endothelial growth factor (VEGF) and FGF-2, only about 8% of cells express a primary cilium, and furthermore exposure of the cells to high USS (~15 dyn/cm^2) disassembled cilia [94]. In summary, primary cilia sense most effectively low USS and OSS.

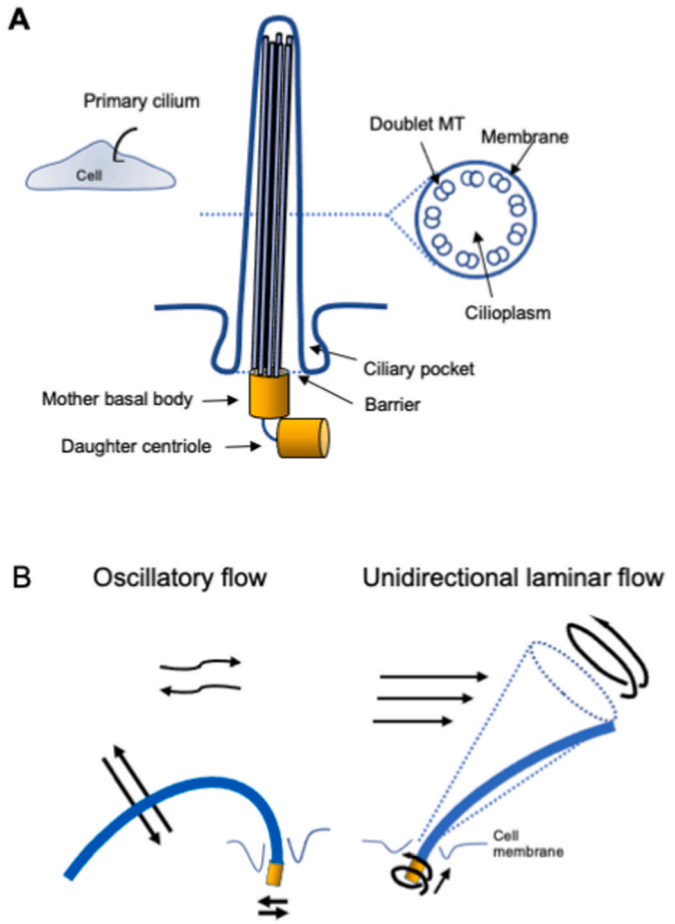

Fig. 3. Structure of primary cilium and its response to flow stress. *A*, Basic skeletal structure of non-motile primary cilium is composed of the "9 + 0" microtubule fibers with a diameter of ~200 nm and several μm in length that emerges from the basal body, a structure derived from the mother centriole of the centrosome. A daughter centriole stays aside the mother basal body forming an asymmetrical structure (modified from Refs. [103,104]). ***B***, Oscillatory flow bends the ciliary rod to cause bending stress on the rod, while unidirectional flow induces directional rotation of the ciliary rod causing stretch-mediated stress around the basal body.

As a primary cilium has a rod-like structure, fluid shear stress could cause structural changes in the rod and thereby amplify the stress. Previous studies discussed the possibility that bending or deflection of the cilium by shear stress triggers the opening of mechanosensitive Ca^{2+} permeable cation channels residing on the cilium [94,151,152]. Oscillatory flow could cause swing movement of ciliary rod with bending of the shaft, causing vibrational movement of the basal part of the rod [153] (Fig. 3 B left). As the basal body is connected to cytoskeletal network [146], shaking of the rod would activate mechanosensitive Ca^{2+} permeable cation channels in cell surface as discussed in Section 11. However, unidirectional flow may cause structural changes in the cilium other than bending, such as pulling or rotation of the cilium rod-like structure (Fig. 3 B right). If we imagine that the cilium has a structure like a string of kites, unidirectional flow would generate a pulling force around the basal body. The concept of cilia rotation is based on the fact that microtubule fibers have an asymmetric spiral-like structure and the primary cilia are designed to rotate. The rotational movement of primary cilia has actually been observed in special cells in the ventral node at the distal tip of the early headfold embryo [154,155]. The nodal pit cells have primary cilia, but exceptionally possess dynein ATPase motors between adjacent microtubule fibers allowing them to generate clockwise rotation (looking down from the tip) of posteriorly tilted cilia, creating leftward fluid flow over the node [156,157]. Given that rotational movements of the cilia can cause directional flow, it is reasonable to assume that directional fluid flow could conversely rotate nonmotile primary cilia like a fan or windmill (Fig. 3 B right). Notably, pulling or rotation of cilia could induce strong physical stress around the basal body. We here propose that exposure of endothelial cells to low USS (0.2–10 dyn/cm^2) induces directional rotation of the cilium rod, causing localized directional stretching stress between the membrane and the basal part of the cilium rod structure (Fig. 3 B). Laminar shear could stimulate mechanosensitive channels residing in the membrane around the basal part of ciliary rod without enhancing cytoskeletal network-mediated Ca^{2+} influx.

5. Cilia-mediated cytoplasmic RyR/Ca^{2+} signaling and IGF-1 secretion

USS induces rapid Ca^{2+} influx into ciliary plasma (cilioplasm) mediated by the cation channel complex comprising PC1 and PC2 TRP type cation channels [89,158]. PC1 is a large membrane-associated protein with a large proportion in the extracellular region [159] and directly contributes to the structure of the Ca^{2+} permeable PC1/PC2 channel complex [160]. The PC1/PC2 complex resides around the transition zone (TZ) membrane, facing ciliary pocket, in addition to along the ciliary outer membrane and appears to play a key role in USS-induced Ca^{2+} influx into the cilioplasm in cultured aortic endothelial cells [89].

Nauli et al. [89] also observed that USS leads to a cilia-dependent rapid increase in cytoplasmic Ca^{2+} levels and eNOS activation in endothelial cells. The highest Ca^{2+} response was observed during challenge of static endothelial cells with a step increase of 7.2 dyn/cm^2. However, a cilia-mediated cytoplasmic Ca^{2+} response was not observed at a USS above 15 dyn/cm^2 [89]. Importantly, cilioplasm and cytoplasm are separated by a diffusion barrier [151,161,162] that prevents the direct transfer of Ca^{2+} between these two compartments [163]. The research group led by Nauli further established that the USS-dependent

rapid increases in cytosolic Ca^{2+} levels were mediated via ryanodine receptors (RyRs) located on the endoplasmic reticulum [151]. These findings suggest that a rapid signal relay from cilia to cytoplasm is mediated via signal mediators, such as ATP and NAD^{+}, presumably released into the ciliary pocket. The ciliary pocket is an invagination of the periciliary membrane and may be an important space for cilia-mediated signal transduction. Basic characterization of the structure of the ciliary pocket has been conducted in fibroblasts and retinal pigment epithelial cells (reviewed in Refs. [86,87]). In articular chondrocytes, Cx43 hemichannels are shown to locate in primary cilium [164]. However, the precise structure and possible functions of the ciliary pocket and transition zone in endothelial cells remains largely unclarified.

Notably, low USS (5 dyn/cm^2) induces secretion of IGF-1 from cultured endothelial cells and IGF-1 gene expression [129]. Wang et al. [129] observed that low USS increases IGF-1 mRNA 6-fold over 12 h compared to high USS (15 dyn/cm^2). Previous reports show that RyR/Ca^{2+} signaling plays a role in glucose-induced insulin secretion from pancreatic β-cells [165,166]. Additionally, various signal receptors localize in primary cilia in β-cells and cilia play a key role in glucose-mediated insulin secretion and signaling [167–170]. Based on these results, we speculate that cilia-mediated rapid increase in cytoplasmic RyR/Ca^{2+} signaling facilitates IGF-1 secretion from endothelial cells, although this hypothesis requires future verification.

