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STATE-OF-THE-ART REVIEW

AMPK, metabolism, and vascular function

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Adenosine monophosphate-activated protein kinase (AMPK) is a cellular energy sensor activated during energy stress that plays a key role in maintaining energy homeostasis. This ubiquitous signaling pathway has been implicated in multiple functions including mitochondrial biogenesis, redox regulation, cell growth and proliferation, cell autophagy and inflammation. The protective role of AMPK in cardiovascular function and the involvement of dysfunctional AMPK in the pathogenesis of cardiovascular disease have been highlighted in recent years. In this review, we summarize and discuss the role of AMPK in the regulation of blood flow in response to metabolic demand and the basis of the AMPK physiological anticontractile, antioxidant, anti-inflammatory, and antiatherogenic actions in the vascular system. Investigations by others and us have demonstrated the key role of vascular AMPK in the regulation of endothelial function, redox homeostasis, and inflammation, in addition to its protective role in the hypoxia and ischemia/reperfusion injury. The pathophysiological implications of AMPK involvement in vascular function with regard to the vascular complications of metabolic disease and the therapeutic potential of AMPK activators are also discussed.

Abbreviations
ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide riboside; AMPK, AMP-activated protein kinase; AMPKK, AMPK kinases; BH4, tetrahydrobiopterin; BK_{Ca}, large conductance calcium-activated potassium channel; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase kinases; CPA, cyclopiazonic acid; CPT1, carnitine palmitoyltransferase 1; EC, endothelial cell; EDH, endothelium-dependent hyperpolarization; EDHF, endothelium-dependent hyperpolarizing factor; eEF2, eukaryotic elongation factor 2; eNOS, endothelial nitric oxide synthase; ER, endoplasmic reticulum; ETC, electron transport chain; FAs, fatty acids; FAO, fatty acid oxidation; FFAs, free fatty acids; FOXO, forkhead transcription factor; GLUT, glucose transporter; GP, glycogen phosphatase; GTPCH, GTP-cyclohydrolase I; Hsp90, heat-shock protein 90; I/R, ischemia/reperfusion; ICAM-1, intracellular adhesion molecule 1; IFNγ, interferon gamma; IK_{Ca} channel, intermediate-conductance calcium-activated K+ channel; IKK, IκB kinase; JAK, janus kinase; JNK, c-Jun N-terminal kinase; K_{ATP}, ATP-sensitive potassium channel; KSR2, kinase suppressor of Ras 2; LKB1, tumor suppressor liver kinase B1; LPS, lipopolysaccharide; MLC, myosin light chain; MLCK, myosin light-chain kinase; MnSOD, manganese superoxide dismutase; mTORC1, mammalian target of rapamycin complex 1; MYPT1, myosin phosphatase targeting subunit 1; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; NO, nitric oxide; Nox, NADPH oxidase enzymes; Nrf2, nuclear factor erythroid 2-related factor 2; $ONOO^-$, peroxynitrite; PGC1 α, peroxisome proliferator-activated receptor γ coactivator 1α; PGs, prostaglandins; Phe, phenylephrine; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKB, protein kinase B or Akt; PKC, protein kinase C; PP, protein phosphatase; PP2A, protein phosphatase 2A; PP2Cα, protein phosphatase 2C α; ROS, reactive oxygen species; RS, reticulum sarcoplasmic; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; SOD, superoxide dismutase; SR, sarcoplasmic reticulum; STAT-1, signal transducer and activator of transcription 1; TAG, triacylglyceride; TAK1, tumor growth factor (TGF) β kinase 1; TNFα, tumor necrosis factor alpha; Trx, thioredoxin; TSC, tuberous sclerosis complex; TXNIP, thioredoxin-interacting protein; UCP2, uncoupling protein 2; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; VOC, voltage-operated Ca^{2+} channels; VSM, vascular smooth muscle; VSMCs, vascular smooth muscle cells.

AMPK, a key regulator of energy metabolism and mitochondrial homeostasis

All living cells use energy from the environment for vital processes such as growth, reproduction, and development. Animal cells extract energy from biomolecules through respiration, which consumes O_2 and produces CO_2 and H_2O. When they take more energy than they require for immediate use, the excess energy is stored in chemical bonds, primarily as glycogen and lipids [1,2]. Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is a main sensor of the cellular energy status. AMPK is activated in response to energy deficiency when the AMP/ATP ratio is increased and restores energy balance by inhibiting anabolism that consumes ATP, while promoting catabolic processes that generate ATP. Several conditions favor depletion of energy levels, such as hypoxia, exposure to mitochondrial respiration toxic agents, and nutrient starvation [3].

AMPK is a heterotrimeric serine/threonine protein kinase complex ubiquitously expressed, consisting of a catalytic α subunit (α_1 and α_2) and two regulatory subunits namely β (β_1 and β_2) and γ (γ_1, γ_2, and γ_3). There are multiple AMPK isoforms composed of one α-subunit, one β-subunit, and one γ-subunit, in any of their possible combinations, that are differentially expressed and regulated throughout the human body [4]. AMP and AMP mimetic small molecules are direct activators of AMPK that trigger a conformational change in the AMPK complex leading to phosphorylation of Thr^{172} in the AMPKα subunit [5–7]. Besides its key role as a gauge energy sensor, AMPK is involved in the regulation of several other cell processes such as apoptosis, cell growth and differentiation, cell polarity, autophagy, immune function, and inflammation [3,8,9].

Regulation of AMPK activity

AMPK is activated by three mechanisms sensitive to small changes in the AMP/ATP ratio: phosphorylation at the Thr^{172} residue of the AMPKα catalytic subunit by upstream protein kinases, dephosphorylation by upstream protein phosphatases (PP), and then allosteric activation by AMP or/and ATP [10]. Under conditions of energy deficit, the Thr^{172} residue is phosphorylated mainly by two distinct upstream kinases: the tumor suppressor liver kinase B1 (LKB1) and the Ca^{2+}/calmodulin-dependent protein kinase 2 (CaMKK2 or CaMKKβ) [11–16]. LKB1 is constitutively active and a key element in the mechanism by which AMPK senses the energy status of the cell. LKB1 activates AMPK in response to energy depletion which is modulated by AMP or ADP binding to the AMPK-γ subunit, especially in metabolic tissues such as muscle, adipose tissue, and liver [12,16]. CaMKK2 is activated by increases in intracellular Ca^{2+} which occurs in response to many hormones in specific cell types such as neurons or endothelial cells (ECs) [10,14,15,17,18] (Fig. 1). In contrast to upstream kinases, PP dephosphorylates the Thr^{172} residue on the AMPKα catalytic subunit. To sense cell energy status, AMPK primarily monitors the AMP : ATP ratio and is activated by AMP binding by three mechanisms that are complementary and antagonized by ATP: increasing the rate of AMPK phosphorylation at Thr^{172} by LKB1, suppressing dephosphorylation of p-Thr^{172} AMPKα by PPs and by allosteric activation increasing the activity of AMPK already phosphorylated on Thr^{172} [7,10,14,15,17]. AMPK can also be regulated by inhibition through an ubiquitination mechanism mediated by the formation of a complex between the AMPKβ subunit and several factors such as the cell death-inducing DNA fragmentation factor α-like effector, or E3 ubiquitin ligases such as MG53, CRBN/CRL, among others, which induces AMPK degradation [19,20]

Role of AMPK in metabolism: downstream pathways

Lipid metabolism

The regulation of lipid metabolism is the first known function of AMPK. AMPK inhibits *the novo* synthesis of fatty acids (FAs), cholesterol, and triacylglycerides (TAG), while it activates FAs uptake by the cell and lipid oxidation pathways. Thus, in response to several cell stressors, AMPK is phosphorylated at Thr^{172} that induces acyl CoA-carboxylase (ACC) phosphorylation, thus inhibiting ACC activity as rate-limiting step in FAs synthesis that converts acetyl-CoA to malonyl-CoA. A second mechanism by which AMPK inhibits lipid synthesis is by inhibition of sterol regulatory element-binding protein 1c, transcriptional factor of enzymes involved in lipid synthesis such as ACC and FA synthase [17,21]. Furthermore, AMPK inhibits phosphorylation of the cholesterol synthesis rate-limiting enzyme 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase [22], thus reducing cholesterol *the novo* synthesis (Fig. 1). Finally, AMPK inhibits the enzyme glycerol-3-phosphate acyltransferase, involved in the synthesis of TAGs, thus avoiding lipid storage [23].

