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Flow signaling and atherosclerosis

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Abstract

Atherosclerosis rarely develops in the region of arteries exposed to undisturbed flow (u-flow, unidirectional flow). Instead, atherogenesis occurs in the area exposed to disturbed flow (d-flow, multidirectional flow). Based on these general pathohistological observations, u-flow is considered to be atheroprotective, while d-flow is atherogenic. The fact that u-flow and d-flow induce such clearly different biological responses in the wall of large arteries indicates that these two types of flow activate each distinct intracellular signaling cascade in vascular endothelial cells (ECs), which are directly exposed to blood flow. The ability of ECs to differentially respond to the two types of flow provides an opportunity to identify molecular events that lead to endothelial dysfunction and atherosclerosis. In this review, we will focus on various molecular events, which are differentially regulated by these two flow types. We will discuss how various kinases, ER stress, inflammasome, SUMOylation, and DNA methylation play roles in the differential flow response, endothelial dysfunction, and atherosclerosis. We will also discuss the interplay among the molecular events and how they coordinately regulate flow-dependent signaling and cellular responses. It is hoped that clear understanding of the way how the two flow types beget each unique phenotype in ECs will lead us to possible points of intervention against endothelial dysfunction and cardiovascular diseases.

Keywords

MAP kinase; SUMOylation; p90RSK; SENP2; DRP-1; NEMO; PKC family; AMPK; ER stress; PPARs; ERK5; DNA methylation; HATs and HDACs

Introduction

Atherosclerosis is a multi-factorial disease that is associated with risk factors, including hypercholesterolemia, hyperlipidemia, hyperglycemia, smoking, hypertension, aging, and diabetes. These risk factors are the so-called systemic factors to which the entire cardiovascular system is exposed in more or less uniformly. However, atherosclerosis is a focalized disease that develops in anatomically identifiable regions of the arterial system. It

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is well known that while atherogenesis rarely occurs in the region of arteries exposed to undisturbed flow (u-flow), lesions frequently arise in areas exposed to disturbed flow (d-flow). This regional specificity of atherogenesis suggests a strong correlation between the local pattern of blood flow (u-flow or d-flow) and the pathogenesis (inhibition or promotion) of atherosclerotic plaque formation. In this regard, it is important to understand how u-flow inhibits, while d-flow promotes atherogenesis, because this understanding will guide us toward therapeutic interventions against this disease.

One of the key events that cause endothelial cell (EC) dysfunction and atherosclerosis is the increased expression by ECs of cell adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin. Normally, healthy ECs do not recruit leukocytes on the luminal surface, but when ECs are placed in atherogenic environments (e.g., disturbed flow), the expression of these adhesion molecules increases, promoting leukocyte recruitment and inflammation. In contrast, u-flow inhibits these proatherogenic events in ECs. Recent studies have reported a number of signaling pathways that are differentially regulated by the two types of flow. In this review, we summarize flow-activated signaling pathways that are either anti-atherogenic or proatherogenic and discuss their regulatory mechanisms.

Mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated-kinase 5 (ERK5) in endothelial cells

Mitogen-activated protein kinases (MAPKs) are highly conserved serine/threonine kinases that are activated by a wide variety of stimuli, including growth factors, G protein-coupled receptor activation, and oxidative stress. Four major groups of the MAPK family, including extracellular signal-regulated-kinase (ERK)1/2, c-Jun N-terminal kinases (JNKs), p38 kinases, and the MEK5-regulated ERK5, have been reported to regulate downstream transcription factors, which mediate cell growth and differentiation [1, 2] (Fig. 1). The activation of these MAP kinases requires dual phosphorylation of threonine and tyrosine residues within the threonine-x-tyrosine motif (T-x-Y motif), which is T-E-Y in ERK1/2, T-P-Y in JNKs, T-G-Y in p38, and T-D-Y in ERK5.

ERK1/2 activity is involved in embryogenesis, cell proliferation, differentiation, and apoptosis, whereas the overexpression or constitutive activation of ERK1/2 can result in the progression of several cancers [3]. In 1995, Bradford Berk's group showed that flow-activated ERK1/2 [4] and that a herbimycin-sensitive tyrosine kinase and PKC may be involved in this activation [5–7]. The role of endothelial ERK1/2 in regulating atherosclerosis is not clearly defined. Chen et al. have reported that an inhibitor of mitogen-activated protein kinase kinase 1/2 (MEK1/2, the upstream kinase of ERK1/2) and a liver X receptor (LXR) ligand can synergistically reduce atherosclerotic plaque formation, but the effect of MEK1/2 inhibition on atherosclerosis was marginal [8].

JNK can phosphorylate several transcription factors, including c-Jun, ATF2, Elk-2, RXR α , NFAT4, HSF-1, and p53, which can contribute to both endothelial inflammation and apoptosis [9–13]. It has been well established that the induction of VCAM-1, ICAM-1, and E-selectin by TNF- α is regulated by JNK [14–16]. JNK can also accelerate apoptosis by the

following two different ways: one mechanism is by regulating nuclear transcriptional factors, including c-Jun and p53. For example, JNK can phosphorylate p53 at Ser6, which inhibits ubiquitin-mediated p53 degradation and increases the p53 expression level, resulting in p53-mediated up-regulation of several pro-apoptotic genes, including Bax and PUMA [17, 18]. The other mechanism is by regulating mitochondrial function. It has been reported that JNK translocates to mitochondria and phosphorylates Bcl-2, Bim, and Bid, all of which are apoptosis-related molecules [9]. These molecules can induce cell apoptosis singly as well as cooperatively. It was first reported that JNK phosphorylated Bcl-2 at Ser70, which inhibited anti-apoptotic effect of Bcl-2 [19]. Then, it was discovered that JNK also phosphorylated Bim at Ser65 [20] or Thr56 [21], which up-regulated Bim's pro-apoptotic activity via activating Bax and Bak, and subsequently inhibited the anti-apoptotic effect of Bcl-2. Finally, the contribution of Bid in JNK-mediated apoptosis was reported, although this was not by direct phosphorylation of Bid by JNK [22]. In this case, JNK played a role in cleaving Bid and generated a JNK-mediated Bid cleavage product (jBid), which then translocated to mitochondria and induced apoptosis by releasing Smac/DIABLO, not cytochrome c [22]. The role of JNK on atherosclerosis likely depends on its isoforms. Ricci et al. reported that ApoE^{-/-} mice also lacking JNK2 (ApoE^{-/-} JNK2^{-/-} mice), but not ApoE^{-/-} JNK1^{-/-} mice, developed less atherosclerosis than ApoE^{-/-} mice [23]. Pharmacological inhibition of JNK efficiently reduced plaque formation [23, 24], indicating that JNK is a pro-atherogenic MAPK.

Possible roles for p38 α in the development and progression of atherosclerosis have been suggested. For example, the activation of p38 α can up-regulate migration [25, 26], proliferation [25, 26], permeability [27], apoptosis [28], and adhesion molecule expression [29] of endothelial cells. It was reported that u-flow increased p38 activation and inhibited matrix metalloproteinase MMP-2 expression [30]. However, Kardakar et al. reported that EC-specific p38 α knockout in ApoE^{-/-} mice had no effect on the atherosclerotic plaque formation [31]. These results suggest that the pathological role of flow-induced p38 activation in ECs during the development of atherosclerosis remains unclear.

MAPK kinase 5 (MEK5) is a dual-phosphorylation kinase and is an immediate upstream activator of ERK5. Hayashi et al. reported that the depletion of endothelial ERK5 impaired vascular integrity via enhancing EC apoptosis [32]. Although the mechanism for the anti-apoptotic property of ERK5 is unclear, it is attributed to possible phosphorylation of BAD (Bcl2 antagonist of cell death) [33].

Berk et al. have earlier found that u-flow activates ERK1/2, p38, and ERK5, while it inhibits TNF- α -induced JNK activation [34]. It appears that the inhibition of JNK signaling can be one of the mechanisms for the u-flow-induced anti-inflammatory and anti-apoptotic effects. In the following sections, we will discuss several possible mechanisms how u-flow could inhibit JNK signaling.

Role of ERK5 in s-flow-induced anti-inflammatory responses in ECs

It has been reported that u-flow increases ERK5 kinase activity [35] but not d-flow [36]. Since u-flow but not d-flow inhibits EC inflammation as we explained above, ERK5 may be

involved in regulating the u-flow-induced anti-inflammatory effect. Tumor necrosis factor- α (TNF α) induces EC inflammation and up-regulates the expression of adhesion molecules including VCAM-1, ICAM-1, and E-selectin in ECs. JNK activation is required for TNF α -induced VCAM-1 expression, and u-flow inhibits JNK activation as well as VCAM-1 expression [37]. Li et al. showed that the MEK5 inhibitor BIX02188 completely reversed u-flow-mediated inhibitory effect without inhibiting ERK1/2, suggesting that u-flow inhibits TNF α -mediated signaling in ECs by a mechanism that depends on MEK5-ERK5 but not MEK1-ERK1/2 activation [38]. The exact molecular mechanism through which u-flow inhibits TNF α -induced JNK activation via activating MEK5/ERK5 needs further investigation [14, 39].