The major source of IGF-1 in plasma are liver hepatocytes, but endothelial and smooth muscle cells are able to synthesize and secrete IGF-1, which may contribute to local metabolism (reviewed in Refs. [171,172]). IGF-1 exerts a wide array of influences in the cardiovascular system, including eNOS activation [173,174] and stimulation of smooth muscle cell proliferation (reviewed in Refs. [172,175–178]). Microvascular endothelial cells and HUVEC express IGF-1 receptors (IGF1R), and treatment of these cells with IGF-1 phosphorylates the IGF1R β-subunit [177,178].

6. USS-dependent secretion of stored neutrophil elastase

USS also induces secretion of the serine protease elastase from endothelial cells [38]. Notably, high USS (16 dyn/cm^2) induces a rapid release of elastase activity from porcine aortic endothelial cells into the medium, peaking after 30 min of USS. Interestingly, detection of a 28 kDa elastase in endothelial cells cross-reacted with an antibody raised against neutrophil elastase. As neutrophil elastase mRNA was undetectable in endothelial cells, this suggests that the elastase gene is not expressed in endothelial cells [38]. These results indicate that endothelial cells store neutrophil elastase in their cytoplasm in static culture conditions and that USS may induce release of the stored neutrophil elastase presumably via ATP/$P2Y_2$ signaling as discussed in Section 3 (Fig. 4 A).

Jerke et al. [179] found that endothelial cells internalize extracellularly supplemented neutrophil elastase. Moreover, other serine proteases such as cathepsin G and proteinase 3 also enter endothelial cells and remain intact without degradation [179]. Given that both neutrophil and pancreatic elastase are present in serum [180,181], and that Hennig et al. [38] pre-incubated endothelial cells in a medium containing 10% fetal calf serum and performed elastase secretion experiments in 1% serum containing medium, it is plausible that cells incorporated bovine neutrophil elastase from the serum during static culture and USS induced release of the stored elastase. Synchronous secretion of elastase and IGF-1 suggests a presence of functional cooperation between these two factors. For instance, elastase is able to cleave IGF binding proteins, which interfere with binding of IGF-1 to its receptor [182]. Elastase could promote IGF-1 signaling via degradation of one of the extracellular matrix proteins fibronectin to produce different sized bio-active fragments [183,184]. These fragments activate αvβ3 integrins [185] facilitating formation of a ternary complex of IGF-1/IGFR1/αvβ3 required for the promotion of IGF-1 signaling [186–188] (Fig. 4 B).

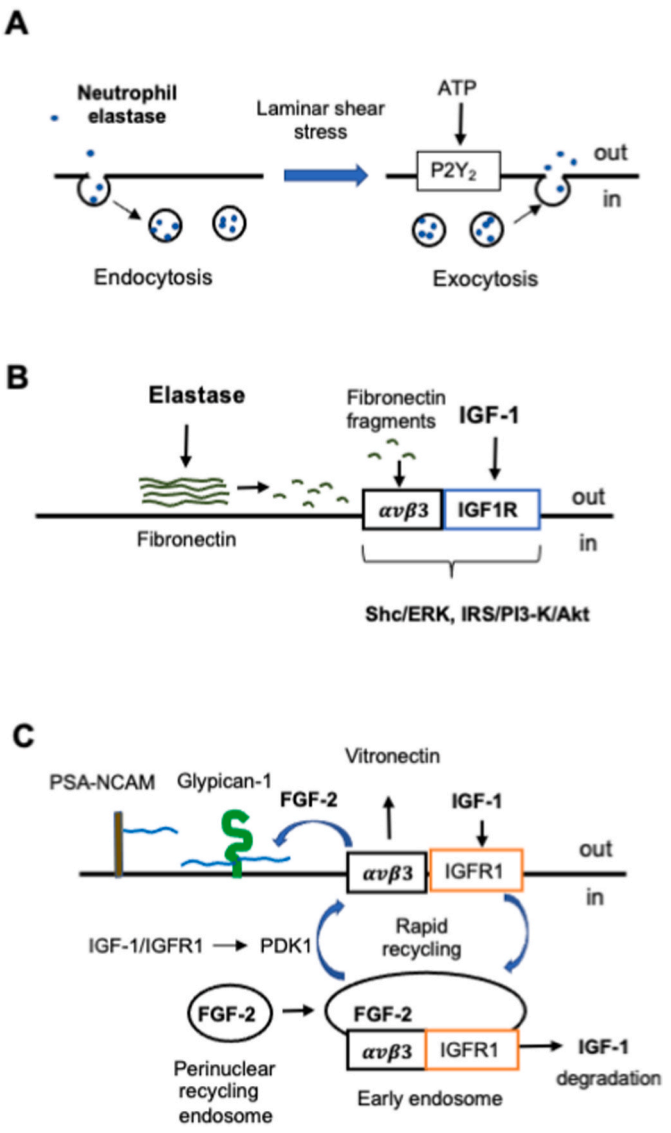

Fig. 4. Neutrophil elastase secretion, elastase-dependent IGF-1 signaling and FGF-2 secretion. ***A***, Laminar shear stress induces release of stored neutrophil elastase via ATP/$P2Y_2$ signaling. ***B***, Elastase degrades fibronectin to produce bioactive fragments, which in turn activates αvβ3 integrin to facilitate IGF-1/IGF1R signaling. ***C***, Proposed mechanism of the complex of αvβ3 integrin/IGF1R-dependent secretion of FGF-2. Recycling of the complex between the membrane and early endosomes is coupled with FGF-2 transport to cell surface, where part of FGF-2 is transferred to glypican-1 HS chains and polysialylated NCAM (PSA-NCAM).

7. Elastase- and αvβ3 integrin-dependent FGF-2 secretion

Gloe et al. [37] first observed that USS (16 dyn/cm^2) induces FGF-2 secretion from endothelial cells into culture medium, noting that release occurred within the first 30 min of USS. The origin of released FGF-2 is not from the matrix but from the cytoplasm, and USS redistributed FGF-2 from nuclear and perinuclear regions to the cell membrane, with intracellular stored FGF-2 decreasing over 2 h. FGF-2 does not contain a conventional secretory signal peptide and accordingly FGF-2 is a poorly secreted protein [189]. Therefore, FGF-2 trafficking to the cell membrane and secretion is a tightly controlled multistep phenomenon, dependent exclusively on both elastase and αvβ3 integrin [38]. Notably, elastase inhibitors suppress FGF-2 secretion [38] and inhibition of αvβ3

integrin by a specific RGD (Arg-Gly-Asp) containing peptide GRGDSP or an antibody to αvβ3 integrin significantly inhibit FGF-2 secretion induced by USS [37,38].

Two experimental results suggest a direct role of αvβ3 integrin in FGF-2 secretion. First, αvβ3 integrin directly binds FGF-2, and the binding is competitively inhibited by vitronectin but not by fibronectin [190]. Second, αvβ3 integrin rapidly recycles between the cell surface and early endosomes [191,192]. These findings suggest that in the presence of IGF-1, the ternary complex IGF-1/IGFR1/αvβ3 integrin may facilitate internalization of the complex into endosomes, where αvβ3 integrin binds FGF-2 and released IGF-1 may be degraded. Recycling of FGF-2 associated αvβ3 integrin back to membrane requires protein kinase D1 (PKD1) activation and association with αvβ3 integrin [192]. As IGF-1/IGFR1/insulin receptor substrate 1 signaling activates PKD1 [193], IGF-1/IGFR1 signaling may facilitate FGF-2-associated αvβ3 integrin/IGFR1 complex back to membrane. At the cell surface, αvβ3 integrin changes its binding partner from FGF-2 to vitronectin to induce cell attachment to the matrix, while part of released FGF-2 presumably associates with cell surface HS side chains present in glypican-1 and polysialylated PAS-NCAM. Thus, we propose elastase- and IGF-1-dependent cycling of IGFR1/αvβ3 between the membrane and endosomes facilitates translocation of FGF-2 from endosomes to the membrane and association of FGF-2 with the glycocalyx to compensate for degradation of IGF-1 and fibronectin fragments (Fig. 4C).