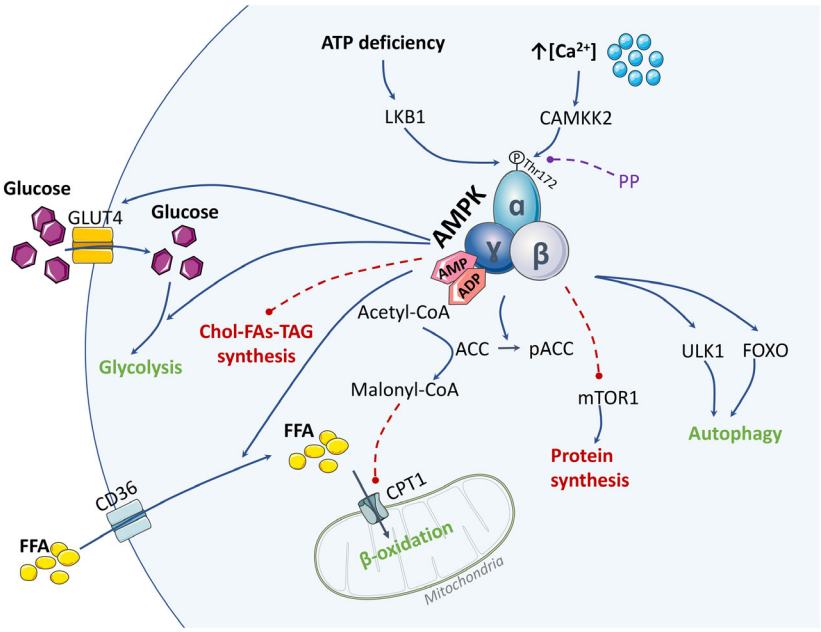

Fig. 1. AMPK signaling pathways. AMPK is a trimeric protein formed by subunits α, β, and γ. During energy starvation (ATP deficiency), AMP or ADP bind to AMPK leading to its activation through phosphorylation at Thr^{172} by AMPK kinases (AMPKK) as LKB1. Increased intracellular calcium levels stimulate CAMKK2 that also phosphorylates AMPK. Other factors inhibit AMPK, such as a PP that reduces AMPK activity by dephosphorylation. AMPK activation restores the energy status through activation of catabolic pathways. First, AMPK stimulates phosphorylation of ACC, preventing transformation of acetyl-CoA to malonyl-CoA which inhibits free FAs (FFA) mitochondrial uptake by CPT1. Consequently, AMPK activation favors FFA mitochondrial uptake to get energy through β-oxidation. AMPK also stimulates FFA cell uptake by the transporter CD36 to supply mitochondrial β-oxidation. Furthermore, AMPK activity inhibits lipid anabolic pathways, preventing synthesis of cholesterol, FFA, and TAG. Second, AMPK activation increases glucose cell uptake into the cell by GLUT4 and stimulates enzymes involved in glycolysis. Third, AMPK activation inhibits the mammalian target of rapamycin 1 (mTOR1) thus preventing protein synthesis. Forth, AMPK activation favors autophagy mainly through the autophagy-related protein ULK1 and forkhead family of transcription factor (FOXO) signaling pathways. (Green color: processes stimulated by AMPK activation, mainly catabolic pathways such as glycolysis, β-oxidation, autophagy. Red color: processes inhibited by AMPK activation, mainly anabolic pathways, such as lipid and protein synthesis).

In addition to inhibiting lipid anabolism, AMPK stimulates lipid catabolic pathways by facilitating FAs uptake and FAs mitochondrial β-oxidation (FAO). By inducing the activity of the FAs transporter CD36, AMPK facilitates FAs uptake through the cell membrane into the cytosol [24]. Moreover, by phosphorylating and inhibiting ACC, AMPK reduces malonyl-CoA levels; malonyl-CoA in turn inhibits carnitine palmitoyltransferase 1 (CPT1) that favors movement of FAs from the cytosol into the intermembrane space of mitochondria to be oxidized, thus resulting in AMPK indirectly facilitating FAs uptake into the mitochondria and FAO [25,26].

Glucose metabolism

As an energy sensor, AMPK favors glucidic catabolic pathways in different organs. AMPK facilitates skeletal muscle glucose uptake by increasing the expression of the glucose transporter 4 (GLUT4), as well as its translocation to the plasma membrane [27,28]. Once glucose is inside the cells, AMPK facilitates its use as an energy source by stimulating enzymes involved in the glycolysis (Fig. 1), as demonstrated in cardiomyocytes, macrophages, and tumor cells [29,30]. AMPK also favors glycogenolysis by activating glycogen phosphorylase (GP) while inhibits glycogen synthase thus preventing glycogen synthesis in skeletal muscle cells. Mutations in the γ_2 and γ_3-AMPK subunits lead to abnormal glycogen storage associated with arrhythmias and hypertrophic cardiomyopathy in human heart [31–34] and also to elevated glycogen levels in skeletal muscle [35]. In addition, AMPK inhibits hepatic gluconeogenesis, essential for maintenance of plasma glucose levels during fasting, by decreasing expression of enzymes such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase [36]. On the other hand, AMPK ensures both normal β-cell glucose

sensing and glucose-stimulated insulin secretion, and reduced AMPK activity has been associated with β-cell dysfunction in diet-induced obese rodents, which increases the therapeutic potential of AMPK activators as antidiabetic drugs [37].

Protein metabolism

AMPK promotes protein catabolism. The tuberous sclerosis complex (TSC)1/2 integrates environmental signals including energy status stressors. Thus, under conditions of energy deficit AMPK is activated and is responsible for inhibition of protein synthesis to keep ATP levels in the cell through phosphorylation of TSC2, which inhibits mammalian target of rapamycin complex 1 (mTORC1) and subsequently the ribosomal protein S6 kinase beta-1 (p70S6K), resulting in the prevented translation of ribosomal proteins [38]. AMPK has also been reported to inhibit mTORC1 through phosphorylation of RAPTOR [39]. Other studies have shown that AMPK directly inhibits translational protein elongation by phosphorylating and activating the eukaryotic elongation factor 2 kinase, resulting in the inhibition of protein synthesis [40] (Fig. 1). However, under conditions of energy depletion AMPK activation can favor the synthesis of various proteins essential for cell survival [41].

Role of AMPK in autophagy and mitochondrial biogenesis

Autophagy is a physiological regulated cell mechanism that removes and reuses unnecessary/dysfunctional cell components through degradation and recycling. Thus, autophagy consists in the self-digestion through lysosome-dependent processes to maintain cellular integrity mainly during starvation [41,42]. Autophagy-related genes codify the proteins responsible for autophagy, mainly ULK1 in mammals, a serine/threonine–protein kinase considered as an initiator in the autophagic cascade that activates phosphorylated phosphoinositide 3-kinase (pPI3K) to regulate the formation of autophagosomes. AMPK is involved in the regulation of autophagy, since AMPK can directly activate autophagy through ULK1 phosphorylation and indirectly through mTORC1 inhibition, since mTORC1 exerts an inhibitory phosphorylation on ULK1, preventing autophagy [43]. AMPK can also activate forkhead family of transcription factor (FOXO) thus favoring upregulation of several autophagy inducers [44]. Autophagy is mainly regulated by feedback regulatory loops. Thus, mTORC1 inhibits ULK1 activity and ULK1 negatively regulates mTORC1 [45,46]. Likewise, AMPK activates ULK1 favoring autophagy, while ULK1 negatively regulates AMPK [47]. The coordinated effects of mTORC1 and ULK1 prevent mitochondrial damage thus preserving mitochondrial integrity during nutrient deficiency states in which AMPK is closely involved [45,46].