Role of the PKC family in EC dysfunction

The protein kinase C (PKC) family consists of ubiquitously expressed homologous serine/threonine kinases, which are divided into three groups according to their second messenger requirements: classical, novel, and atypical [40–43]. Classical PKCs consist of α , β_1 , β_2 , and γ isoforms and require Ca^{2+} , diacylglycerol (DAG), phosphatidylserine, and phorbol esters for activation. Novel PKCs are δ , ϵ , η , and θ isoforms. These isoforms only need DAG for activation and are Ca^{2+} insensitive. Atypical PKCs consist of ζ and ι/λ isoforms, and they lack the calcium-sensitive C2 domain and contain only one atypical C1 domain. Thus, atypical PKCs are unresponsive to DAG and calcium [40–43].

In terms of the role of classical PKCs in regulating EC functions, ox-LDL reduces PKC α activity and subsequently de-phosphorylates eNOS. De-phosphorylation of eNOS causes its dissociation from the plasma and Golge membrane and also increases the production of eNOS-derived superoxide anion [44]. A PKC β antagonist, CGP53353, inhibits JNK and mitochondrial gene p66Shc activation as well as ROS production in ECs [45–52]. The role of endothelial PKC β in diabetes has been extensively studied. Baseline vascular endothelial PKC β expression is increased in diabetes compared to non-diabetics. The reduced PKC β expression impairs eNOS activation, which can be reversed by a PKC β specific synthetic inhibitor, LY379196 [53]. The possible contribution of PKC β to insulin resistance has also been suggested; insulin-induced eNOS expression is impaired by PKC β -mediated phosphorylation of IRS2 (insulin receptor substrate 2) at S303 and S375 in vascular endothelial cells [54]. The depletion of PKC β in ApoE $^{-/-}$ mice results in decelerated plaque formation with reduced expression of Egr-1 (early growth response protein-1) and inhibits the expression of endothelial cell adhesion molecules and matrix metallo-proteinase-2 (MMP2) [55]. PKC β inhibition by ruboxistaurin reduces plaque lesion size in diabetic mice [56]. However, clinical trials with ruboxistaurin have not shown improved endothelial function, including endothelium-dependent vasodilatation [57]. In terms of the novel PKCs family, treatment with a PKC δ inhibitor or a PKC ϵ activator decreases eNOS phosphorylation and increases EC survival, but the role of this family of kinases in atherosclerosis remains unclear [58].

Among the PKC family members, PKC ζ has recently emerged as an important isoform in ECs. PKC ζ has three functional domains: (1) a PB1 (Phox and Bem1p) domain which constitutes a recently recognized protein–protein interaction domain found in atypical

protein kinase C (aPKC) isoenzymes, PKC ζ , and PKC λ/ι , certain members of MAPKs, such as MEK5, MEKK2, and MEKK3 [59, 60], and in several scaffold proteins involved in cellular signaling; (2) a zinc-finger (ZF) domain for DNA interaction; and (3) a kinase domain. The important role of PKC ζ in regulating EC inflammation via nuclear factor kappa B (NF- κ B) has been reported [61, 62]. Reduced PKC ζ activity has been reported in ECs exposed to u-flow than those under d-flow [63]. The activity of PKC ζ is regulated by proteolytic cleavage. PKC ζ (72 kDa) is processed by caspase 3 to form a 50 kDa fragment (CAT ζ , catalytic domain of PKC ζ) that consists of the catalytic domain [64]. This caspase 3-dependent processing occurs at three aspartate residues (Asp 210, 222, and 239) and frees the enzyme from the auto-inhibitory state by separating the kinase domain (aa 268–335) from the pseudosubstrate auto-inhibitory sequence (aa 116–122) [65]. Since PKC ζ activity is also required for TNF-mediated activation of JNK and caspase-3 in ECs, a positive feedback loop for PKC ζ -caspase 3 activation via regulating CAT ζ formation has been proposed [66]. The inhibitory effect of u-flow on this PKC ζ -caspase 3 positive feedback loop has also been reported, but the exact regulatory mechanisms remain unclear [66] (Fig. 2).

Undisturbed flow (u-flow) induces anti- inflammatory effects in ECs by inhibiting SHP2 phosphatase activity

One of the important regulators of the TNF α pathway is SHP-2, whose activity is required for the activation of JNK and NF- κ B [67, 68]. SHP-2 is a ubiquitously expressed cytosolic phosphatase that contains 2 NH₂-terminus tandem SH2 domains and a carboxyl-terminus catalytic domain [69]. MEKK3 is required for the TNF α -induced c-Jun and NF- κ B transcriptional activity [70, 71]. Our group has found that a constitutively active form of MEKK3 (CA-MEKK3) stimulates c-Jun and NF- κ B transcriptional activity and that SHP2C/S (phosphatase inactive mutant with cysteine 495 mutated to serine), but not wild-type SHP2, inhibits this transactivation [72]. We also found that Gab1 associated with MEKK3 and inhibited MEKK3-induced c-Jun and NF- κ B activation [70]. Since the binding between Gab1 and MEKK3 in vitro was enhanced by the presence of SHP-2C/S, it appears that SHP-2 phosphatase activity regulates the Gab1-MEKK3 association and consequently TNF α signaling in ECs. U-flow significantly inhibited TNF- α -induced SHP-2 phosphatase activity [72]. Therefore, it is possible that u-flow inhibits SHP-2 phosphatase activity and subsequent EC inflammation via keeping the MEKK3-Gab1 interaction intact (Fig. 3).

ERK5 and its transcriptional activity

Compared to other MAPKs, ERK5 has a unique feature, because it has a long COOH-terminus with transcriptional activity domains that are necessary for activating ERK5 downstream transcription factors, including peroxisome proliferator-activated receptors (PPARs) and Kruppel-like factor 2 (KLF2) (Fig. 4) [33, 34, 73]. Members of the nuclear hormone receptor family, PPARs, are known for their anti-inflammatory properties [74]. Currently, there are three known PPAR isotypes, including PPAR α , PPAR δ , and PPAR γ , all of which are expressed in vascular ECs. Activation of PPARs via a ligand-dependent or ligand-independent mechanism has been shown to reduce inflammation by down-regulating

the expression of TNF- α -induced inflammatory adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin [75]. PPARs consist of two transcriptional activation domains residing in the NH₂-terminus A/B domain (activation function-1) and the COOH-terminus E domain (activation function-2). MAPK family members have different effects on these receptors. While Camp et al. reported that the phosphorylation of PPAR γ 1 S82 by ERK1/2 decreased PPAR γ 1 transcriptional activity [76], our study showed that ERK5 up-regulated PPAR γ transcriptional activity through binding to PPAR γ and not by phosphorylating PPAR γ (Fig. 5) [77]. Through intra-molecular bonds, the ERK5 NH₂-terminus inhibits its own transcriptional activity located in the COOH-terminus. U-flow activates MEK5 and promotes MEK5-ERK5 binding, which disrupts the inhibitory effect of the ERK5 NH₂-terminus on its COOH-terminus domain-containing PPAR γ binding site and transcriptional activity. Subsequently, the ERK5 COOH-terminus can bind the hinge-helix 1 region of PPAR γ , which releases SMRT co-repressor from PPAR γ and increases PPAR γ transcriptional activity [77]. Both releasing SMRT and increasing PPAR γ transcriptional activity induced by ERK5 transcriptional activation and subsequent ERK5-PPAR γ binding contribute to u-flow-induced anti-inflammatory effects and inhibit TNF α -induced adhesion molecule expression [77]. Besides its association with PPAR γ , ERK5 is also reported to bind and increase PPAR δ transcriptional activity in skeletal muscle. The ERK5-PPAR δ association appears to be mediated by heme oxygenase 1-induced carbon monoxide, which is known to have a cytoprotective effect. Unlike PPAR γ , PPAR δ has no hinge-helix 1 region. However, the binding of PPAR δ with the middle region of ERK5 is still required to increase PPAR δ transcriptional activity. As noted, ERK5 increases transcriptional activity of both PPAR γ and δ , and thus has an anti-inflammatory property [78].