8. HB-EGF-dependent FGF-2 secretion and FGF-2 gene expression

Under high USS (16 dyn/cm^2), elastase may induce FGF-2 secretion without the aid of IGF-1/IGFR1 signaling [38]. Elastase is known to induce release of heparan-binding epidermal growth factor-like growth factor (HB-EGF) from matrix-laden rat pulmonary fibroblast cultures [194]. HB-EGF is a mitogen and chemotactic factor expressed in endothelial cells, smooth muscle cells and other cells. Endothelial cells express EGF receptor family members ErbB2, ErbB3, and ErbB4 but not EGFR [195]. As HB-EGF receptors are able to interact with αvβ3 integrin [196–198], HB-EGF may facilitate ErbBs/αvβ3 integrin complex-mediated FGF-2 secretion in a similar manner as IGF-1 (Fig. 5 A). In the absence of IGF-1/IGFR1 signaling, other signaling pathways that induce PKD1 activation are required for the efficient transport of FGF-2 via ErbBs/αvβ3 integrin complex cycling. PKD1 can be activated by other signaling pathways such as protease-activated receptor-1 (PAR-1) [199]. Kim et al. [25] showed that activation of PAR-1 is required for USS (12 dyn/cm^2) dependent activation of Nrf2 in endothelial cells. These authors observed that PAR-1 knockdown in HUVEC, significantly reduced USS mediated Nrf2 activation [25]. Notably, in addition to thrombin, neutrophil elastase can activate PAR1 in lung epithelial cells [200], suggesting the presence of elastase/PAR-1/PKD1 signaling could potentially support αvβ3 integrin-mediated FGF-2 secretion in endothelial cells (Fig. 5 A).

USS not only induces secretion of stored FGF-2, but also activates FGF-2 gene expression. Malek et al. [125] reported that USS of 36 dyn/cm^2 induces a 4-5-fold increase in FGF-2 mRNA in 3–6 h in bovine

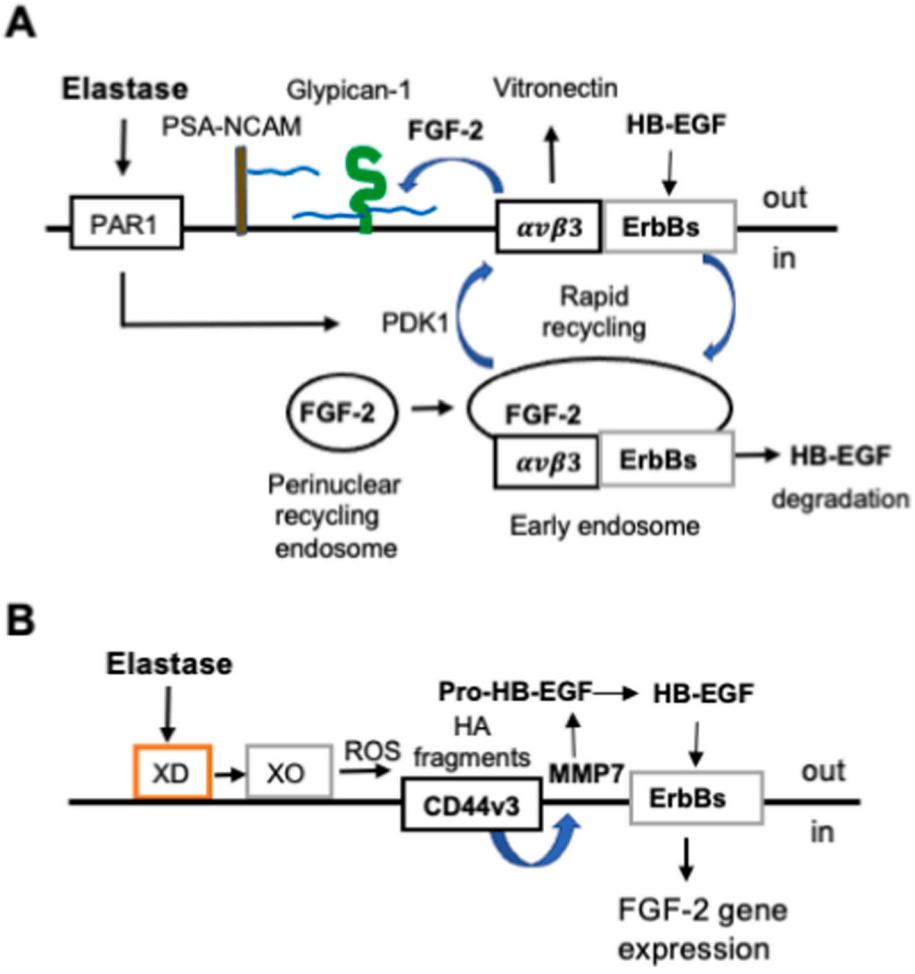

Fig. 5. Roles of HB-EGF in elastase-mediated FGF-2 secretion and gene expression. ***A***, HB-EGF/ErbBs facilitates recycling of αvβ3 integrin to enhance FGF-2 secretion. Elastase activates protease-activated receptor 1 (PAR1) to induce PKD1 activation, important for membrane transfer of the αvβ3/ErbBs complex. ***B***, Elastase cleaves cell surface xanthine hydrogenase (XD) to generate xanthine oxygenase (XO). XO-mediated ROS degrade hyaluronan (HA) into small fragments, which interact with CD44 variant 3 (CD44v3) to induce MMP-7-mediated cleavage of Pro-HB-EGF to release mature HB-EGF.

aortic endothelial cells. Exposure of HUVEC to USS (15 dyn/cm^2) for 24 h increases FGF-2 mRNA levels 3.9-fold [201]. These results are consistent with the observation of Hennig et al. [38] that USS increases FGF-2 levels in culture media to 360 and 1200 pg/ml after 2 and 16 h, respectively. Notably, elastase can induce transcriptional activation of the FGF-2 gene through HB-EGF, as evidenced in rat vascular smooth muscle cells [202]. Interestingly, both low USS (8 dyn/cm^2) [203] and IGF-1/IGFR1 signaling [204] can induce HB-EGF gene expression, suggesting IGF-1 and elastase could cooperate to enhance HB-EGF gene expression and HB-EGF-mediated FGF-2 gene expression in endothelial cells.

HB-EGF is synthesized as the membrane-anchored precursor, which is shed by proteases to release mature soluble HB-EGF. Proteolytic cleavage of pro-HB-EGF to generate HB-EGF is mediated by matrix metalloproteinases (MMPs) (reviewed in Refs. [205–207]), and elastase is able to convert some pro-MMPs to active MMPs [208–212]. Notably, MMP-7 is expressed in endothelial cells and plays a role in proliferation and angiogenesis [213,214]. CD44v3 variants interact with HB-EGF and modulate HB-EGF signaling, which requires depolymerization/degradation of high molecular weight HA into HA fragments [215,216]. It has been reported that superoxide can degrade high molecular HA (~3400 kDa) into small fragments (~14 kDa) *in vitro* [217–221]. Interestingly, neutrophil elastase can lead to HA degradation in endothelial cells, as elastase can convert xanthine dehydrogenase (XD) to XO