Autophagy processes are essential for the proper functioning of mitochondria, the main organelle in charge of energy production through mitochondrial respiration. Mitochondrial integrity requires elimination of damaged mitochondria through autophagy and the production of fresh mitochondria through biogenesis. Therefore, mitochondrial biogenesis is a crucial process of energy production and cellular response during nutrient starvation. Several studies have demonstrated that chronic treatment with AMPK activators such as 5-aminoimidazole-4-carboxamide riboside (AICAR) or β-guanidionapropriate can stimulate mitochondrial biogenesis, and this effect disappears in mice lacking AMPK [48–51] suggesting that AMPK plays a key role in the regulation of mitochondrial biogenesis. Accordingly, genetic and pharmacological evidence reveals the importance of AMPK in mitochondrial biogenesis through activation of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), an important cofactor which favors transcription of genes involved in mitochondrial biogenesis [52]. Accordingly, the stimulatory effect of AMPK on PGC1α is reduced in mice lacking AMPK [51]. Several mechanisms have been proposed through which AMPK can upregulate PGC1α. First, through activation of SIRT1, a nicotinamide adenine dinucleotide (NAD^+)-dependent protein/histone deacetylase sirtuin, which in turn activates AMPK by deacetylating LKB1, thus suggesting a positive feedback loop between AMPK and SIRT1 [53–56]. Moreover, AMPK can activate PGC1α by increasing nicotinamide phosphoribosyltransferase which favors NAD+/NADH ratio or through upregulation of FAO or p38-MAPK [57].

Role of AMPK in vasculature

The cardiovascular system has mechanisms responsible for coupling blood flow and organ perfusion in response to tissue metabolic demand. AMPK regulates vascular function and structure in EC, vascular smooth muscle cells (VSMCs), and immune cells, and it is activated by harmful stimuli including oxidative stress, ischemia, and hypoxia to restore vascular homeostasis [58–62]. In the vasculature, each AMPK isoform is layer-specific: α_1 and α_2 AMPK subunits are expressed in both endothelium and VSM, α_1-subunit

being the most predominant isoform. VSM also expresses both β_1 and β_2 subunits, preferentially AMPKβ_1 [63–67].

AMPK in endothelial cells: regulation and activation mechanisms

Activity of AMPK in ECs can be regulated by various stimuli including low ATP levels, as occurs in hypoxia, ischemia or nutrient deprivation conditions, shear stress, exercise, elevation of intracellular calcium by agonists and hormones such as adiponectin, angiotensin II and ghrelin and by pharmacological activators [64], as illustrated in Fig. 2. Endothelial AMPK is activated by LKB1, that is upregulated in AMP-rising physiological and pathological conditions [11–13], and also by CaMKK2 in response to increases in intracellular Ca^{2+} levels produced by endothelial agonists such as thrombin, bradykinin, or histamine in an AMP-independent manner [68,69] or by vascular endothelial growth factor (VEGF) [70]. Concerning AMPK regulation by PP in ECs, although protein phosphatase 2C α (PP2Cα) is the most accepted isoform that dephosphorylates the Thr^{172} residue on the AMPKα catalytic subunit in several tissues, it is hardly expressed in human ECs and VSMCs [71]. In contrast, protein phosphatase 2A (PP2A) is abundantly expressed in ECs wherein it inactivates endothelial nitric oxide