The KLF family is a group of zinc-finger DNA-binding transcriptional factors that have an important role in regulating vascular homeostasis and EC function [79]. The crucial role of KLF2 in regulating EC function has been well established. First, Kuo et al. have reported that KLF2 is necessary for blood vessel formation and vascular integrity, because KLF2 null mice died of embryonic hemorrhage [80]. Second, See Banerjee et al. have demonstrated that u-flow-induced KLF2 up-regulation increases eNOS expression via binding to the eNOS promoter [81]. Therefore, KLF2 is important for regulating EC-dependent vaso-reactivity. In addition, through competition with NF- κ B for transcriptional coactivator CBP/300, KLF2 inhibits the NF- κ B-dependent expression of VCAM-1 and E-selectin [81]. Third, Lin et al. have reported that KLF2 inhibits inflammation-mediated vascular permeability by its effect on tight junctions [79]. Finally, statins up-regulate the eNOS and thrombomodulin expression via KLF2 [82], and KLF2 expression is induced by u-flow and inhibited by d-flow [83]. These data suggest that KLF2 mediates the u-flow-and statin-induced athero-protection.

Parmar et al. have shown that ERK5 activation is required for u-flow-induced KLF2 up-regulation using a dominant negative form of MEK5 (DN-MEK5) [84]. ERK5/Myocyte enhancing factor 2 (MEF2) is an upstream regulator of KLF2 (KLF2 promoter has a MEF2 binding site) and is activated by ERK5. Young et al. have reported that u-flow activates the AMP-activated protein kinase cascade, which then regulates KLF2 through activating MEF2 [73]. These data suggest that in addition to PPARs, the MEF2-KLF2 module is one of the

important down-stream targets of ERK5 and up-regulates eNOS expression and inhibits EC inflammation, especially under u-flow.

To determine the role of endothelial ERK5 in vivo, our group has generated tamoxifen (4-OHT, 4-hydroxytamoxifen)-inducible EC-specific ERK5 knock-out mice (ERK5-EKO) [85]. We found that eNOS expression in ECs was decreased, whereas the expression of VCAM-1 and E-selectin was increased in ECs isolated from 4-OHT-treated mice in comparison with vehicle-treated mice. To evaluate the functional role of endothelial ERK5, we studied leukocyte rolling and acetylcholine-induced vasodilation in mesenteric venules and arterioles of the inducible ERK5-EKO^{+/-} mice. We found that leukocyte rolling in the venule was increased in the 4-OHT-treated group in comparison with the vehicle-treated group, and acetylcholine-induced dilation of arterioles was significantly decreased in both inducible heterozygous ERK5-EKO^{+/-} and homozygous ERK5-EKO^{-/-} mice in comparison with the four-OHT-treated control mice. To examine the role of endothelial ERK5 in atherogenesis, inducible ERK5-EKO-LDLR^{-/-} mice were fed a high-cholesterol diet for 16 weeks. We found a significant increase in both the surface area and the size of lesions in the aortic root of these induced mice in comparison with non-induced mice. These data suggest a critical role of ERK5 in EC dysfunction and atherosclerotic plaque formation. Although we have shown that ERK5 is anti-inflammatory using EC-specific ERK5 KO mice [85], Wilhelmssen et al. reported that ERK5 was pro-inflammatory when TLR2 (Toll-like receptor 2) was activated [86]. This controversy may be reconciled by different ERK5 post-translational modifications, as we will discuss in the following sections.

Flow and hippo pathway

The vital role of the hippo pathway in the regulation of organ size, cardiac development and hypertrophy, stem cell pluripotency, and tumorigenesis has been extensively studied [87–89]. However, to our knowledge, the role of this pathway in EC dysfunction as well as in atherosclerosis remains largely unknown. The core of the mammalian hippo pathway is composed of two pairs of kinases: mammalian Ste20-like kinases 1 and 2 (MST1/2) and LATS1/2, which are localized in the cytoplasm [90, 91]. MST1/2 kinases bind to their regulatory protein WW45 to form active enzymes and phosphorylate and activate LATSS [92]. In addition, MST1/2 phosphorylate MOB1 (the Mps One Binder kinase activator protein 1), which strongly interacts with LAST, thereby enhances the interaction between Mob1 and LATSS [93]. The MST1/2-LATSS Hippo pathway controls the activity of two closely related transcriptional coactivators: YAP and a transcriptional coactivator with a PDZ-binding motif (TAZ). Activated LATSS phosphorylate YAP S127, which allows 14-3-3 proteins to bind and sequester YAP to the cytoplasm [90, 91]. De (or un)-phosphorylated YAP and TAZ translocate into the nucleus and act as transcriptional coactivators by binding to the TEA domain-containing transcription factor (TEAD) and drives a transcriptional program that promotes proliferation [94] and inhibits apoptosis [90, 95, 96], which are key events for controlling organ size.

The contribution of YAP and TAZ to mechano-signaling has been suggested. Dupont et al. have reported that when a cell spreads over the extracellular matrix, the cytoskeletal adaptation to the spread-out cell shape (including formation of filamentous actin [F-actin]

stress fibers) induces YAP/TAZ nuclear accumulation and activation, which promotes proliferation and inhibits apoptosis [97]. In contrast, when adhesion is restricted to a very small extracellular adhesive area, its anti-apoptotic functions are lost [88, 97]. Interestingly, the MST1/2-LATSs pathway is dispensable for the YAP/TAZ nuclear translocation and activation, and the inhibitory role of LATSs in YAP activation is cell-type dependent [87]. Since the role of LATSs and YAP/TAZ activation in EC mechano-response remains unclear, a detailed evaluation using ECs is necessary.

Flow, SREBP2, and inflammasome

The inflammasome is a protein complex that serves as a platform for the maturation of caspase-1, leading to the proteolytic maturation and secretion of IL-1 β and IL-18. There are three essential components of the inflammasome, which are (1) a sensor protein, (2) the adapter protein ASC, and (3) the inflammatory protease caspase-1. The response of the inflammasome to various stresses is decided by sensor protein, such as the Nod-like receptor (NLR) family (NLRP1, NLRP3, and NLRP4) and AIM2, which function as pathogen sensors and form an inflammasome complex. NLRP3 is unique in that it responds to numerous diverse physical and chemical triggers, unlike other NLRP members, for example, extracellular ATP, reactive oxygen species (ROS), uric acid crystals, cholesterol crystals, and IAPP (islet amyloid polypeptide) [98, 99]. Shyy and colleagues reported that unlike u-flow, d-flow increased NLRP3 expression and subsequently cleaved caspase-1 and increased IL-1 β expression [100, 101]. Not only increasing IL-1 β expression but also pyroptosis (a regulated form of necrosis) can be induced by caspase-1 activation as we will explain later in this review. Shyy's group also reported the crucial role of SREBP2 transactivation in the d-flow-induced expression of NLRP3 and Nox2 [100, 101]. In fact, they also showed that the levels of cleaved caspase-1 and expression of IL-1 β and NLRP3 were higher in the aortic arch (predominantly exposed to d-flow) than in thoracic aorta (area exposed to u-flow), supporting the crucial role of d-flow-induced NLRP3 inflammasome activation in the development of atherosclerosis.

SREBPs, including SREBP-1a, -1c, and -2, are regulators of transcription of a number of genes involved in the cellular sterol and lipid homeostasis. In particular, SREBP2 is ubiquitously expressed, is a relatively selective activator of cholesterol synthesis, as opposed to fatty acid synthesis, and regulates HMG-CoA reductase and low-density lipoprotein receptor genes [102, 103]. SREBP2 regulates endothelial inflammation via increasing NLRP3 and Nox2 expression, which is not directly related to the regulation of lipid homeostasis [104]. In addition to SREBP2, d-flow can also cause sustained SREBP1 activation with nuclear localization. In contrast, u-flow induces only a transient nuclear translocation of SREBP [105]. It appears that SREBP family of proteins exhibit different responses to u-flow and d-flow, suggesting that they may be involved in determining EC responses to different flow types.

SREBPs are the bHLH-LZ family of transcription factors, and inactive newly synthesized SREBPs (pre-SREBPs) are cleaved by proteases, releasing the NH₂-terminus portion (SREBP-N). This mature form of SREBPs (SREBP-N) enters the nucleus and binds the sterol regulatory element 1 (SRE-1), and activates transcription of target genes [102]. There

are two possible steps at which flow can regulate SREBP activation: one is when pre-SREBPs are cleaved in the cytoplasm and the second is to regulate nuclear SREBP-N transcriptional activity. It has been suggested that d-flow can affect both steps and increase SREBPs activation. For example, d-flow may increase SREBP-N transcriptional activity via increasing Akt and mammalian target of rapamycin complex 1 (mTORC1) kinase activation [106]. In addition, Akt activation can also attenuate ubiquitination and proteosomal degradation of active SREBP-N, because Akt can inhibit GSK3 kinase activity, which directly phosphorylates SREBP-N and enhances SREBP-N degradation [106].