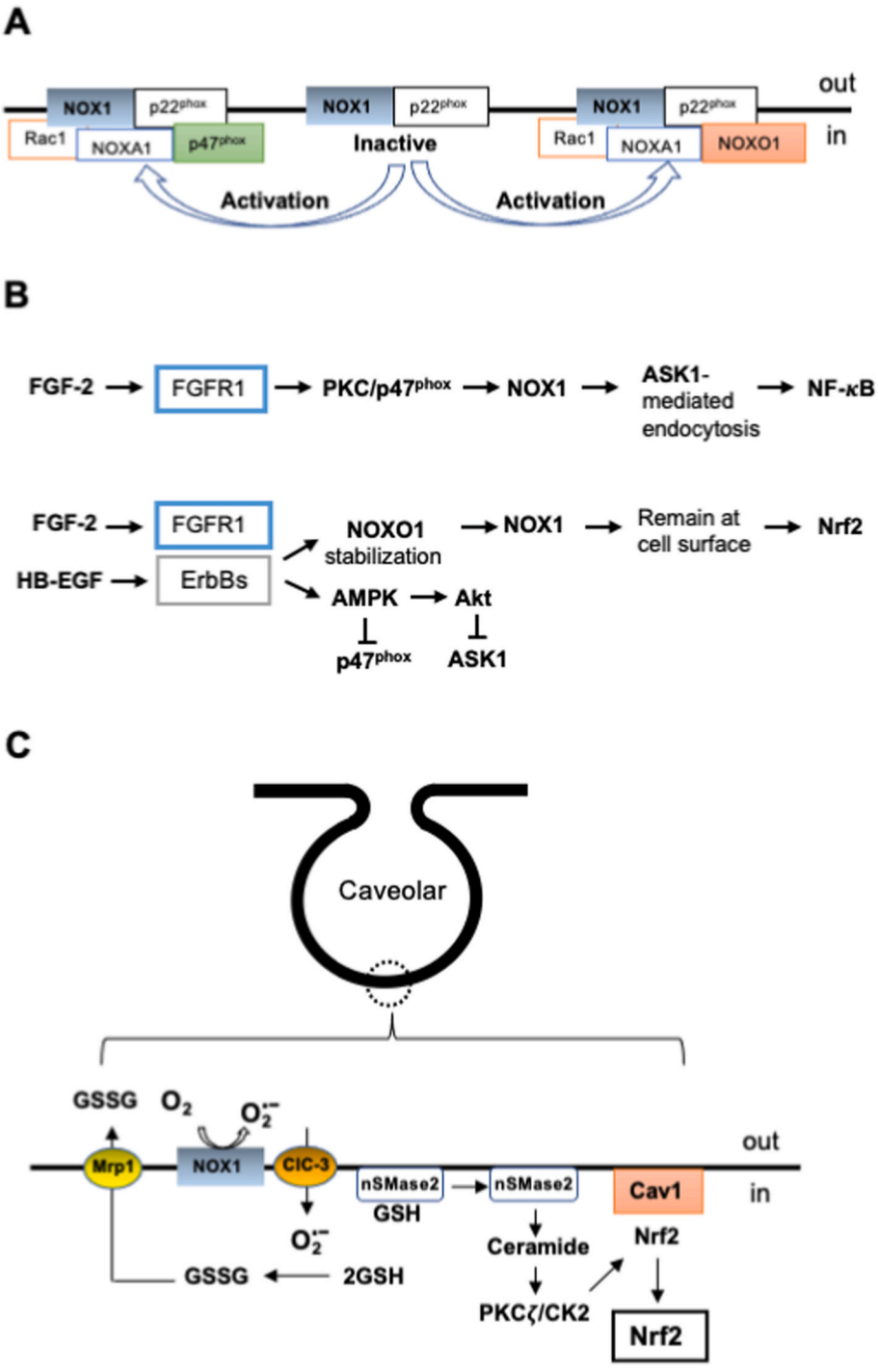

Fig. 6. Cooperation of FGF-2 and HB-EGF in NOX1-dependent Nrf2 activation. ***A***, NOX1 activation is controlled by association of NOXA1, Rac1 and either p47^{phox} or NOXO1 with NOX1/p22^{phox} complex in the membrane. ***B***, FGF-2/FGFR1 signaling induces p47^{phox}-mediated NOX1 activation leading to ASK1-mediated endocytosis of NOX1 to activate NF-κB. HB-EGF/ErbBs signaling stabilizes NOXO1, while AMPK inhibits p47^{phox} activation and Akt inhibits ASK1 activation, which results in Nrf2 activation. ***C***, Activated NOX1 formed signaling complexes containing chloride channel CIC-3 and Mrp1 reside in caveolae membranes and induces superoxide-dependent oxidation of GSH to GSSG and export of GSSG across the membrane, leading to local decrease in GSH and activation of nSMase2 to generate the signaling lipid ceramide. Ceramide/PKCζ/CK2 signaling phosphorylates Cav1-associated Nrf2, resulting in Nrf2 translocation to the nucleus to activate the expression of target genes.

via proteolysis [222,223], noting that XD/XO is present on the cell surface of endothelial cells [224] associated with proteoglycans [225]. In summary, we propose that elastase may contribute to HB-EGF signaling via at least two mechanisms, conversion of pro-MMPs to active MMPs and cell surface conversion of XD to XO to generate superoxide to degrade HA. Both are prerequisites to trigger HA/CD44v3/HB-EGF signaling to upregulate FGF-2 gene expression (Fig. 5 B).

9. HB-EGF modulates FGF-2/NOX1 signaling to favor Nrf2 activation

Endothelial cells express NADPH oxidases NOX1, NOX2, NOX4 and NOX5 [226,227]. Among these NOXs, NOX1 activation may be key for USS-induced Nrf2 activation, based on reports that FGF-2 can activate NOX1 in vascular smooth muscle cells [40] and FGF-2 activates Nrf2 in cardiomyocytes [41,42]. However, FGF-2/NOX1-mediated reactive oxygen species seems to result in NF-κB activation in the smooth muscle cells [228–230], suggesting that FGF-2/NOX1 signaling can activate two redox sensitive transcription factors NF-κB and Nrf2. To elucidate the mechanism of FGF-2/NOX1-mediated Nrf2 activation, we propose a novel concept that cross-talk with HB-EGF signaling shifts FGF-2/NOX1 signaling from NF-κB activation toward Nrf2 activation in endothelial cells.

NOX1 localizes plasma membrane associated with $p22^{phox}$. Activation of NOX1 requires assembly with other components such as NOX activator 1 (NOXA1), NOX organizer 1 (NOXO1) and the small GTPase Rac1 [231,232]. NOXA1 and NOXO1 are homologs of $p67^{phox}$ and $p47^{phox}$, respectively. Importantly, NOX1 can also be activated via association of $p47^{phox}$ instead of NOXO1 [231]. Human endothelial cells [233] and smooth muscle cells [234] express NOXA1. Plasma membrane targeting of NOXA1 depends on either NOXO1 or $p47^{phox}$ (Fig. 6 A). A simple hypothesis to explain the differential NOX1 signaling pathways is that the $p47^{phox}$/NOXA1 axis is associated with NF-κB activation, while NOXO1/NOXA1 axis with Nrf2 activation (Fig. 6 B).

Previous studies suggest that $p47^{phox}$-mediated NOX1 activation seems to be associated with NF-κB activation (reviewed in Ref. [235]). OSS induces expression of bone morphogenic protein 4 (BMP4) in endothelial cells [235,236]. BMP4 stimulates expression of $p47^{phox}$ and NOX1 in an autocrine-like manner [235]. The mechanism underlying $p47^{phox}$/NOX1-dependent NF-κB activation has been studied extensively in coronary microvascular endothelial cells stimulated with TNF-α [237,238]. In the case of TNF-α, NOX1 is endocytosed together with its receptor TNFR1 leading to endosomal generation of ROS, which are critical for c-Src mediated tyrosine-phosphorylation of IκBα and NF-κB activation [237,238]. TNF-α-mediated endocytosis of NOX1 is controlled by the activation of apoptosis signal-regulating kinase 1 (ASK1) [239,240]. In summary, PKC/$p47^{phox}$ and ASK1 signaling pathways likely induce endocytosis of NOX1 leading to NF-κB activation (Fig. 6 B). In vascular smooth muscle cells, $p47^{phox}$ plays a key role in NOX1 activation [241,242] and FGF-2 activates NOX1 leading to NF-κB activation in these cells [228–230]. In endothelial cells, FGF-2 increases pro-angiogenic activity via activation of FGF receptor 1 (FGFR1) [201,243–245], and NOX1 is important for endothelial cell migration and tube-like structure formation [246]. The effects of FGF-2 on the endothelial mesenchymal transformation and proliferation, migration and tube formation also depend on NF-κB activation [247,248].