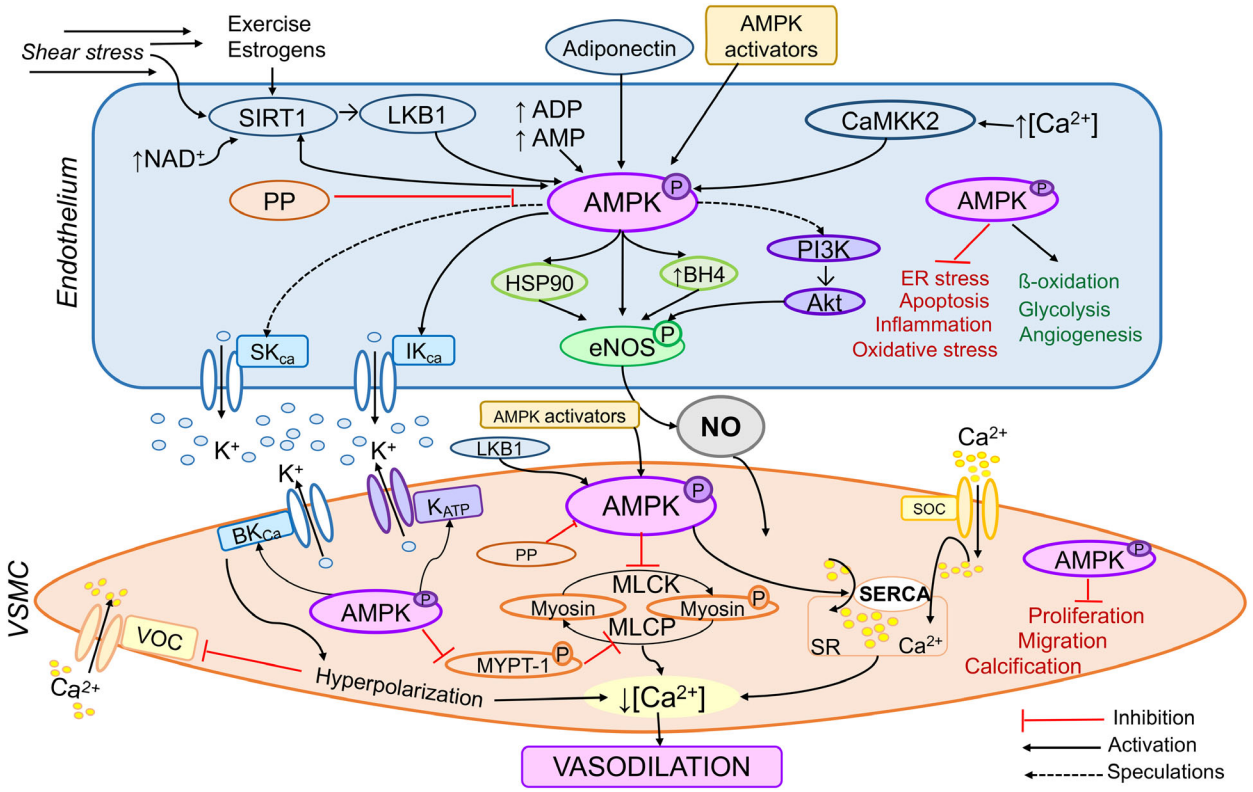

Fig. 2. AMPK in the vasculature: activation mechanisms of AMPK and main targets in endothelium and VSMCs. Endothelial AMPK can be activated by upstream AMPKK such as LKB1 and CaMKK2, hormones such as adiponectin, pharmacological activators (metformin, AICAR, A769662), as well as the energy-sensing molecule, SIRT1. In contrast, PP dephosphorylates and inactivates AMPK. AMPK activation leads to phosphorylation and activation of eNOS, increase in BH4 levels and Hsp90 association with eNOS, which results in NO production. Another eNOS stimulation mechanism by AMPK is through AMPK-PI3K-Akt pathway. AMPK is also involved in EDH-mediated relaxation of resistance arteries through activation of endothelial K_{Ca} channels. Activation of endothelial AMPK inhibits oxidative stress, inflammation, ER stress, and apoptosis, and also activates endothelial metabolism by upregulation of β-oxidation of FFAs and glycolysis and promotes angiogenesis. In VSMCs, AMPK can be activated by LKB1 and pharmacological activators that induce relaxation through endothelium-independent mechanisms. AMPK directly relaxes VSM by Ca^{2+}-lowering mechanisms, increasing SERCA activity, activation of K_{ATP} and BK_{Ca} channels resulting in hyperpolarization and inhibition of Ca^{2+} entry through VOC channels. AMPK activation can directly interfere with VSM contractile machinery and induce VSM relaxation by decreasing VSM Ca^{2+} sensitivity through inhibition of MLCK and phosphorylation of MYPT1. In addition to its role in VSM relaxation, AMPK exerts antiproliferative, antimigratory actions and inhibits vascular calcification (straight black line: processes stimulated by AMPK activation; straight red line: processes inhibited by AMPK activation; discontinuous lines: speculation).

synthase (eNOS) by dephosphorylation of Ser^{1177} [67,72]. Chronic activation of PP2A under pathological conditions triggers AMPK inactivation which results in endothelial dysfunction [67]. On the other hand, acute exercise has been reported to induce SIRT1-LKB1 cascade to activate AMPK and eNOS [73].

Endothelium is constantly exposed to shear stress, an important physiological stimulus to maintain and modulate vascular tone. Laminar blood flow enhances NO production by phosphorylation of eNOS and AMPK is one of the protein kinases implicated in this process along with the PI3K/Akt pathway [74–76]. Shear stress activates LKB1-AMPK signaling cascade in endothelium to activate eNOS through phosphorylation at Ser^{1177} thus increasing bioavailability of NO and vasodilatation [77]. Other studies suggest that SIRT1 is activated in response to shear stress independently of AMPK but both act synergistically on eNOS to increase NO bioavailability [78]. Furthermore, there is evidence that NO itself can activate AMPKα_2 in ECs [79].

An important physiological activator of AMPK in ECs is adiponectin, an adipokine that exerts cardioprotective actions in part through activation of AMPK and eNOS phosphorylation at Ser^{1177}. Hypoadiponectinemia has been associated with endothelial dysfunction and insulin resistance in metabolic and cardiovascular diseases [80]. The mechanism by which adiponectin stimulates AMPK has been studied by different research groups, suggesting that adiponectin binds to its receptor (adipoR1) and activates adaptor protein–phosphotyrosine interacting with PH domain and leucine zipper (APPL1)–LKB1-AMPK–eNOS pathway [81–83]. Other authors reported that adiponectin stimulates eNOS through AMPK/PI3K7Akt pathway [84,85]. Another reported mechanism of AMPK activation in ECs is through peroxynitrite ($ONOO^-$) formation by the reaction of $O_2^{\cdot-}$ and NO, which activates protein kinase Cζ and leads to activation of LKB1 and subsequent AMPK phosphorylation [86]. Moreover, estrogens have been identified as activators of AMPK and eNOS in human ECs via CaMKK2 [87] and via SIRT1, since treatment with 17β-estradiol upregulates the expression of SIRT1 and increases production of NO, protecting against endothelial dysfunction in ovariectomized rats [88,89].

Pharmacological activation of vascular AMPK

Besides its physiological regulation, activation of vascular AMPK can be achieved by direct or indirect pharmacological approaches. In fact, the cardiovascular protective effects of many antidiabetic drugs traditionally used in the treatment of metabolic diseases such as statins, metformin, and thiazolidinediones involve AMPK activation [58–67]. Thus, metformin indirectly activates AMPK through inhibition of complex I of the mitochondrial respiratory chain and subsequent increase in the AMP/ATP ratio [90,91]. In the heart, metformin-induced AMPK activation following ischemia/reperfusion (I/R) enhanced AMPK activity by phosphorylation at the Thr^{172} residue, upregulated eNOS phosphorylation, and increased NO bioavailability [92]. Direct AMPK activators have been developed to selectively bind AMPK-specific subunit isoforms independently on AMP or ATP levels, although some of these compounds may have off-side target effects in vascular tissues and limited potency and selectivity. AICAR is a pro-drug that is converted into the AMP analog ZMP inside cells to bind AMPKγ subunit [93], while the thienopyridine A769662 is a selective allosteric activator that preferentially binds the AMPKβ_1 subunit and increases activity of the α_1 catalytic subunit by protecting against Thr^{172} dephosphorylation [7,90,93–95]. AICAR is a vasodilator in aorta through AMPKα_1 subunit activation [65] and acutely lowers blood pressure and relaxes mesenteric resistance arteries of hypertensive rats [96] but it causes bradycardia when administered intravenously which makes it unsuitable for clinical use [97]. A7669662 has been reported to be 3 orders of magnitude more potent than AICAR as *in vitro* vasodilator of resistance arteries, its vasorelaxant effects in intrarenal arteries being associated with phosphorylation of the AMPKα_1 subunit at Thr^{172} and phosphorylation of the AMPK downstream target ACC, thus supporting the specificity of A7669662 vascular action and linking AMPK vasoactive and metabolic actions in the arterial wall [65,66,98,99]. New synthetic compounds such as MT63–78 and PF-249 interact with the allosteric drug and metabolite (ADaM) site located between AMPK α and β subunits and allosterically activate both β_1- and β_2-containing AMPK trimers [100,101]; concerning cardiovascular protective therapeutic potential of these agents, cardiac hypertrophy and glycogen accumulation have been reported as off-side target effects [101]. On the other hand, the novel pan-AMPK activator O304 has recently been shown to increase AMPK activity by suppressing PP2C-mediated dephosphorylation of the Thr^{172} residue in human recombinant AMPK and trimers [102]. This compound not only reduced plasma glucose and ameliorated insulin resistance but also improved microvascular perfusion and blood pressure in both obese mice and type II diabetic patients, which suggests a great therapeutic potential as antidiabetic drug due to its beneficial

metabolic and vascular effects. Further studies are needed to elucidate the mechanisms underlying the vascular actions of these new agents.

Endothelial AMPK: main targets and physiological actions

Endothelium is the key modulator of vascular tone and tissue perfusion and releases vasoactive factors in response to different stimuli; as a metabolic sensor, vascular AMPK has been proposed to be involved in coupling blood flow to energy demand. Conduit arteries are more dependent on NO than resistance arteries. As the diameter of the artery becomes smaller, endothelium-derived hyperpolarization (EDH) turns into the dominant endothelium-dependent vasodilator mechanism, regulating peripheral resistance and blood pressure [89,103,104].