D-flow and regulated form of necrosis

As we discussed above, d-flow activates inflammasomes, and recently, it has been shown that activation of the caspase 1-inflammasome induces pyroptosis, a regulated form of necrosis (canonical inflammasome pathway) [107] (Fig. 1). Although pyroptosis has not been well studied in ECs, it is reasonable to discuss the possible role of the regulated form of necrosis in endothelial dysfunction. Necrosis was once recognized as an “accidental” or “mechanical” cell death induced by physio-chemical stresses [108]. This view has been greatly changed, and it is now clear that necrosis can also take place in a genetically and well controlled manner defined as the “regulated form of necrosis” [108]. Vanden Berghe et al. have defined regulated necrosis as “a genetically controlled cell death process, which leads to cellular leakage” [108]. Pyroptosis is a form of necrosis and is characterized by pore formation in the plasma membrane, cell swelling, and membrane disruption [108, 109]. Jorgensen et al. have defined pyroptosis by the following four criteria [107]: (1) programmed by an inflammatory caspase activation; (2) pore formation in the plasma membrane; (3) TUNEL positivity for DNA damage at a lower intensity than apoptosis; and (4) ADP-ribose polymerase activation following the pyroptosis-mediated DNA damage. Not only caspase 1 but also the possible involvement of caspase 4/5/11 in regulating pyroptosis was also suggested [110]. Caspase 1 can be activated by various extracellular stimuli, including ATP, cholesterol crystals, double-strand DNA, and flagellin via several different types of inflammasomes, which has recently been reviewed [107, 111]. In contrast, caspase 4/5/11 in the noncanonical inflammasome has been reported to directly sense cytosolic LPS (Fig. 6). The caspase 4/5/11 CARD binds LPS, presumably via the acidic phosphate moiety of lipid A, and causes caspase 4/5/11 CARD oligomerization and activation [107, 112, 113]. GSDMD and caspase 4/5/11 are required for cytosolic LPS-induced pyroptosis and the processing and secretion of IL-1 β . Both caspase 1 and caspase 4/5/11 directly cleave the 53-kDa inactive precursor form of GSDMD (Pro-GSDMD) to generate the pro-pyroptotic NH₂-terminus fragment (GSDMD p30) [109, 114].

In the canonical pathway, caspase 1 activation induced by various types of inflammasomes triggers pyroptosis by releasing GSDMD p30, which bears intrinsic pyroptosis-inducing activity (Fig. 6) [109, 114]. In the non-canonical pathway, a direct activation of caspase 4/5/11 induced by cytosolic LPS cleaves pro-GSDMD and converts it to the mature GSDMD p30 form, which is sufficient for inducing pyroptosis [109]. However, it remains unclear how GSDMD p30 can induce pyroptosis. Pore formation in the plasma membrane is a feature of pyroptosis, and it is suggested that GSDMD p30 may directly induce the pore formation

[115], but this needs further investigation. Since d-flow increases NLRP3-caspase1 activity, it will be intriguing to investigate if d-flow can induce pyroptosis.

ER stress and flow

The accumulation of misfolded proteins during protein synthesis in the endoplasmic reticulum (ER) is an endogenous cellular source of stress (ER stress). As the cellular defense mechanism against ER stress, three axes of signal transduction pathway termed the unfolded-protein response (UPR) exist in higher eukaryotic cells. In the unstressed state, immunoglobulin-heavy-chain-binding protein [BIP, also known as heat-shock protein A5 (HSPA5) and glucose-related protein 78 (GRP78)] associates with and inhibits the function of the three ER-proximal UPR transmembrane proteins (UPR's three pronged signaling network axes), including (1) activating transcription factor 6 (ATF6), (2) inositol-requiring transmembrane kinase/endonuclease 1 (IRE1), and (3) pancreatic ER kinase (PERK, also known as EIF2AK3). Under the unstressed condition, a newly synthesized and properly folded proteins transit into the ER lumen through the SEC61 channel. When cells are stressed, unfolded, and misfolded proteins start to accumulate in the ER. This causes BIP to detach from ATF6, IRE1, and PERK, releasing its inhibitory effect on them and activates the three axes of UPR signaling.

1. **ATF6:** ATF6 contains a Golgi-localization sequence (GLS), which becomes exposed after ATF6 is released from BIP, and ATF6 translocates to the Golgi apparatus, where it is cleaved by S1P and S2P, respectively). The ATF6 fragment (ATF6f) transcription factor is released from the Golgi body and translocates to the nucleus, where it binds ER stress response elements (ESRE) and induces the expression of BIP, CHOP (CCAAT/enhancer-binding protein homologous protein), and X-box binding protein (XBP-1).

Civelek et al. have reported that d-flow induces the activation of adaptive UPR through ATF6 α and IRE1 α . In contrast, PERK, the third regulatory axe of UPR, as we will explain later, appeared not to be involved in this adaptive response [116].

2. **IRE1:** Dissociation of IRE1 from BIP results in homodimerization of IRE1 and activation of its endoribonuclease activity, which excises a 26 base-pair fragment from XBP-1 mRNA and forms spliced XBP1 (sXBP1) mRNA. sXBP1 protein translocates to the nucleus and binds UPR elements (UPREs), which induces the transcription of a broad array of UPR genes, thereby increasing the synthesis and secretion of UPR proteins, including ER-associated degradation (ERAD) protein EDEM, ER-localized DNAJ4 (ERdj4), and p58^{IPK} [117–119]. It has been suggested that d-flow-induced EC proliferation and apoptosis are mediated by sXBP1-induced VE-cadherin down-regulation. For example, in ApoE^{-/-} mice, BrdU incorporation was significantly higher in ECs exposed to d-flow at the aortic arch than those exposed to laminar flow in the descending aorta, and sXBP-1 expression was higher in proliferating ECs than in quiescent cells. Furthermore, EC proliferation was inhibited when XBP1 and IRE1 were depleted [120]. It has been reported that d-flow decreases VE-cadherin

expression via activating XBP1 splicing, leading to VE-cadherin transcriptional repression and increased degradation [120]. The reduced VE-cadherin at the adherens junction releases β -catenin which then translocates into the nucleus and promotes transcription of genes responsible for cell proliferation. In addition, the reduction of VE-cadherin can activate a multiple number of caspases and up-regulate apoptosis, which can form a positive feedback loop and further decreasing VE-cadherin expression by shedding [120–122]. In addition to its endoribonuclease activity, phosphorylated IRE1 also leads to activation of JNK, through TRAF2 (tumor necrosis factor-receptor-associated factor 2) and ASK1 (apoptosis signal-regulating kinase 1), which can lead to apoptosis via caspase 12 activation and inflammation by association of IKK with the IRE1-TRAF2 complex and activating IKK-NF- κ B signaling.

3. PERK: BIP dissociation causes dimerization and autophosphorylation of PERK and activated PERK phosphorylates Ser51 of eukaryotic translation initiation factor 2a (eIF2a), causing translation arrest of most proteins except ATF4. The phosphorylation of eIF2a up-regulates the expression of ATF4, which translocates to the nucleus and induces apoptosis via increasing CHOP expression. Phosphorylation of eIF2a induced by PERK activation induces NF- κ B activation [123]. Although it has been reported that u-flow can inhibit eIF2a phosphorylation [124] and subsequent NF- κ B activation, the exact mechanism remains unclear.

In summary, a low level of ER stress can be a protective mechanism for cells, because it accelerates degradation of misfolded proteins (via ERAD), impairs new protein synthesis (via PERK-mediated eIF2a phosphorylation), and induces the expression of chaperons and folders to promote protein folding (via ATF6 and IRE1-mediated sXBP-1 induction). However, excess ER stress enhances apoptosis signaling via the two axes of UPR signaling networks of PERK and ATF6/IRE1. In addition, d-flow-induced EC proliferation can be explained by sXBP-1-mediated VE-cadherin reduction.