Notably, USS-induced HB-EGF/ErbBs signaling could modulate FGF-2/NOX1 signaling. Firstly, HB-EGF/ErbBs signaling activates AMPK [249,250], which inhibits activation of $p47^{phox}$ [251,252]. Secondly, a recent study shows that NOXO1 is unstable due to rapid proteasomal degradation, but EGF stimulation induces Ser154 phosphorylation of NOXO1, resulting in stabilization and rapid accumulation of NOXO1 protein in colon cancer cells [253]. These studies suggest a possibility that HB-EGF signaling promotes NOXO1 accumulation, while inhibiting activation of $p47^{phox}$ leading to NOXO1-centered NOX1 activation in USS-exposed endothelial cells. Thirdly, AMPK/Akt signaling could inhibit ASK1 activation by phosphorylation of ASK1 by Akt [254,255], resulting in inhibition of NOX1 endocytosis (Fig. 6 B).

We recently proposed a mechanism by which NOX1-mediated superoxide could lead to CK2-dependent Nrf2 activation [256]. Briefly, superoxide rapidly reacts with glutathione (GSH) to produce disulfide of GSH (GSSG) to downregulate local GSH levels. Decrease in GSH leads to activation of membrane-associated neutral sphingomyelinase 2 (nSMase2) and generation of lipid signal molecule ceramide. nSMase2 functions as a GSH sensor as GSH inhibits its enzyme activity, highlighting an inverse relation between cellular GSH levels and enzyme activity [257]. Membrane topology and the signaling compartment are important factors in understanding the activation of Nrf2 by NOX1-dependent superoxide. NOX1 [258] and nSMase2 [259] are localized in lipid rafts/caveolae, where the substrate of nSMase2 sphingomyelin is enriched. Activation of NOX1 generates superoxide in the extracellular space, and superoxide can pass into cells through chloride ClC-3 channels [260] to oxidize cellular GSH forming GSSG [261], which is rapidly exported from cells via ABC transporters such as Mrp1 [262,263]. The hypothetical NOX1/nSMase2/ClC-3/Mrp1 functional complex should reside in caveolae in order to efficiently activate nSMase2. Notably, Nrf2 is concentrated in caveolar membrane domains scaffolded by Cav1 [264,265]. The Cav1-associated pool of Nrf2 is thought to suppress Nrf2 activation, as over-expression of Cav1 suppresses Nrf2 activation by 4-hydroxynonenal [264] and H_2O_2 [265]. However, we suggest Cav1-associated Nrf2 is a special pool protected from Keap1-mediated degradation but available for phosphorylation by ceramide/PKCζ/CK2 signaling. Notably, Nrf2 can be stabilized and/or activated through direct phosphorylation by CK2 [266,267]. This hypothesis requires the NOX1 containing signaling complex to remain in the plasma membrane without endocytosis in order to facilitate GSSG efflux to extracellular space (see Fig. 6C).

10. BDNF-mediated Nrf2 activation depends on TrkB.T1 and $p75^{NTR}$ receptors

BDNF transduces signals through its receptors, full length TrkB, truncated TrkB.T1 and $p75^{NTR}$. TrkB and TrkB.T1 have a high affinity for BDNF, but TrkB.T1 lacks the tyrosine kinase domain. BDNF/TrkB signaling induces NOX-derived ROS generation through the activation of $p47^{phox}$ and in HUVEC promotes angiogenic tube formation depending on the TrkB tyrosine kinase activity [268]. Furthermore, BDNF/TrkB signaling induces relaxation of resistance arteries in rats via PI3–K/Akt-mediated eNOS activation [269], and generation of NO contributes to prolonged activation of the BDNF/TrkB axis [270]. BDNF also interacts with $p75^{NTR}$, which binds all neurotrophins [271,272]. $p75^{NTR}$ mediates many distinct cellular functions, including cell survival and apoptosis, axonal growth and cell proliferation, depending on the cellular context. $p75^{NTR}$ enhances the affinity and specificity of BDNF binding to TrkB [272]. These results suggest that BDNF/TrkB/$p75^{NTR}$ signaling induces $p47^{phox}$-dpendent NOX activation and NF-κB-dependent neovascularization (Fig. 7 A).

However, endothelial cells seem to express TrkB.T1 at high levels. For example, cardiac microvascular endothelial cells primarily express TrkB.T1 [273,274]. BDNF/TrkB.T1/$p75^{NTR}$ signaling does not lead to angiogenesis. Instead, previous studies suggest that neurotrophins may generate the bioactive lipid ceramide via nSMase2 in various cells heterologously expressing $p75^{NTR}$ [275,276]. Importantly, Trk receptors suppress $p75^{NTR}$-mediated ceramide generation with its tyrosine kinase activity [277]. Kosaka et al. [278] first showed that nerve growth factor activates Nrf2 in PC12 cells. We proposed that neurotrophin's common receptor $p75^{NTR}$ plays a key role in the activation of Nrf2 via generation of ceramide [45]. As astrocytes predominantly express TrkB.T1, we further postulated that the BDNF/TrkB.T1/$p75^{NTR}$/ceramide/PKCζ/CK2 signaling phosphorylates Nrf2 to enhance nuclear translocation in astrocytes [45]. As endothelial cells express TrkB.T1 as

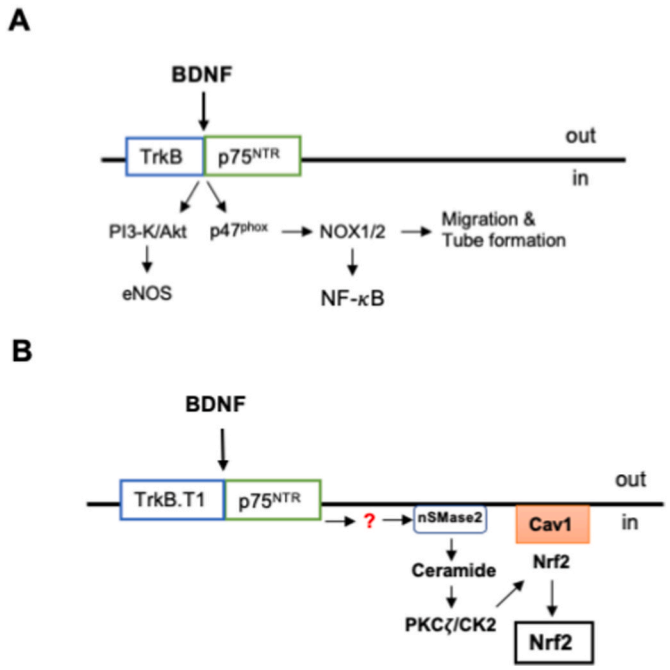

Fig. 7. BDNF-mediated activation of NF-κB and Nrf2. ***A***, BDNF/TrkB/$p75^{NTR}$ signaling induces $p47^{phox}$-mediated activation of NOX1 and probably NOX2, inducing NF-κB activation and cell migration and tube formation. eNOS is also activated by PI3–K/Akt signaling. ***B***, BDNF/TrkB.T1/$p75^{NTR}$ signaling enhances nSMase2-mediated ceramide generation leading to PKCζ/CK2-mediated Nrf2 activation.

the major form of TrkB receptors [273,274], we propose that high USS induces secretion of BDNF, which activates Nrf2 in endothelial cells via a TrkB.T1/$p75^{NTR}$-mediated signaling the pathway we proposed for astrocytes (Fig. 7 B).