Endothelial NOS is the major source of vasodilator NO and is activated by AMPK through different mechanisms (Fig. 2): (a) phosphorylation of eNOS at Ser^{1177} or Ser^{633}, as depicted from the increased eNOS Ser^{1177} and/or Ser^{633} phosphorylation concomitant with increased Thr^{172} pAMPK and AMPK activity found in human ECs or intact arteries in response to various stimuli including shear stress, adiponectin, or pharmacological activators [58–61,99]; (b) suppression of GTP-cyclohydrolase I (GTPCH) degradation and the subsequent increase in tetrahydrobiopterin (BH4) levels, supported by the reduced levels of GTPCH I and BH4 in aortas from the AMPKα_2(−/−) mice [105]; (c) increase in heat-shock protein 90 (Hsp90) association with eNOS, as demonstrated by the augmented eNOS Ser^{1179} phosphorylation, NO bioactivity, and coimmunoprecipitation of eNOS with hsp90 induced by AICAR and metformin in wild-type C57BL6 mice but not in AMPKα_1 knockout mice [106]. On the other hand, several studies have shown that activation of AMPK can phosphorylate eNOS at the inhibitory Thr^{495} site, inactivating eNOS and reducing NO production, these effects being attenuated by specific endothelial AMPKα_1 deletion [107,108]. AMPK-induced cardiovascular effects by phosphorylation of eNOS were first reported by Chen *et al* [109] in the heart. In this study, eNOS was shown to be activated in high calcium conditions with Ca^{2+}-calmodulin presence by AMPK phosphorylation at Ser^{1177} and, in contrast, to be inhibited in low calcium conditions by AMPK phosphorylation at Thr^{495} site. As mentioned above, physiological stimuli such as adiponectin [110] or leptin [111], acute exercise [112], shear stress [77] and antidiabetic drugs such as metformin [113] and rosiglitazone [114] increase endothelial NO production through AMPK activation and eNOS phosphorylation at Ser^{1177}.

AMPK activation has also been implicated in the EDH-mediated relaxations of resistance arteries and in the regulation of blood pressure in mice *in vivo*, since this EDH-type vasodilator response is lost after specific knockout of the endothelial AMPKα_1 subunit [115]. However, other studies have contradictorily shown that activators of AMPK (AICAR and A769662) inhibit EDH-like relaxations, through blocking preferentially small conductance Ca^{2+}-activated K^+ (K_{Ca}) channels in mesenteric arteries [108]. In a recent study, our group first demonstrated that AMPK activation in kidney resistance arteries induced a potent renal vasodilation through endothelium-dependent mechanisms involving activation of both eNOS by eNOS Ser^{1177} phosphorylation concomitant with increased Thr^{172} pAMPK, and endothelial intermediate-conductance calcium-activated K^+ channels, the latter inducing hyperpolarization of ECs that further spreads through myoendothelial gap junctions to the underlying VSM to reduce calcium and induce relaxation [99,116], as shown in Fig. 2. Further investigations are needed to elucidate the therapeutic potential of the AMPK-induced potentiation of EDH-relaxant responses in resistance arteries under pathological conditions of elevated blood pressure and/or endothelial dysfunction involving impaired NO bioavailability.

Besides its beneficial role in endothelium-dependent vasodilation, activation of endothelial AMPK has been implicated in the regulation of other important physiological functions (Fig. 2) such as reduction of oxidative stress and inflammation [117,118], promotion of angiogenesis [119], inhibition of apoptosis [120], suppression of endoplasmic reticulum (ER) stress [121,122], and regulation of endothelial metabolism by upregulation of β-oxidation of FAs, a major energy source in ECs, via an AMPK–ACC–malonyl-CoA–CPT1 mechanism [63,123,124].

AMPK in vascular smooth muscle: regulation and main targets

Vascular smooth muscle cells produce vasoconstriction or vasodilation in response to physiological or pathological stimuli. In blood vessel injury, VSM changes the contractile to a synthetic phenotype thus increasing proliferation and migration, a critical step for development of atherosclerosis [125]. As in the endothelium, AMPKα_1 and α_2 subunits are located in VSM, α_1 subunit being the most abundantly expressed isoform [66]. VSM also expresses both β_1 and β_2 subunits, predominantly β_1-AMPK [65,66]. AMPK has been involved in

endothelium-independent vasodilation of both large and small arteries [65,66,98] and in the regulation of VSM proliferation and inflammation [126]. AMPK was initially reported to be activated by metabolic stress in arterial smooth muscle [66]. Acetylcholine [127] and reactive oxygen species (ROS) such as H_2O_2 [128] activate LKB1-AMPK pathway in VSM. On the other hand, a PP2A heterotrimer, PP2APpp2r2d, has been shown to inhibit AMPK activity by dephosphorylating AMPKα at Thr^{172} in rat and human VSMCs [129,130].

Activation of $AMPK\alpha_1$ by AICAR was demonstrated to induce a potent relaxation in mice aorta in an endothelium- and eNOS-independent manner [65]. It has been proposed that AMPK directly relaxes VSM by Ca^{2+}-lowering mechanisms, as shown in Fig. 2. Accordingly, our group has recently demonstrated that AMPK activation produces renal vasodilation by reducing VSM $[Ca^{2+}]_i$ and to a lesser extent through Ca^{2+}-independent mechanisms by decreasing VSM Ca^{2+} sensitivity of the contractile machinery. Moreover, we also demonstrated that activation of AMPK by A769662 in renal resistance arteries increased sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA) activity in VSM, thus inducing Ca^{2+} sequestration to SR and producing endothelium-independent relaxations [99]. In other small arteries such as mesenteric arteries, A769662 was shown to activate SERCA by AMPK-dependent phosphorylation of phospholamban [98], thus supporting that SERCA stimulation by AMPK activation is a common mechanism to induce VSM-mediated relaxations in small arteries. In relation to endothelium-independent hyperpolarization of VSM, our group also demonstrated that vascular AMPK activation by A769662 induced a potent vasodilation through activation of VSM ATP-sensitive K^+ (K_{ATP}) channels in renal resistance arteries [99], whereas other authors proposed that A769662 activates large conductance K_{Ca} (BK_{Ca}) channels in VSM of mesenteric resistance arteries, resulting in hyperpolarization (Fig. 2) [98].

On the other hand, AMPK activation can directly interfere with VSM contractile machinery and signaling to induce VSM relaxation through a Ca^{2+}-independent mechanism that decreases Ca^{2+} sensitivity [131–133]. This relaxing mechanism plays a key role when vasodilation is needed for prolonged periods without lowering cytosolic Ca^{2+} concentrations, such as occurs in hypoxia. Thus, AMPK activation by metformin decreased phenylephrine (Phe)-induced contraction in rat aortic rings and inhibited Phe-mediated myosin light-chain kinase (MLCK) phosphorylation of myosin light chains (p-MLC) in cultured rat VSMCs (Fig. 2) [132]. Other studies reported that AMPK activation by AICAR in cultured human VSMCs abolished Phe-induced phosphorylation of myosin phosphatase targeting subunit 1 (MYPT1) and inhibited p-MLC (Fig. 2) [131]. AMPK activation by A769662 or PT-1 has also been shown to mediate Ca^{2+} desensitization of the contractile machinery and VSM relaxation through depolymerization of the actin cytoskeleton [133].

In addition to its role in VSM relaxation, AMPK exerts antiproliferative and antimigratory actions in VSMCs [58,134,135] and inhibits vascular calcification [136,137]. The fact that AMPK can have vascular endothelium-independent VSM-mediated vasorelaxant and antiproliferative effects confers an additional therapeutic potential to AMPK activators in the treatment of metabolic disease-associated vascular complications wherein endothelium dysfunction and impaired eNOS/NO signaling pathway compromise NO-mediated vasodilator, antinflammatory, and antiproliferative actions [138].