SUMOylation

SUMOylation is a post-translational modification in which an SUMO (small ubiquitin-like modifier) protein is covalently conjugated to a lysine residue of a target protein. SUMOylation regulates various protein functions, including sub-cellular localization, protein–protein interaction, and DNA binding amongst others. SUMOylation is also important for a wide range of cellular processes including transcriptional regulation, epigenetic regulation, signal transduction, cell proliferation, apoptosis, and cell cycle regulation [125]. There are four SUMO isoforms (SUMO 1–4) in human, and SUMO1 shares ~50% sequence identity with SUMO2, while SUMO2 and three differ by only three NH2-terminus residues (97% identity). SUMO2/3 are considered to form a subfamily, and their function is thought to be different from that of SUMO1 based on the sequence difference. SUMO1-3 are ubiquitously expressed, whereas SUMO4 is limited to kidneys, lymph nodes, and spleen [126, 127]. The functional role of SUMO4 remains controversial, because the COOH-terminus proline residue, which is unique to SUMO4, inhibits the

maturation of SUMO4 into the conjugatable form (or modifier) [128]. SUMOylation is achieved via a three-step enzyme process that involves deconjugation and conjugation enzymes (Fig. 7) [129]. The first step is to convert precursor SUMO to mature SUMO by SUMO proteases sentrin/SUMO-specific proteases (SENPs), whose proteolytic action exposes the di-glycine COOH-terminus motif. The processed SUMO is then activated by the E1-activating enzyme (SAE1–SAE2 heterodimer) in an ATP-dependent manner [130]. In the second step, activated SUMO is transferred to the E2-conjugating enzyme (Ubc9), forming a thioester bond between Ubc9 and SUMO. In the third and final steps, SUMO E3 ligases catalyze the transfer of SUMO from the Ubc3-SUMO complex to the target substrate containing the free ε-amino group of a lysine residue [131]. Reversibility of protein SUMOylation is achieved by de-SUMOylation enzymes called sentrin/SUMO-specific proteases (SENPs; SENP1–7), which are also important for SUMO processing. SENP1 and 2 contain both nuclear localization and export signal domains, and shuttling of SENPs from one compartment of the cell to another has an effect of altering SUMOylation levels in different sub-cellular regions as we will discuss in the subsequent sections in this review. SUMO modification of substrates is known to play a major role in the following molecular events and processes: (1) SUMOylation provides a platform to recruit molecules which non-covalently bind to SUMO via the SUMO-interacting motif (SIM); (2) SUMOylation can promote or block the association of molecules that interact with SUMOylated substrates; (3) SUMOylation can regulate substrate stability by competing for the lysine site with ubiquitination or degradation by recruiting the SUMO-targeted ubiquitin ligase (STUbL) family of proteins to the SUMOylated substrates; and (4) SUMOylation induces conformational changes in proteins, so that their interaction with other molecules can be regulated [132, 133].

ERK5 SUMOylation

As we have summarized earlier in this review, u-flow-mediated ERK5 activation leads to the up-regulation of ERK5 downstream transcription factors KLF2 and PPARs with resulting anti-inflammatory and athero-protective effects [134]. On the contrary, hydrogen peroxide (H₂O₂), the most important reactive oxygen species, and advance glycation end products (AGE) promote EC inflammation via inhibiting ERK5 transcriptional activity. To elucidate the molecular mechanism of H₂O₂/AGE-mediated inhibition of ERK5 transcriptional activity, our group investigated the role of ERK5 SUMOylation (Fig. 8) [135]. When cultured ECs were treated with H₂O₂, ERK5 was SUMOylated at Lys6 and 22 (K6/22), which subsequently inhibited ERK5 transcriptional activity and the expression of KLF2 and eNOS. We also found that d-flow increased, but u-flow decreased, ERK5 SUMOylation, suggesting that ERK5 SUMOylation can be a key switch to distinguish the response of endothelial cells to these two different types of flow.

p53 SUMOylation

In addition to promoting EC inflammation, d-flow is known to induce EC apoptosis. The increased EC apoptosis results in increased EC turnover [136, 137]. In contrast, u-flow inhibits EC apoptosis [138]. Transcription factor p53 has been demonstrated to play a key role in promoting cell death by up-regulating the expression of pro-apoptotic factors or promoting cell arrest (for DNA repair) when DNA damage occurs to prevent the further

DNA damage [139]. The role of p53 in EC proliferation under u-flow was investigated by Lin et al. who observed that the expression of p53 was increased via JNK activation with resulting increases in p21 expression and GADD45 (growth arrest and DNA damage inducible protein 45), causing EC growth arrest [140]. p21 expression is known to inhibit RB phosphorylation thus inhibiting progression of cells into S-phase. GADD45 causes cell arrest in the G0–G1 and G2–M phases of the cell cycle. Of note, in addition to inhibiting cell proliferation, p21 can inhibit apoptosis via down-regulating glutathione peroxidase and superoxide dismutase anti-oxidant activity [139, 141, 142]. Taken together, p53 causes EC growth arrest during u-flow which could also inhibit EC apoptosis [140]. Importantly, it should be noted that these effects of p53 on ECs occur when p53 is localized within the nucleus.

In contrast to u-flow, when cultured ECs are exposed to d-flow, we have found that p53 is exported from the nucleus to the cytoplasm and that this nuclear export is regulated by PKC ζ -mediated p53 SUMOylation (Fig. 9) [137]. First, d-flow activates PKC ζ via increasing reactive oxygen species production. The activation of PKC ζ enables its COOH-terminus kinase domain (aa401–587) to associate with the RING domain of PIASy (protein inhibitor of activated STATy). The PIASy RING domain contains the catalytic SUMO ligase site, and the PIASy-PKC ζ association increases PIASy enzymatic activity, probably by changing its tertiary structure. As the result, the PIASy-PKC ζ association causes an increase in p53 SUMOylation. Once SUMOylated, p53 is exported to the cytoplasm from the nucleus [137]. In the cytoplasm, p53 induces apoptosis through a direct interaction with Bax and Bcl-2, which blocks the Bax/Bcl-2 anti-apoptotic function and subsequently induces EC apoptosis (Fig. 8) [137, 143]. These data suggest the crucial role of p53 SUMOylation in regulating d-flow-induced EC apoptosis (Fig. 9).

Role of SENP2 in d-flow-induced ERK5 and p53 SUMOylation

SUMO-specific proteases (SENPs) are required for processing inactive pro-form SUMO proteins to mature forms for conjugation as well as controlling deconjugation from substrate proteins [129]. Of the six SENP isoforms that exist in humans (SEN1–3 and SEN5–7), we characterized the functional role of SENP2 in controlling d-flow-induced SUMOylation of ERK5 and p53 [135]. When SENP2 is deleted, d-flow-induced EC inflammation and apoptosis are up-regulated. In addition, the accelerated EC inflammation and apoptosis are likely due to increased SUMOylation of p53 and ERK5, respectively. This notion is supported by experiments using SUMOylation mutants of ERK5 and p53, which, when overexpressed in cultured ECs, inhibit d-flow-induced EC inflammation, EC apoptosis as well as the expression of inflammatory cell adhesion molecules. Moreover, aortas from SENP2 knock-out mice exhibit increased atherosclerotic plaque formation. As such, one might expect d-flow to decrease SENP2 expression. However, the expression of SENP2 is not regulated by d-flow. Because SENP2 has several nuclear localizations (NLS) and nuclear export signals (NES), one possibility is that SENP2 localization could affect its ability to de-SUMOylate substrates in different sub-cellular compartments. Our group has reported the significant role of p90RSK (p90 kDa ribosomal S6 kinases) in accelerating SENP2 nuclear export [144] via up-regulating SENP2 T386 phosphorylation [135, 144].

p90RSK is a unique serine/threonine kinase that contains two functional catalytic domains: the NH₂-terminus and the COOH-terminus kinase domains [145]. The NH₂-terminus kinase domain appears to belong to the AGC group of kinases (i.e., PKC and PKA) and phosphorylates its substrates, while the COOH-terminus kinase domain is a member of the calcium/calmodulin-dependent kinase group and regulates mainly the activation of the p90RSK NH₂-terminus kinase [146]. Recently, we identified de-SUMOylation enzyme SENP2 as one of the substrates for p90RSK [144]. p90RSK phosphorylates SENP2 T368, and increases SUMOylation of p53 and ERK5 in the nucleus (Fig. 8). We assume that the nuclear export of SENP2 induced by p90RSK may also contribute to up-regulate nuclear p53 and ERK5 SUMOylation. In this study, we also established the role of the endothelial p90RSK-SENP2 module in regulating d-flow-induced atherosclerotic plaque formation in mice [144].

Other SUMOylation events, which are crucial for regulating endothelial functions

MK2 (MAPK-activated protein kinase-2)

MK2 is a pro-inflammatory kinase that enhances NF- κ B activity by blocking nuclear retention of p38 and preventing excessive phosphorylation of MSK1 (mitogen-and stress-activated protein kinase 1). Since MSK1 phosphorylates p65 to facilitate the nuclear export of the latter, reduced MSK1 activity results in the nuclear retention and sustained activation of NF- κ B. In fact, it has been reported that the depletion of MK2 significantly decreases TNF- α -induced inflammatory responses in ECs and plaque formation in Ldlr^{-/-} mice with the reduced expression of VCAM-1 and MCP-1, suggesting that activation of endothelial MK2 is pro-atherogenic [147]. Our group has previously reported that MK2 K339 SUMOylation inhibits its kinase activity and subsequent actin filament remodeling and TNF α -mediated inhibition of EC migration [148]. Although the role of MK2 SUMOylation in endothelial inflammation remains unclear, these data suggest that MK2 SUMOylation can regulate endothelial functions.