Notably, a natural compound 7,8-dihydroxyflavone, a TrkB agonist, induces Nrf2 activation in 8 h in lung fibroblasts [279], keratinocytes [280], myoblasts [281] and chondrocytes [282]. Wang et al. [283] observed that pre-treatment of HUVEC-derived cells with 7,8-dihydroxyflavone protected against H_2O_2-mediated cell damage and suppressed NF-κB activation. However, these authors found that the HO-1 level returned to a basal level in 24 h, suggesting the effect of 7,8-dihydroxyflavone on the Nrf2/HO-1 signaling is relatively rapid and transient. Thus, BDNF could activate Nrf2 in various cells similar to the actions of the TrkB agonist 7,8-dihydroxyflavone. $p75^{NTR}$-mediated activation of nSMase2 occurs relatively fast [275,276] and may not depend on NOX1 activation. Czarny et al. [284,285] observed that exposure of rat lung vasculature to high pressure (14–15 mm Hg) caused a transient increase in nSMase2 activity with ~1.8-fold increase in ceramide levels in 2–5 min. These results suggest a possible role of BDNF in pressure-mediated activation of nSMase2 in the vasculature. Currently, studies on the functions of $p75^{NTR}$ in BDNF signaling in endothelial cells are limited, and further research is required to verify our concept that USS-mediated BDNF secretion activates Nrf2 via the TrkB.T1/$p75^{NTR}$-mediated signaling pathway in endothelial cells.

11. Summary of cilia-mediated mechanotransduction in cultured endothelial cells

We propose that primary cilia sense differences between USS and OSS as summarized in Fig. 8. Primary cilia sense low USS and amplify the signal by changing flow stress into rotational stress at the base of cilium rod (see Fig. 3 B right). We speculate that this stress leads to secretion of IGF-1 and elastase (see Sections 5 and 6). IGF-1 and elastase cooperate in FGF-2 secretion and HB-EGF-dependent FGF-2 gene expression (see Section 7), in which elastase contributes to superoxide-dependent degradation of HA polymers via conversion of cell surface XD to XO (see Section 8). We hypothesize that FGF-2/NOX1 signaling

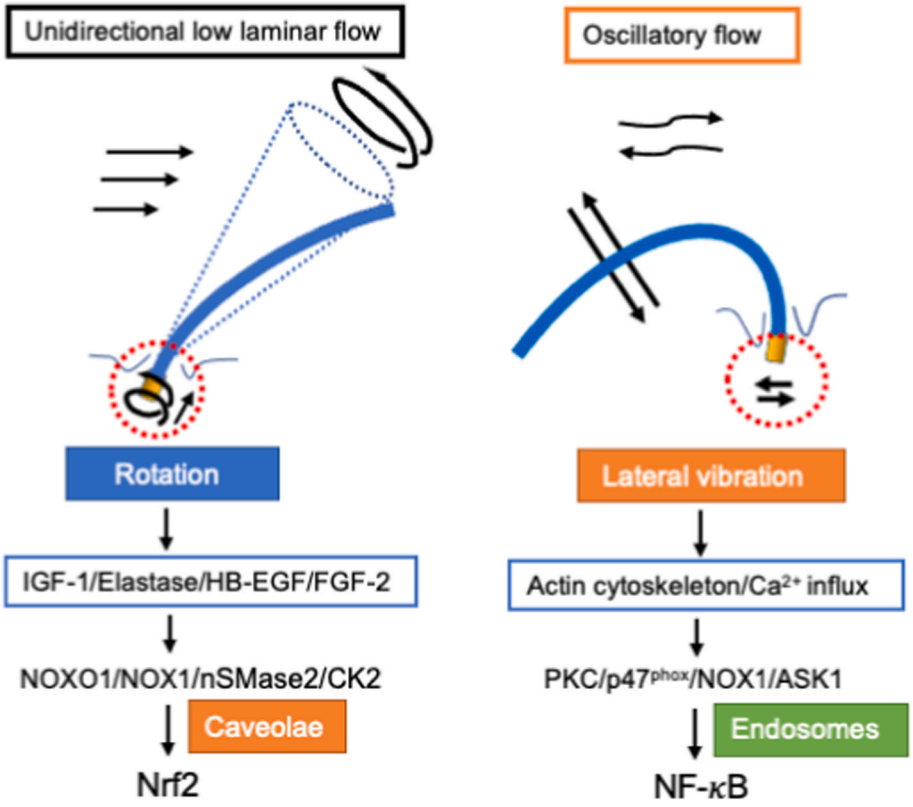

Fig. 8. Primary cilia amplify low laminar shear stress and oscillatory flow signals leading to activation of Nrf2 and NF-κB activation, respectively. Low shear stress rotates the ciliary rod leading to release of insulin-like growth factor-1 (IGF-1) and ATP/$P2Y_2$-mediated elastase release. IGF-1, elastase and proteoglycans cooperate in FGF-2 secretion, expression and receptor signaling, which results in the activation of NOX1/CK2-dependent Nrf2 activation (Left). Oscillatory flow-induced vibrational stress shakes the actin cytoskeleton network, enhancing Ca^{2+} influx through mechanosensitive cation channels, which causes $p47^{phox}$-mediated NOX1 activation leading to NF-κB activation (Right).

modified by HB-EGF plays a major role in the early phase of Nrf2 activation (see Section 9). Prior to activation of eNOS and Nrf2 via phosphorylation, both are associated with Cav1 in endothelial cell surface signaling compartments in caveolae. In addition to HA and HS chains and CD44v3, cell surface glycocalyx components glypican-1 [102] and sialic acids [26], presumably PSA-NCAM [108], are important for retaining FGF-2 and enhancing FGF-2 signaling through FGFR1. Moreover, αvβ3 plays a key role in USS-dependent FGF-2 secretion (see Fig. 4C and 5 A).

In contrast to USS, OSS induces different vascular remodeling processes through the activation of TGF-β and NF-κB [10–12]. We postulate that the primary cilium also amplifies OSS and transduces it into specific Ca^{2+} signaling (Fig. 8 Left). As illustrated in Fig. 3 B, oscillatory flow could cause strong vibrational stress with a lever-like effect over the basal structure of the cilium, which is tightly connected with actin cytoskeleton [146]. Shaking of the cytoskeletal network by the cilium rod would be transmitted to modulate tension within the cell via focal adhesion sites, integrins, cellular junctions and the extracellular matrix [286,287], augmenting the opening of mechanosensitive Ca^{2+} permeable cation channels such as Piezo1 [288,289] and TRPV4 [290–292]. These cell surface cation channels can be directly activated by physical stress such as deflection and/or stretching [293], and Piezo1 plays an essential role in shear stress-induced rapid increases in cytoplasmic Ca^{2+} levels [294]. In contrast, TRPV4 channels are slowly activated under high shear stress [295]. Flow-mediated Ca^{2+} influx induces hyperpolarization and Ca^{2+}/calmodulin-dependent eNOS activation and NO generation, inducing vasodilation [296,297]. However, enhanced influx of Ca^{2+} through Piezo1 and TRPV4 channels in response to high OSS would lead to activation of Ca^{2+}-dependent PKC, which phosphorylates $p47^{phox}$, inducing $p47^{phox}$ translocation to membrane to bind $p22^{phox}$ to activate NOX1 [298,299]. Notably, studies in mice have shown that high shear stress induces generation of peroxynitrite, resulting from $p47^{phox}$-dependent NOX and eNOS activation [300]. Notably, $p47^{phox}$ and hyaluronidase2 form a complex in cells and PKC phosphorylation of $p47^{phox}$ induces dissociation of $p47^{phox}$ from hyaluronidase2 in endothelial cells resulting in the activation of hyaluronidase2 and degradation of HA [301]. Oscillatory flow also increases MMP-9 mRNA as well as secretion of MMP-9 protein [302], which facilitate remodeling of proteoglycans (reviewed in Ref. [303]). Notably, oscillatory flow can shed ectodomains of syndecans with HS chains [304] and PSA-NCAM [305], whilst degradation of the glycocalyx disrupts Cav1 expression and function [306]. These responses suppress Nrf2 activation by FGF-2 and BDNF.