AMPK and reactive oxygen species in the vasculature

Oxidative stress or excessive ROS production is a key pathogenic factor underlying endothelial and VSM dysfunction in cardiovascular diseases. Nevertheless, redox signaling plays a critical role in homeostasis and cell survival, and ROS such as the superoxide anion ($O_2^{\cdot-}$), the hydroxyl radical ($OH^{\cdot-}$), or the cell-permeable nonradical H_2O_2 are produced in small amounts during normal cell function. ROS sources in the vascular wall include complex I and III of the mitochondria, NADPH oxidases (Nox), xanthine oxidase, and uncoupled eNOS. Reduction of molecular O_2 by Nox in complexes I and III of the mitochondria electron transport chain (ETC) during metabolic processes produces $O_2^{\cdot-}$, being the mitochondrial respiration a major source of ROS generation under basal physiological conditions. ROS production is counterbalanced and modulated by the cell antioxidant systems including superoxide dismutase (SOD), catalase, glutathione peroxidase, and peroxiredoxins, which block harmful effects of ROS [104,139–142]

The role of AMPK in redox homeostasis is well established. While ROS can regulate AMPK activity, AMPK in turn modulates antioxidant defense gene expression and ROS production in the vascular wall. In addition to its activation by changes in metabolic pathways and AMP/ATP ratio, AMPK can be directly or indirectly influenced by cellular redox status (Fig. 3). Thus, AMPK is directly activated by reversible oxidation of critical cysteine residues (Cys-299/Cys-

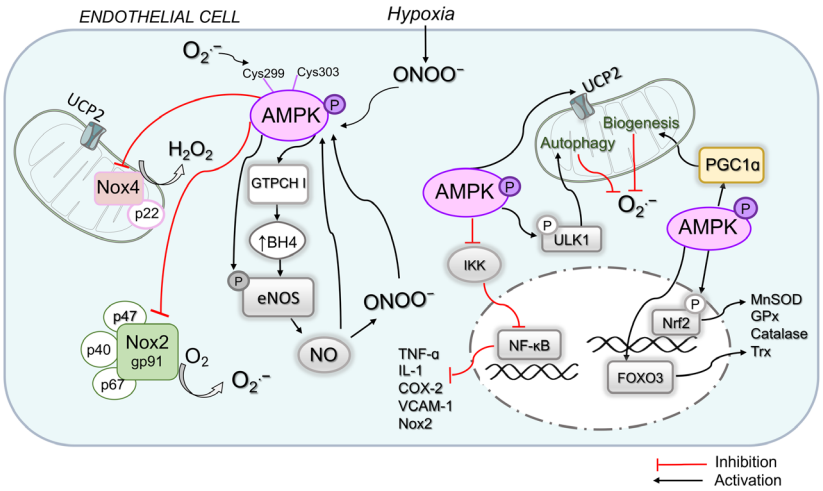

Fig. 3. Role of AMPK in redox homeostasis of ECs. AMPK activity can be regulated by direct oxidation of cysteine residues (Cys-299/Cys-303) and by ischemic signals, NO, and hypoxia through the formation of peroxynitrite $ONOO^-$. AMPK in turn modulates antioxidant defense gene expression through transcription factors FOXO3 and Nrf2 and by promoting mitochondrial biogenesis through activation of the PGC1α. AMPK downregulates oxidant production by increasing levels of GTPCH I and the synthesis of the eNOS cofactor BH4, thus preventing eNOS uncoupling and $O_2^{\cdot-}$ generation and by suppressing Nox2 and mitochondrial Nox4 ROS generating activity and expression, and by inhibiting activation of the redox-sensitive transcription factor NF-κB. AMPK reduces mitochondrial ROS production by promoting biogenesis through activation of PGC1α, by upregulating UCP2 and by regulating autophagy via by direct phosphorylation of the autophagy kinase ULK1.

303) in the AMPKα$_1$ catalytic subunit without ATP depletion [143]. However, changes in AMPK activity in response to redox variations have also been reported to be indirect and secondary to redox effects on mitochondrial ATP production [144]. In the vascular wall, AMPK is responsive to numerous oxidative stress and ischemic signals including NO, the potent oxidant peroxynitrite formed by interaction of NO and $O_2^{\cdot-}$ and hypoxia. In bovine aortic ECs, peroxynitrite activated AMPK through phosphorylation at Thr^{172} [145], and mitochondrial ROS stimulated by berberine activated AMPK through peroxynitrite formation and AMPKα phosphorylation [146] (Fig. 3). In pulmonary arterial smooth muscle cells, hypoxia-induced ROS elevation increased AMPK activity independently of changes in AMP/ATP ratio, through intracellular Ca^{2+} release from ER and enhanced store-operated Ca^{2+} entry, activation of CaMKKβ and AMPKα phosphorylation at Thr^{172}, [147]. ROS generated within the mitochondrial ETC and not oxidative phosphorylation are involved in hypoxic activation of AMPK [148].

Elevated ROS levels produce energy stress and metabolic failure by oxidizing enzymes from the ETC, tricarboxylic acid cycle, and glycolysis [149]. AMPK functions as a redox sensor and its activation maintains redox status by inhibiting oxidant production or by increasing the antioxidant potential (Fig. 3). In human ECs, activation of AMPK by AICAR inhibits palmitate-induced ROS elevation by upregulating expression of thioredoxin (Trx) through FOXO3 [150], and reduces hyperglycemia-induced increase in mitochondrial ROS by upregulating manganese SOD (MnSOD) and promoting mitochondrial biogenesis through activation of the AMPK-PGC1α [151]. Silencing of AMPKα$_1$ in cultured human umbilical ECs reduces the expression of MnSOD, catalase, γ-glutamylcysteine synthase, and Trx genes [118]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an antioxidant transcription factor activated by ROS under both physiological and stress conditions and stimulates the expression of antioxidant genes as an adaptive response to oxidative stress [152]. AMPK positively regulates and directly phosphorylates Nrf2 at the Ser^{550} residue, which promotes nuclear accumulation of Nrf2 for antioxidant response element-driven gene transactivation [153].

Reduction of oxidant production by AMPK is mainly achieved through inhibition of Nox, uncoupled eNOS, and mitochondria-derived ROS [58,154] (Fig. 3). *In vivo* activation of AMPK in diabetic rats, or AMPK overexpression in ECs, normalized endothelial function and increased levels of GTP-cyclohydrolase (GTPCH I) and the synthesis of the essential eNOS cofactor BH4, thus preventing eNOS uncoupling and superoxide generation [105].

NADPH oxidases from the Nox family primarily generate ROS and catalyze the conversion of O_2 into $O_2^{\cdot-}$ using NADPH as electron donor. Seven Nox homologues have been identified in the human genome based on the number of big transmembrane catalytic subunits, Nox1-Nox5, as well as Duox1 and Duox2 [155], and from these, Nox1, Nox2, Nox4, and Nox5 are expressed in the vascular endothelium but also in other vascular cells such as VSMCs, adventitial fibroblasts, and various leucocytes present in vascular wall under pathological conditions [156]. AMPK was first shown to inhibit Nox activity in human neutrophils wherein AMPK activation with AICAR suppressed phorbol ester-stimulated $O_2.^-$ generation and the respiratory burst by reducing translocation to the cell membrane and phosphorylation of the cytosolic Nox adaptor p47phox, protein crucial for Nox2 activation [157] (Fig. 3). In human umbilical vein ECs, activation of AMPK with rosiglitazone quenched high glucose-induced Nox-derived oxidative stress through inhibition of p22phox protein expression, but also inhibition of the protein kinase C (PKC)-dependent translocation of p47phox and Rac1 to the membrane [158]. In cardiac myocytes, glucagon-like peptide-1 activates AMPKα_2, the major isoform expressed in cardiomyocytes, thus blocking high glucose-induced PKC-β_2-dependent phosphorylation of p47phox, translocation to the plasma membrane and Nox2-derived oxidative stress and glucotoxicity [159]. Endothelial Nox2 can also be a physiologically relevant source of H_2O_2 generation that contributes to the endothelium-dependent vasodilatation in the heart and kidney [104,141,160]. Our group has recently demonstrated that AMPK is a physiological regulator of Nox2 activity in intrarenal arteries, since acute exposure to the AMPK activator A769662 suppressed $O_2^{\cdot-}$ generation stimulated by phorbol esters and did not further inhibit Nox-derived ROS production under conditions of Nox2 blockade [99]. On the other hand, AMPK does not only regulate Nox2-derived ROS production by inhibiting its activation but also through regulation of Nox2 expression. AMPKα_1 knockout mice displayed Nox2 upregulation, endothelial dysfunction, and increased vascular oxidative stress in response to angiotensin II [161]. Although the major isoform of AMPK in ECs is AMPK$\alpha_1\beta_1\gamma_1$, both AMPKα_1 and AMPKα_2 are equally important in maintaining endothelial function. The early studies of Wang *et al.* [162] demonstrated that AMPKα_2 functions as a physiological suppressor of Nox2 activity and that AMPKα_2 deletion leads to endothelial dysfunction and enhanced expression (p47phox, p67phox, and gp91phox) and activity of Nox2 mediated by increased nuclear factor (NF)-kappaB activation in ECs, whereas AMPK activation with AICAR inhibited endothelial Nox2 expression and ROS production in an AMPKα_2-dependent manner [162,163].