I κ B and NEMO/IKK γ

In endothelial cells, NF- κ B plays a crucial role in increasing EC inflammation and has a pro-atherogenic role. NF- κ B is the collective name for a family of transcription factors with five members: c-Rel, RelB, p65 (RelA), p105/p50, and p100/p52. NF- κ B dimers are kept inactive by association with inhibitory proteins, the inhibitors of NF- κ B (I κ Bs). When inactive, NF- κ B is in the cytoplasm and is bound to I κ B. To activate NF- κ B, I κ B kinase (IKK) is first activated by pro-inflammatory stimuli, including d-flow and cytokines, and phosphorylates I κ B which is then ubiquitinated and subjected to proteasomal degradation. Freed NF- κ B then translocates into the nucleus and transactivates pro-inflammatory genes [149]. IKKs form a complex with two kinases, called IKK1 (or IKK α) and IKK2 (or IKK β), and a regulatory subunit named NF- κ B essential modulator (NEMO or IKK γ). NEMO has no catalytic activity but is required for both IKK activation and its subsequent phosphorylation of I κ B [150]. When NEMO is deleted, IKKs cannot efficiently phosphorylate I κ B and activate NF- κ B signaling [151]. Gareus et al. have reported that

endothelial specific depletion of NEMO or dominant negative I κ B α inhibited atherosclerotic plaque formation, demonstrating the crucial role of NF- κ B signaling in atherosclerosis [152].

SUMOylation of I κ B α and NEMO has been reported. SUMO1 modified I κ B α is protected from ubiquitination and degradation, thus down-regulating NF- κ B activation [153]. In contrast, modification of I κ B α by SUMO2/3 confers the opposite effect and dissociates I κ B α from NF- κ B, leading to NF- κ B activation [154]. However, the exact vascular pathophysiological role of I κ B α SUMOylation remains unclear.

Miyamoto and his colleagues have reported that genotoxic stress induces SUMOylation (SUMO1) of NEMO, leading to increased IKK-NF- κ B activation [155–161]. Interestingly, a de-SUMOylation enzyme, SENP2, is a downstream transcriptional target of NF- κ B thereby creating a negative feedback mechanism for NF- κ B activation through NEMO de-SUMOylation [162]. SENP6 inhibits Toll-like receptor-triggered inflammation via de-SUMOylation of NEMO at K277 [163]. However, the role of NEMO SUMOylation in flow-induced signaling and atherosclerotic plaque formation has not been well-characterized and awaits further investigation.

Adenosine monophosphate-activated protein kinase (AMPK) and LKB1

AMPK is a stress-activated kinase which can orchestrate the cellular response to a variety of stresses in the heart by regulating metabolism, protein synthesis, degradation, autophagy, and apoptosis [164]. AMPK is a complex of three subunits: a catalytic subunit (α) containing a serine–threonine kinase domain (KD) with a Thr172 phosphorylation site which is the target of liver kinase B1 (LKB1) and calcium-calmodulin-activated protein kinase kinase- β (CAMKK β) and two regulatory subunits (β and γ) [164]. In unstressed cells, AMPK is mainly localized in the cytoplasm but can translocate to the nucleus after its activation [164]. It has been reported that u-flow but not d-flow activates AMPK. The s-flow-induced AMPK activation inhibits Akt-mTOR-S6K signaling and subsequently causes EC arrest in the G0/G1 phase [165, 166]. In addition, AMPK can suppress ER stress by decreasing mitochondrial ROS production and activating eNOS [166, 167]. The role of AMPK in regulating EC inflammation and subsequent atherogenesis is isoform dependent; AMPK α 1 is pro-inflammatory and AMPK α 2 is anti-inflammatory. Indeed, reduced atherosclerotic plaque formation was reported in AMPK α 1^{-/-}/ApoE^{-/-} [168], while it increased in AMPK α 2^{-/-}/LDLR^{-/-} mice [166]. One of the substrates of AMPK α 2 is poly [ADP ribose] polymerase 1 (PARP1) [169]. Originally, PARP1 was recognized as the molecule that utilized NAD⁺ to synthesize poly(ADP)-ribose (PAR) and “PARylated” itself and other nuclear proteins [170]. In addition to PARylation, PARP1 is now known to directly associate with a specific DNA sequence TTGATATAAAT within target genes via its zinc-finger domain [171]. PARP1 is involved in multiple cellular functions, such as repair of single- and double-strand DNA breaks, RNA interference, mitochondrial function, and cell division. In terms of inflammation, PARP1 can play as a coactivator of NF- κ B and activate pro-inflammatory pathways, such as p38 MAPK and JNK pathways, and inhibit B cell lymphoma-6 (Bcl-6)-induced anti-inflammatory function [169, 172–176]. In addition, PARP-1 represses Bcl-6 transcription by sequence-specific binding to the first intron of the

Bcl-6 gene, and PARP-1 knockdown induces *Bcl-6* expression [171]. Shyy and his associates have reported that AMPK α 2 phosphorylates PARP1, which then dissociates from the *Bcl-6* intron 1. This up-regulates *Bcl-6* transcription, and in ECs, the increased *Bcl-6* expression inhibits the expression of VCAM-1, MCP-1, and MCP-3 and down-regulates inflammatory responses [169]. In contrast, AMPK α 1 has been suggested to mediate the expression of pro-inflammatory cytokines by activating NF- κ B [177, 178]. Further investigation is necessary to determine how AMPK α 1 and α 2 play opposite roles in regulating NF- κ B activation.

U-flow increases LKB1 activity. Zhang et al. have reported that endothelium-specific LKB1 deletion decreases eNOS activity and impairs endothelial functions, causing hypertension and cardiac hypertrophy in mice [179]. They also found that the depletion of LKB1 inhibited AMPK activation and induced human antigen R (HuR) nuclear export. HuR binds to and stabilizes caveolin-1 mRNA and enhances caveolin-1 expression, and at the same time, it inhibits eNOS catalytic activity. It is unclear what the functional relationship is between LKB1 and the two AMPK isoforms in ECs exposed to d-flow.

Rubio et al. have reported that AMPK β 2 SUMOylation by SUMO2 activates AMPK and inhibits its ubiquitin-dependent degradation [180]. In contrast, AMPK α 1 SUMOylation inhibits AMPK activity specifically for activating mTORC1 signaling [181]. It remains unclear how AMPK α 1 and AMPK α 2 SUMOylation coordinately regulate AMPK kinase activity. Yeh and his group have recently shown that LKB1 K178 SUMO1 modification promotes LKB1 association with AMPK α SUMO-inter-acting motif (SIM) and accelerates AMPK activation [182].

Dynamin-related protein 1 (DRP1)

It has been reported that aberrant flow inhibits the function of mitochondrial respiratory complexes I, II/III, and IV as early as 5 min from the flow onset, and this inhibition is due to increased production of ONOO⁻ [183]. In addition, aberrant flow can induce mitochondrial fission via regulating DRP-1 sub-cellular localization. Mitochondria are dynamic organelles, and their overall morphology changes in response to cellular activity via processes called fission and fusion (i.e., elongation) [184]. These morphological changes are under the tight regulation dictated by the physiological state of the cell [185, 186]. Mitochondrial fusion is described as integrating the outer and inner membranes of one mitochondrion with those of another mitochondrion, while mitochondrial fission is achieved by pinching off of a mitochondrion. The crucial role of the GTPase called DRP1 in the process of fission has been well established [187, 188].

SUMOylation of DRP1 regulates this GTPase and thus also regulates mitochondrial fission. DRP1 SUMOylation (SUMO1) up-regulates mitochondrial fission and causes hyper-fragmentation of mitochondria [189–191]. In Cos7 and HeLa cells, mitochondrial hyper-fragmentation resulted in apoptosis [192–194]. Cytochrome c functions as the terminal trigger for apoptotic cell death and is located in the intermembrane space of mitochondria [195]. Release of cytochrome c has been shown to depend on SUMOylation (SUMO1) of DRP1 [196]. The level of DRP1 SUMOylation can be reduced by de-SUMOylation enzymes, including SENP5, SENP3, and SENP2 [197, 198]. DRP1 can be SUMOylated not

only by SUMO1 but also by SUMO2 and 3. The functional consequence of DRP1 SUMO1 vs SUMO2/3 modification can be different.