12. Summary of mechanisms of Nrf2 activation by USS

In HUVEC, USS induces Nrf2-dependent expression of many genes including HO-1, the cystine transporter (xCT), sequestosome1 (SQSTM1/ZIP/A170), NQO1, glutamate-cysteine ligase modifier subunit (GCLM), ferritin heavy chain and thioredoxin reductase 1 [18,20]. These proteins are important for the protection of endothelial cells against oxidative damages. We propose that FGF-2, BDNF and 15d-PGJ_2 are potential Nrf2 activators induced by USS (see Section 3) and that activation of Nrf2 by these factors is mainly mediated by two stress sensors Keap1 and nSMase2. The Keap1-Nrf2 system is well-known as the electrophile-dependent Nrf2 activation system [49,307,308]. The cytoplasmic protein Keap1 interacts with Nrf2 and facilitates proteasomal degradation of Nrf2 under normal conditions [48,49]. Keap1 has sulfhydryl residues highly reactive with electrophiles, which react with Keap1 forming Michaelis adducts and induce structural changes in Keap1. This in turn results in inhibition of Keap1-mediated proteasomal degradation of Nrf2 leading to stabilization and nuclear accumulation of Nrf2 (see reviews in Refs. [49,307,309–311]) (Fig. 9 A). 15d-PGJ_2 is an electrophile and has been shown to react with Keap1 inducing Nrf2 stabilization [48,49,308].

In contrast to Keap1, nSMase2 senses decreases in GSH. The activity

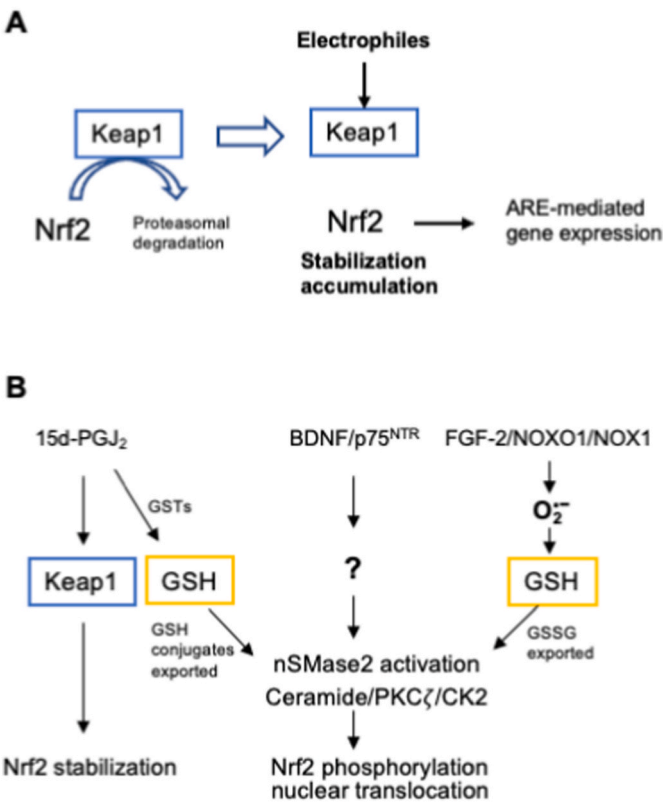

Fig. 9. Summary of mechanisms underlying activation of Nrf2 by 15d-PGJ_2, FGF-2 and BDNF. ***A***, Keap1 interacts with Nrf2 and facilitates proteasomal degradation of Nrf2. Electrophiles react with cysteine residues in Keap1 leading to stabilization/accumulation of Nrf2 [309–311]. ***B***, In addition to the interaction with Keap1, 15d-PGJ_2 forms conjugates with GSH mediated by GSH transferases (GSTs) leading to depleted GSH levels, which could cause nSMase2 activation. FGF-2/NOXO1/NOX1 signaling induces nSMase2 activation. BDNF/$p75^{NTR}$ signaling also induces nSMase2 activation although the molecular mechanisms remain to be elucidated.

of nSMase2 is inhibited by cellular GSH under normal conditions, but depleted GSH levels induce nSMase2-mediated generation of the lipid signal molecule ceramide [257]. Overgeneration of ceramide is toxic to cells, but low levels of ceramide induce activation of PKCζ [312]. We proposed that nSMase2/ceramide/PKCζ signaling activates CK2 [45,256]. Notably, stabilization and/or activation of Nrf2 can be controlled through direct phosphorylation of Nrf2 by CK2 [266,267] and PKC [313]. As nSMase2 and its substrate sphingomyelin reside in lipid rafts/caveolae, we propose that PKCζ/CK2 signaling induces preferential phosphorylation of Nrf2 associated with Cav1, leading to nuclear translocation of Nrf2 and Nrf2/ARE-mediated gene expression. Thus, Nrf2 activation induces upregulation of GSH synthesis through expression of GCLM and the membrane cystine transporter xCT [18,20]. We propose that the FGF-2/NOXO1/NOX1 signaling induces superoxide-dependent local oxidation of GSH to GSSG leading to depletion of GSH levels in the vicinity of nSMase2 leading to the generation of ceramide (Fig. 6). Notably, BDNF/TrkB.T1/$p75^{NTR}$ signaling also induces activation of nSMase2 and ceramide generation (Fig. 7). Fig. 9 B summarizes these signaling pathways.

These two sensor systems actually work together to induce rapid and efficient Nrf2 activation by electrophiles such as 15d-PGJ_2. Notably, GSH transferases catalyze formation of conjugates of electrophiles with GSH leading to depletion of GSH at high levels of electrophiles [314]. There are reports that 15d-PGJ_2 treatment induces depletion of intracellular GSH [315], dependent on the export of 15d-PGJ_2-GSH conjugates via Mrp1 [316], and that 15d-PGJ_2 increases ceramide generation in human cancer cells [317]. These studies suggest the cooperation of

Keap1 and GSH/nSMase2 sensor systems to counteract the effect of electrophiles to restore GSH levels and the cellular redox balance through Nrf2 activation (Fig. 9 B).

13. USS-induced activation of Nrf2 and future research perspectives

Primary cilia are not uniformly distributed in the main arterial vasculature but are concentrated in disturbed flow regions, such as at bifurcations and inner curvatures, and are barely detected in regions exposed to USS [318–320] (Fig. 10). In disturbed flow regions, vascular enlargement and angiogenic processes may be enhanced via activation of hyaluronidase2 [301,321], MMP-9, TGF-β and NF-κB [322,323], and primary cilia presumably contribute to these cellular responses as discussed in Section 11 (see Fig. 10). Dai et al. [22] observed that Nrf2 is activated in endothelial cells in regions of the aorta exposed to pulsatile USS, where cilia are sparsely distributed [319] (Fig. 10). As FGF-2 levels in plasma of healthy human adults are only a few pg/ml [324,325], and those of 15d-PGJ_2 are about 2.6 ng/ml or 8.2 nM [326], effects of circulating FGF-2 and 15d-PGJ_2 on Nrf2 activation in aortic endothelial cells may be limited. However, non-ciliated endothelial cells are able to synthesize and secrete FGF-2 dependent on circulating levels of elastase and IGF-1. Bailey-Downs et al. [327] analyzed adult-onset endocrine IGF-1 deficient mice and showed that the liver-derived circulating IGF-1, nearly a half of the total IGF-1 in serum, contributes to Nrf2 activation in the aorta. Furthermore, these authors showed that incubation of human coronary artery endothelial cells with IGF-1 (200 ng/ml) for 24 h induced Nrf2 activation, suggesting that circulating IGF-1 may be involved in the induction of FGF-2/Nrf2 signaling in aortic endothelium (Fig. 10). However, IGF-1 secreted by hepatocytes into the circulation may be unrelated to endothelial shear stress.