Nox4 is the most abundantly expressed Nox enzyme in ECs, is constitutively active, localized in the mitochondria and ER, and generates H_2O_2 in contrast to Nox1 and Nox2 that primarily produce $O_2^{\cdot-}$, [155,156]. While AMPK was first shown to negatively regulate Nox4-dependent oxidative stress and apoptosis in kidney podocytes and tubular cells in diabetes and obesity [164,165], we have recently shown that acute treatment of healthy renal interlobar arteries with the AMPK activator A769662 induced a powerful inhibition of NADPH-derived ROS production associated with a marked downregulation of Nox4 expression and reduced H_2O_2 generation in the vascular wall, which suggests that AMPK is a regulator of arterial Nox4 activity under physiological conditions [99] (Fig. 3).

Mitochondria are major sources of ROS generation and AMPK regulates mitochondrial ROS production through several mechanisms including upregulation of the mitochondrial anion carrier protein uncoupling protein 2 (UCP2) and by modulating autophagy and elimination of dysfunctional mitochondria [154]. Exposure of human ECs to either AICAR or metformin caused AMPK-dependent upregulation of both UCP2 mRNA and UCP2 protein, while overexpression of UCP2 suppressed both $O_2^{\cdot-}$ and prostacyclin synthase nitration induced by high glucose [166]. Damaged proteins and DNA or dysfunctional mitochondria cause enhanced ROS production. Dysfunctional mitochondria are eliminated from cells through autophagy mechanism, autophagy deficiency leading to enhanced ROS [167]. AMPK regulates mitochondrial ROS production through the regulation of autophagy via inactivation of the mTORC1 pathway (by phosphorylation and activation of TSC2 exchange factor) [168] or by direct phosphorylation of ULK1 thus favoring autophagy [169] (Fig. 3).

Mitochondrial dysfunction is involved in the pathogenesis of the vascular complications of metabolic disease, usually associated with enhanced ROS production induced by atherosclerotic stimuli such as hyperglycemia, hyperlipidemia, and oxidized low-density lipoproteins [166,170–172]. Pharmacological activation of AMPK in ECs has been shown to attenuate oxidative stress evoked by hyperglycemia [166] and to ameliorate palmitate-induced endothelial dysfunction by suppression of mitochondrial ROS-associated ER stress and subsequent Trx-interacting protein (TXNIP)/NLRP3 inflammasome activation [173]. AMPK activation can also protect against EC damage under oxidative stress via inhibition of EC apoptosis

and mitochondrial superoxide production mediated by phosphorylation of eNOS [174].

Pathophysiological significance of the AMPK-ROS axis in vascular disease

Abnormal elevated levels of ROS, in particular $O_2^{\cdot-}$, produced by Nox, mitochondria or uncoupled eNOS in response to hyperglycemia, hyperlipidemia, or proinflammatory cytokines in diabetes, obesity and insulin resistance, are involved in endothelial and vascular dysfunction associated with metabolic disease [138,142,175]. As described above, AMPK is an oxidative sensor and redox regulator in the cardiovascular system, AMPK activity can be regulated by oxidative stress and AMPK in turn is able to modulate both mitochondrial ROS generation and gene expression of antioxidant defenses in ECs. Hence, pharmacological activation of AMPK in the vascular endothelium was early proposed to be beneficial in metabolic disease not only because of its bioenergetic effects but also due to its ability to counteract oxidative stress [118]. The powerful beneficial effects of AMPK activation on vascular oxidative stress are consistent with the cardiovascular protective actions of drugs used in the treatment of metabolic disorders that activate AMPK such as AICAR, metformin, or thiazolidinediones [64].

AMPK, hypoxia, and ischemia/reperfusion

Ischemia is the restriction of normal blood flow caused by narrowing of blood vessel lumen which decreases O_2 supply (hypoxia) to affected tissues. Hypoxia is one of the most potent physiological activators of AMPK in tissues and organs including heart and the vascular endothelium [29,149]. The mechanisms underlying AMPK activation by hypoxia include reduced ATP production due to inhibition of FAs β-oxidation [176], and ROS from the mitochondrial ETC and reactive nitrogen species such as peroxynitrite that activate AMPK under conditions of nonsevere hypoxia [177,178]. Hypoxia combined with glucose deprivation in ECs induces AMPKα_1 activation and protects these cells by preventing apoptosis via both upregulation of the Nrf2-dependent expression of anti-apoptotic gene products and maintaining intracellular ATP in ECs [179].

Hypoxia phosphorylates and activates endothelial AMPK, specifically AMPKα_2 subunit, to regulate angiogenesis [119]. Although AMPKα_2 is the least expressed AMPK subunit isoform in ECs, it is essential for angiogenesis in response to hypoxic stress. Angiogenesis can also be mediated via AMPKα_1 activation by VEGF, independently of eNOS and possibly by providing energy through inhibition of ACC [70]. Angiogenesis is induced by ischemia-derived hypoxia and is the growth of new capillaries consisting of EC tubes without VSMCs or adventitia from pre-existing vessels, to recover blood perfusion in the ischemic zones [180–182]. Angiogenesis is required for migration and proliferation of ECs and morphological differentiation of ECs into capillary-like tubes under hypoxia conditions. Therefore, endothelial AMPK may be determinant for blood vessel recruitment and maintenance of blood perfusion in ischemic tissues [119]. AMPK inhibition by transfection of AMPKα-specific siRNA reduced tube formation in human umbilical vein ECs, whereas in mouse aortic ECs isolated from AMPKα_1 or AMPKα_2 knockout mice there was a reduced expression of UCP2, and tube formation was impaired. Ischemia increased angiogenesis, Ser^{1177} phosphorylation of eNOS, and UCP2 in ischemic muscles from WT mice, but not from either AMPKα_1 or AMPKα_2-deficient mice [183]. Downregulation of AMPK attenuates EC differentiation/migration and angiogenesis induced by hypoxia [119], VEGF [70], or adiponectin [85]. Overexpression of UCP2 increased angiogenesis in ECs of AMPK-deficient mice indicating that downregulation of ROS may be critical [183]. The mechanism proposed for AMPK-mediated angiogenesis is that hypoxia activates endothelial AMPK that in turn stimulates the Akt/eNOS/NO signaling cascade. This endothelial AMPK pathway was not implicated in EC migration and tube formation under normoxic conditions [119]. However, direct AMPK-mediated eNOS phosphorylation at Ser^{1177} was produced under prolonged hypoxia conditions or peroxynitrite-derived ischemia–reperfusion (I/R) [119,145].

AMPK and I/R

When a tissue recovers the normal blood flow (reperfusion) after a hypoxic–ischemic period, an I/R injury is produced rather than the recovery of normal function. I/R damage is produced mainly because of apoptosis of ECs and subsequent obstruction of capillaries and microvascular flow, which finally produces injury to target organs such as brain (stroke) [184], heart (myocardial damage) [185], or kidneys (renal failure) [186], being a major cause of morbidity and mortality worldwide. In this context, AMPK activation has been demonstrated to have protective effects during I/R via regulation of energy metabolism, oxidative stress, inflammation, mitochondrial function, autophagy, apoptosis, and ER stress [149,187].