Other post-translational modifications of ERK5

ERK5 S496 phosphorylation by p90RSK

Upon activation, p90RSK is able to phosphorylate transcription factors, such as CREB, NF- κ B, and c-fos, and increases their transcriptional activity [85, 144, 199]. In our study, we found that H₂O₂ (used as ROS) activated p90RSK, leading both to p90RSK binding to the COOH-terminal transcriptional domain (aa 571–807) of ERK5 and to phosphorylation of ERK5 S496 with the subsequent inhibition of ERK5 transcriptional activity [85]. The inhibition of ERK5 transcriptional activity decreased the expression of KLF2 and eNOS but increased adhesion molecule expression, leading to EC dysfunction with accelerated atherosclerotic plaque formation. The same phenotypes were observed in EC-specific ERK5 knock-out mice. The role of p90RSK in EC dysfunction was further studied using the specific p90RSK inhibitor, FMK-MEA. In this study, we found that the inhibitor decreased adhesion molecule expression and increased eNOS expression, which are anti-atherogenic phenotypes. However, when EC-specific ERK5 knock-out mice were treated with FMK-MEA, the anti-atherogenic effects of FMK-MEA were no longer observed. Our study suggests that ERK5 is necessary for the p90RSK inhibitor to exert its anti-atherogenic effect. Taken together, we have demonstrated that d-flow-induced p90RSK activation (higher expression noted in aortic arch) inhibits ERK5 transcriptional activity, which is critical for atherosclerotic plaque formation, and the inhibition of this process by FMK-MEA prevented atherosclerotic plaque formation [85].

ERK5 S486 phosphorylation by PKC ζ and p62

PKC (protein kinase C) is a family of serine–threonine kinases that phosphorylate numerous proteins, thus regulating their intercellular signaling. One isomer of this family is PKC ζ , which, as previously mentioned, has been associated with d-flow-induced p53-SUMOylation with resulting EC apoptosis. In addition, we found that PKC ζ can phosphorylate ERK5 S486, which then triggers degradation of eNOS protein, although the precise mechanism that ties these events will require future studies. Our group has also found that p62 is an important molecule implicated in TNF α -mediated PKC ζ activation [200]. p62, a scaffold protein, contains a PB1 (Phox/Bem1p) domain on its NH₂-terminus region that can interact with other PB1 domain-containing proteins [201]. PKC ζ also has a PB1 domain, and we have found that TNF α induces p62 association with PKC ζ , thus activating PKC ζ and resulting in EC apoptosis. The p62-PKC ζ interaction is required for PKC ζ -induced JNK activation and caspase three cleavage, but how this association fits into the d-flow and u-flow-induced signaling needs further investigation [200]. Evidence provided by these studies suggests that PKC ζ activation mediates EC inflammation and apoptosis and plays an important role in atherogenesis.

Blood flow and epigenetics

Epigenetics is defined as the modification in gene expression, usually through alterations in the DNA conformation without changing the DNA code itself [202]. The most studied epigenetic alterations are DNA methylation and histone modifications. Genomic DNA is found in two forms: heterochromatin and euchromatin. Heterochromatin is a tightly packed, highly methylated DNA associated with the events of histone deacetylation. In contrast, euchromatin consists of unmethylated DNA arranged in a more loose conformation in association with acetylated histones [203]. Fluid shear stress has been shown to regulate chromatin remodeling and histone modifications through several signaling events involving methylation, phosphorylation, SUMOylation, and acetylation [204, 205]. In this section, we will focus on the role of d-flow in regulating DNA methylation and histone acetylation.

DNA methylation

In mammals, DNA methylation occurs in a 5' carbon of a cytosine base pair usually in the setting of CpG (cytosine-phosphate-guanine) dinucleotides [206, 207]. CpG islands are regions made of at least 200 base pairs, a CpG percentage greater than 50%, and an observed CpG/expected CpG in excess of 0.6 [208]. Around 50% of human genes contain CpG islands in their promoter regions, these islands are usually unmethylated and, therefore, transcriptionally active [209, 210]. Methylation of a cytosine residue at the promoter region results in transcriptional gene silencing [211]. Apart from CpG islands, CG sites are scant. This occurs, because genomic 5-methylcytosine residues are hotspots for spontaneous deamination into thymine, a phenomenon known as CG suppression [212]. DNA methyltransferases are the enzymes that catalyze the transfer of methyl groups to DNA. There are three main enzymes located on different chromosomes: DNMT1, DNMT3A, and DNMT3B. DNMT3A and DNMT3B are known as de novo methyltransferases. These enzymes can add methyl groups to cytosine residues of completely unmethylated DNA. DNMT1 is known as a maintenance methylase, because this enzyme can only add methyl groups to cytosine residues of partially methylated DNA [213]. DNMT1 has also been found to have de novo methyltransferase activity [214]. Jiang et al. reported a down-regulation in KLF4 expression in swine and human aortic ECs exposed to d-flow [215]. They found that d-flow increases the expression of DNMT3A, which methylates the CpG islands at the myocyte enhancer factor binding site within the KLF-4 promoter. Hyper-methylation of this site leads to decreased interaction of MEF2 with the KLF-4 promoter, abolishing KLF4 expression. In addition to KLF2, KLF4 is also one of the zinc-finger regulatory transcription factors for the gene networks that confer the athero-resistant, anti-inflammatory, and anti-thrombotic properties to the endothelium [216–218]. Therefore, the reduction of KLF4 induced by the hyper-methylation can be pro-atherogenic. The inhibitory effect of d-flow on KLF4 transcription is mitigated by RG-108 and 5-Aza (DNMT inhibitors) and DNMT3A knockout [204, 215]. In addition to DNMT3A, Dunn et al. have found that d-flow created by a partial carotid ligation in mice also up-regulates DNMT1 expression in ECs [204] (Ref). They have also reported that 5-Aza is able to inhibit d-flow-induced atherosclerosis and that d-flow induces an increase in DNA methylation in 11 gene promoters (HOXA5, KLF3, TMEM184B, ADAMTS15, CMKRL1, PKP4, ACVRL1, DOK4, SPRY2, ZFP46, and F2RL1), which can be reversed by 5-Aza. Interestingly, the methylated region in 5 of the 11

genes (HOXA5, KLF3, CMKLR1, ACVRL1, and SPRY2) contained a cAMP response element (CRE) in their promoters, and these investigators were able to confirm that THOXA5, KLF3, CMKLR1, and ACVRL1 genes were hyper-methylated by d-flow at the CRE CG site. Zhou et al. using human umbilical vein endothelial cells (HUVECs) also found that d-flow increased DNMT1 expression and DNMT1 nuclear translocation [219]. These data suggest that DNA methylation plays a crucial role in regulating d-flow-induced endothelial inflammation and dysfunction.

Histone acetylation

Histones are the basic proteins associated with DNA, forming nucleosomes. Eight core histone proteins consisting of two H2A/H2B dimers and two H3/H4 dimers form nucleosomes. Each nucleosome is made of DNA wrapped around a spool-like structure of the eight histones. The chain of nucleosomes is then wrapped into a 30 nm spiral called a solenoid, where additional H1 histone proteins are associated with each nucleosome to maintain the chromosome structure. Post-translational modifications of the histone NN2-terminus can modulate its interaction with DNA, which is known as the “histone code”.

Certain lysine residues of histone are subjected to acetylation and deacetylation and each catalyzed by histone acetyltransferases (HATs) and deacetylases (HDACs), respectively [205, 220]. There are multiple proteins with HAT activity, which are divided into three family groups: GNAT, MYST, and CBP/p300 families. Usually, histone acetylation is associated with transcription activation. For example, the possible role of p300 HAT in s-flow-induced eNOS expression has been reported [221]. U-flow increases the ability of NF- κ B subunits p50 and p65 to bind the eNOS promoter. p300 HAT activation induced by u-flow increases acetylation of both p65 and histones 3 and 4 localized in the proximity of the eNOS shear stress response element (SSRE). This acetylation of p65 and histones opens the chromatin structure at the SSRE and induces eNOS mRNA expression [221]. D-flow-induced H3 lysine 14 acetylation has been reported, [205], but the pathological meaning and regulatory mechanism remain unclear. In addition, roles of GNAT and MYST in flow-mediated signaling are not clear.

In contrast to histone acetylation, histone deacetylation is associated with gene silencing. HDACs are categorized into class I (HDAC1/2/3) and class II (HDAC5/7), and both expression and nuclear accumulation of class I and II HDACs were induced by d-flow [222]. In addition, the association of HDAC-1/2/3 with NF-E2-related factor-2 (NRF2) and HDAC-3/5/7 with myocyte enhancer factor-2 (MEF2) is induced by d-flow, and these associations down-regulate the activation of NADPH quinone oxidoreductase-1, an anti-oxidant gene, and KLF2, an anti-inflammatory gene, respectively [222]. In contrast, u-flow dissociates HDAC5 from MEF2C and promotes MEF2-dependent KLF2 gene transcription [223]. These data suggest that HDACs play crucial roles in regulating flow-induced EC responses.