Notably, circulating BDNF has been implicated in the control of cardiovascular disease including atherosclerosis [327]. Serum BDNF levels in wild type mice are about 1.7 ng/ml [328], and those in human adults are about 28.5 ng/ml [329,330]. These BDNF levels are much higher than those of FGF-2. These results suggest a potential role of circulating BDNF in Nrf2 activation in endothelial cells as well as autocrine effects of BDNF secreted in response to high USS. Intriguingly, the majority of BDNF in blood is concentrated in platelets. The BDNF concentration in platelet-poor human plasma is about 1.7 ng/ml and USS induces a rapid release of BDNF from platelets [331]. Therefore, the close contact or direct interaction of platelets with endothelial cells seems to be important not only for the BDNF/TrkB signaling-dependent vascularization [331], but also for the promotion of BDNF/TrkB. T1-dependent signaling *in vivo*. Another regulatory point of BDNF is the conversion of a precursor to a matured form by proteolysis [332]. The secretion of BDNF may depend on strong mechanical stress such as stretching but not strictly on USS. In addition to shear stress, hypoxia increases expression of BDNF and its secretion from mouse brain microvascular endothelial cells [333] and human pulmonary artery endothelial cells [334]. Helan et al. [335] reported that exposure of pulmonary artery endothelial cells to hypoxia (1–3 kPa O_2) for 24 h induces hypoxia inducible factor-1α (HIF-1α) dependent expression of BDNF. As the BDNF receptor TrkB gene has HIF-1 binding elements [336], hypoxia seems to enhance the expression of BDNF and TrkB, and BDNF/TrkB signaling leading to neovascularization. Thus, BDNF secretion and signaling is complicated and further studies are required to clarify the role of BDNF in USS-dependent activation of Nrf2 in endothelial cells in culture systems and the vasculature *in vivo*.

Studies of atherosclerosis are usually performed under extremely unusual conditions such as ApoE-deficient mice fed a hyperlipidemic diet. USS-dependent activation of Nrf2 may protect the arterial

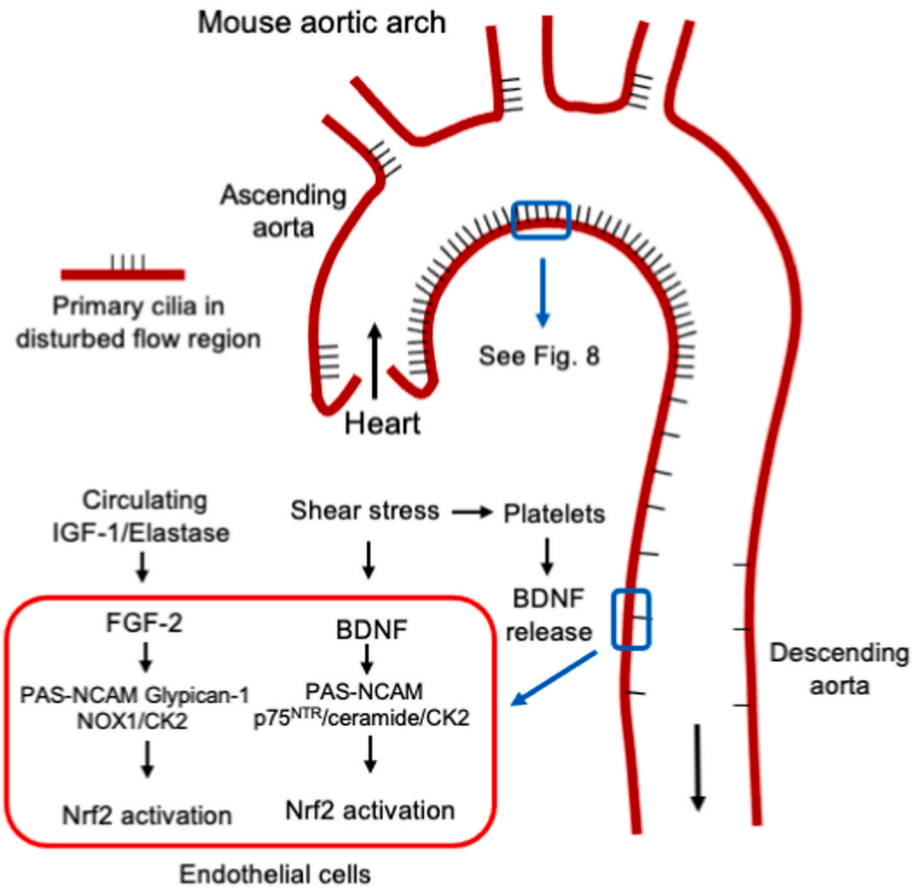

Fig. 10. Schematic of the distribution of primary cilia in mouse aortic arch and descending aorta. Ciliated endothelial cells are primarily localized in regions of disturbed/oscillatory flow (modified from Refs. [307–309]), and disturbed/oscillatory flow activates cilia-mediated Ca^{2+} influx leading to activation of NF-κB (Fig. 8). In contrast, ciliated cells are sparse in vascular regions exposed to unidirectional laminar flow, where Nrf2 is activated [22]. We propose two possibilities for the activation of Nrf2 in the aorta: (i) circulating IGF-1/elastase-mediated FGF-2 signaling and (ii) circulating BDNF and/or secreted BDNF-mediated signaling. Interaction of endothelial cells with platelets, which contain high levels of BDNF may also be important for the activation of Nrf2.

vasculature from atherosclerosis [337,338]. However, the role of Nrf2 in the pathogenesis and progression of atherosclerosis observed in these mouse models is not simple. Enhanced activation of Nrf2 by treating mice with the Nrf2 activator sulforaphane suppresses expression of adhesion molecules such as VCAM-1 and protects arteries exposed to high USS from inflammation and atherosclerosis [339]. Atherosclerosis develops as a consequence of arterial wall injury following interaction of platelets and monocytes/macrophages. Uptake of oxidized low-density lipoproteins (oxLDL) via CD36 in macrophages facilitates foam cell formation [340], but notably oxLDL activates Nrf2, which then upregulates expression of CD36 in macrophages [32]. Therefore, the presence of Nrf2 may enhance plaque formation in the advanced stages of atherosclerosis [341,342]. Notably, FGF-2 and BDNF can activate both Nrf2 and NF-κB depending on receptor-mediated signal transductions. Liang et al. [343] showed that a low molecular weight isoform of FGF-2 promotes atherosclerosis by enhancing macrophage infiltration. Moreover, activation of NOX1 facilitates macrophage infiltration resulting in the increase in lesion area [344]. Neutrophil elastase is present within atherosclerotic plaques where it contributes to matrix degradation and weakening of the vessel wall associated with the complications of aneurysm formation and plaque rupture [345]. Further research on the effects of bioactive factors such as IGF-1, elastase, FGF-2 and BDNF on plaque formation and development is warranted and we encourage that studies using model systems consider the interaction of endothelial cells with platelets and monocytes and recapitulate physiological shear stress [26] and oxygen levels encountered *in vivo* [346,347] to improve clinical translation.

Declaration of competing interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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