Conclusion

It is clear that ECs are differently regulated by different blood flow patterns: d-flow activates pro-atherogenic signaling, while u-flow induces athero-protective responses in ECs. Each

flow type activates different signaling molecules, leading to different sub-cellular localizations of the same molecule and different patterns of gene expression, both of which regulate the proliferation, differentiation, apoptosis, and inflammation of endothelial cells. Thus, the two types of flow are great experimental tools for investigating the pathogenesis of atherosclerosis and other vascular dysfunctions. In fact, as we have discussed in this review, many fruitful accomplishments have been achieved from various flow-based studies, including differential kinase activation, inflammasome activation, DNA methylation, and post-translational modifications of signaling proteins. One of the important future goals will be to determine the interplay among molecular events activated by a specific flow type. How they coordinately regulate and decide the overall cell response under u-flow and d-flow. Although this will be a very complex process, the approach by comparing the signaling events under u-flow and d-flow is still offering us a great opportunity to understand the process of atherosclerosis induced by endothelial dysfunction.

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Fig. 1.

MAPK signaling. *ATF2* activating transcription factor 2, *BAD* BCL2 associated agonist of cell death, *Bim* Bcl-2 interacting mediator of cell death, *cdc25B* cell division cycle 25B, *GLK* germinal center kinase-like kinase, *HGK* hepatocyte progenitor kinaselike/germinal center kinase-like kinase, *HPK1* hematopoietic progenitor kinase1, *HSF1* heatshock transcription factor 1, *LPS* lipopolysaccharide, *MK1/2* MAPK-activated protein kinase 1/2, *MLK* mixed lineage kinase, *MNK* menkes disease-associated protein, *MSK1/2* mitogen-and stress-activated protein kinase1/2, *NFAT* nuclear factor of activated T-cells, *NHE-1* Na⁺/H⁺ exchanger-1, *Nur77* nuclear hormone receptor 77, *PAK1/2* p21 (RAC1)-activated kinase 1/2, *PKC* protein kinase C, *PPARs* peroxisome proliferator-activated receptors, *Sap1* serum response factor accessory protein 1, *TAB 1/2* TGF-beta-activated kinase1-binding protein1/2, *TAK/MAP3K7* TGF-beta-activated kinase, *TRAF* TNF receptor-associated factor 6

Fig. 2.

Flow reduces catalytic domain of PKC ζ (CAT ζ) pro-apoptotic effects. Bovine aortic endothelial cells (BAECs) overexpressing CAT ζ were preexposed or not to flow for 24 h before TNF/cycloheximide (CHX). **a** Caspase-3 activity was estimated by the detection of its cleaved form by Western blot. Anti-HA was used to detect the CAT ζ transfected form. **b** Western blots were quantified by densitometry. All figures are representative of 3 to 4 independent experiments. * $P < 0.05$ vs static TNF/CHX-treated cells. **c** Model: Flow inhibits PKC ζ cleavage and prevents apoptosis. TNF/CHX, via induction of caspase-3 activation, induces PKC ζ cleavage to yield CAT ζ . CAT ζ enhances TNF α -induced JNK and caspase-3 activation leading to a death pathway, where CAT ζ stimulates caspase-3 activation and subsequent PKC ζ cleavage. In contrast, s-flow mediates prosurvival effects via inhibiting caspase-3 and JNK, thus decreasing PKC ζ cleavage [66] (Obtained permission to reproduce from Wolters Kluwer Health)

Fig. 3.
Model of flow and TNF α -mediated signal transduction pathways to regulate adhesion molecule expression in endothelial cells. Reprinted from Lerner-Marmarosh et al. [72], and obtained permission to reproduce from Wolters Kluwer Health

Fig. 4.

Primary structure of extracellular-signal-regulated-kinase 5 (ERK5). ERK5 is the largest member of the mitogen-activated protein kinase family. This Ser/Thr protein kinase has a catalytic NH₂-terminus domain, which contains the MAPK-conserved motif threonine/ glutamate/tyrosine (TEY) in the activation loop that shares about 50% sequence identity with ERK1/2 and a unique long COOHterminus tail. ERK5 activation is induced by the interaction with MEK5, which subsequently dually phosphorylates the TEY motif. In contrast, inflammatory stimuli or athero-prone flow (d-flow) leads to ERK5 deactivation via phosphorylation of Ser486 or Ser496, respectively. The K6 and K22 sites at the NH₂-terminus region with small ubiquitin-like modifier (SUMO) modification inhibit ERK5 transactivation.

Fig. 5. Model of the ERK5-PPAR γ 1 interaction activating PPAR γ 1 activity. The activation of PPAR γ is significantly regulated by Hinge-helix 1, which is sterically altered by ligand binding. When the PPAR γ ligand binds to the receptor, Helix 12-folds back to form a part of the coactivator binding surface, and inhibits co-repressor (such as SMRT) binding to PPAR γ [224]. The co-repressor interaction surface requires Helix 3–5 [225]. We found a critical role of the PPAR γ Hinge-helix 1 domain on ERK5-mediated PPAR γ transactivation [77]. The inactive NH2-terminus kinase domain of ERK5 inhibits its own transactivation and PPAR γ binding. After ERK5 activation, the inhibitory effect of the NH2-terminus domain of ERK5 decreases, and subsequently, the middle region of ERK5 can fully interact with the Hinge-helix 1 region of PPAR γ . The association of ERK5 with the Hinge-helix 1 region of PPAR γ releases co-repressor of SMRT and induces full activation of PPAR γ . AF-1/2: Activating Function (AF)-1/2 transactivation domain, DBD DNA-binding domain. Reprinted and modified from Akaike et al. [77]

Fig. 6.

Schemes for canonical and noncanonical inflammasome signaling. *dsDNA* indicates double-strand DNA, *HHcy* hyperhomocysteinemia, *JNK* c-Jun N-terminal protein kinase, *LPS* lipopolysaccharide, *NLRP3/ASC*, *NOD* (nucleotide oligomerization domain)-like receptor P3/apoptosis-associated speck-like protein containing a caspase recruitment domain, *pro-GSDMD* 53-kDa inactive precursor form of GSDMD, and *ROS* reactive oxygen species. Reprinted from Abe and Morrell [226], and obtained permission to reproduce from Wolters Kluwer Health. Modified from Kayagaki et al. [114] with permission of the publisher. Copyright © 2015, Nature Publishing Group

Fig. 7.

Regulation of SUMOylation pathway. Protein SUMOylation is regulated by a recycling system that consists of conjugation and deconjugation pathways. SUMO conjugation to target substrates involves an enzymatic cascade that is comprised of three classes of enzymes (E1, E2, and E3). The sentrin/SUMO-specific proteases (SENPs) are responsible for the deconjugation pathway as well as the maturation process of newly synthesized SUMO protein. The primary subcellular localization of each SENP is also listed [228]. Reprinted from Heo, Berk, and Abe [36]. Modified from Woo et al. with permission of the publisher [227]

Fig. 8.

p90RSK regulates SENP2 de-SUMOylation. The increase of ERK5 transcriptional activation is vaso-protective via up-regulating KLF2 and eNOS expression. ERK5 SUMOylation inhibits ERK5/MEF2 transcriptional activity and subsequent KLF2 promoter activity and KLF2-mediated eNOS expression [134]. p53 in the nucleus can be protective against cell death by up-regulating p21 expression [139]. p53 SUMOylation mediates p53 nuclear export, and cytoplasmic p53 directly interacts with the Bcl-2 (B cell lymphoma/leukemia-2) family of proteins, and attenuates anti-apoptotic function of these proteins [137]. p90RSK regulates SENP2 de-SUMOylation function by the association at aa131–300 and phosphorylating SENP2-T368, which subsequently induces SENP2 nuclear export. The nuclear export of SENP2 increases nuclear ERK5 and p53 SUMOylation, resulting in d-flow-induced EC inflammation, apoptosis, and consequent plaque formation [144]

Fig. 9.

Athero-prone flow (dflow) increases p53SUMOylation via PKC ζ -PIAS4 binding. **a** Athero-prone flow uniquely induces PKC ζ activation, which increases PKC ζ -PIAS4 binding and PIAS4-SUMO E3 ligase activity, and subsequently increases p53-SUMOylation. p53-SUMOylation causes p53 nuclear export and binds to Bcl-2, which inhibits anti-apoptotic function of Bcl-2 and increases apoptosis. Reprinted from Abe and Berk [229], and obtained permission to reproduce from Wolters Kluwer Health. **b** Athero-prone flow uniquely activates PKC ζ , which increases PKC ζ -PIAS4 binding at the SPRING domain and PIAS4 small ubiquitin-like modifier (SUMO) E3 ligase activity, subsequently increasing p53

SUMOylation. *PIAS* protein inhibitor of activated STAT, *SAP* scaffold attachment factor-A/B, acinus, and PIAS domain, *PINIT* Pro-Ile-Asn-Ile-Thr motif, *SP-RING* Siz/PIAS-RING domain [227, 229]. Reprinted from Heo, Berk, and Abe [36]

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