The heart is highly sensitive to changes in O_2 supply, hypoxia, or anoxia being detrimental for cardiac function. Intrinsic AMPK activation by hypoxia protects the heart against ischemic injury and apoptosis, but also pharmacological AMPK activation has been shown to protect cardiomyocyte function under myocardial I/R injury [185]. Hearts from AMPKα_2 deficiency mice failed to increase glucose uptake and glycolysis during ischemia and FA oxidation during reperfusion and exhibited more myocyte apoptosis and less contractile function [188]. Moreover, adiponectin knockout mice had an increased myocardial infarct size and apoptosis compared to WT after I/R, and administration of adiponectin reduced ischemic damage via AMPK activation, which suggests therapeutic potential for acute treatment of obesity-related ischemic heart disease [189]. On the other hand, acute metformin administration before I/R, activated myocardial AMPK, increased eNOS phosphorylation at Ser^{1177} and decreased myocardial infarction in the heart of *db*/*db* diabetic mice [92]. Pretreatment with A769662 also increased eNOS activation, attenuated infarct size and reduced myocardial apoptosis and necrosis [190], confirming that pharmacologic AMPK stimulation mitigates I/R damage. Interestingly, long-term caloric restriction has been reported to produce a high AMPK activation that profoundly protects the aged heart from I/R injury [191]. Increasing experimental evidence supports that natural antioxidants protect against myocardial I/R by suppressing Nox and enhancing antioxidant enzymes activity via AMPK [192,193] and melatonin has been shown to be a cardioprotective agent by enhancing mitochondrial biogenesis and reducing mitochondrial-derived ROS production in I/R hearts from diabetic rats [194]. In addition to the antioxidant protective action of AMPK activation in I/R injuries, activated protein C [195] and antithrombin [196] have been shown to activate AMPK and to exert anti-inflammatory effects during myocardial I/R through inhibition of c-Jun N-terminal kinase (JNK) and the NF-κB signaling pathways.

In the kidney, AMPK activation also confers protection during renal I/R, a major cause of acute kidney injury in which severe cellular damage and kidney dysfunction are produced [197]. There is evidence that intrinsic AMPK activation alone during ischemia is not able to stop progression of I/R damage but AMPK activation by metformin or AICAR exerts renoprotective actions such as inhibition of oxidative stress, apoptosis, and profibrotic markers, and activation of autophagy, thus better preparing the kidney to respond to and manage injury [186]. On the other hand, AICAR has been shown to decrease monocyte/macrophage infiltration and to ameliorate acute tubular necrosis after kidney I/R injury [198]. Moreover, preactivation of AMPK by metformin preserves cellular integrity and ameliorates the epithelial cell damage caused by renal ischemia [199]. However, it seems that AMPK activation after renal I/R does not increase phosphorylation of eNOS or ACC, in contrast to that observed in the ischemic heart [200].

In conclusion, AMPK activation during I/R leads to increased glycolysis and FAs uptake and oxidation in the mitochondria to restore ATP levels, promotes cell survival by inducing autophagy and inhibiting ER stress, inflammation, and oxidative stress [185,187]. Therefore, AMPK activators may be potential therapeutic drugs for protection against I/R injuries.

AMPK and vascular inflammation

Low-grade inflammation of the vascular wall is associated with endothelial dysfunction and involved in the pathogenesis of atherosclerotic cardiovascular disease. AMPK activation can exert vasoprotective effects not only through augmentation of endothelial NO availability or through reduction of vascular oxidant production, but also through its anti-inflammatory effects on ECs by suppressing the NF-κB signaling pathway and thus reducing levels of cytokines such as tumor necrosis factor α (TNF-α) and adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1), and E-selectin [201,202] (Fig. 3). The NF-kB is a redox-sensitive transcription factor that regulates expression of a large number of inflammatory genes. NF-kB is inactive in the cytoplasm bound to IκB protein under basal conditions; however, upon cytokine stimulation, IκB is phosphorylated by IκB kinase (IKK) that targets its degradation allowing NF-κB translocation into the nucleus, its activation, and the expression of proinflammatory genes [203]. AMPK has been implicated as a modulator of the inflammatory response since AMPK activators suppress lipopolysaccharide (LPS)-, TNFα-, and palmitate-induced NF-kB activity and expression of proinflammatory cytokines in various cell types including endothelial and mesangial cells [204,205], but also in glial cells [206], retinal pericytes [112], and skeletal myocytes of obese/type 2 diabetic patients [207].

AMPK contributes to the vascular anti-inflammatory effects of NO, since NO activates AMPKα_2 in ECs which in turn phosphorylates (at Ser^{177} and Ser^{181}) and inactivates IKK thus attenuating activation of NF-κB signaling and the expression of inflammatory genes [79]. Vascular anti-inflammatory effects of

AMPK were early shown to be related to AMPK interactions with ROS metabolism, and AMPK was demonstrated to negatively regulate NF-κB activation through inhibition of the 26S proteasome-dependent IκBα degradation and the subsequent expression of Nox2 (Fig. 3). Thus, in aortic ECs from AMPKα$_2$−/− mice and human umbilical vein EC expressing dominant negative AMPK or AMPK α$_2$-specific siRNA, loss of AMPK activity caused endothelial dysfunction and increased subunit expression of gp91phox (Nox2), p47phox, p67phox, superoxide production, 26S proteasome activity, IκBα degradation, and nuclear translocation of NF-κB (p50 and p65); in contrast, AMPK activation by AICAR or AMPK overexpression reversed these effects suggesting that AMPK maintains the nonatherogenic and noninflammatory phenotype of ECs [162]. Furthermore, *in vivo* AMPKα$_1$ deletion leads to Nox2 upregulation and to Nox2-derived oxidative stress and to increased mRNA expression of pro-inflammatory mediators such as iNOS, cyclooxygenase-2, and VCAM-1, resulting in endothelial dysfunction and vascular inflammation, and supporting that basal AMPK activity is a protective, redox-regulating element in vascular homeostasis [161]. Recent investigations have elucidated the mechanism by which endothelial AMPKα$_1$ modulates vascular inflammation and oxidative stress and has involved vascular infiltration of phagocytic cells. Thus, specific endothelial AMPKα$_1$ deletion aggravated endothelial dysfunction induced by chronic angiotensin II infusion, and enhanced recruitment of infiltrating leukocytes that contributed to augmented oxidative stress derived from Nox2 (phagocytic NADPH oxidase) by upregulating of cytokines and vascular adhesion molecules including VCAM-1, monocyte chemoattractant protein 1, and chemokine (C–C motif) ligand 5 [208]. Furthermore, mice lacking endothelial AMPKα$_1$ lost heme oxygenase 1-mediated antioxidant defense [208].

Interactions between circulating monocytes and ECs, which is regulated by adhesion molecules such as selectins, ICAM-1, and VCAM-1 expressed on the surface of ECs in response to inflammatory stimuli, are crucial for the progression of atherogenesis. AMPK and AMPK activating drugs such as metformin and AICAR have been reported to attenuate EC pro-inflammatory atherogenic response by inhibiting monocyte adhesion [209,210] through phosphorylation at Ser^{89} of the transcriptional coactivator p300; AMPK thus blocks p300-mediated acetylation of NF-κB and its activation and reduces the expression of adhesion molecules and, consequently, adhesion of monocytes to ECs in TNF-α-activated human aortic ECs [210]. As mentioned above, NF-κB pathway can also be inhibited through AMPKα$_2$-mediated phosphorylation and inactivation of IKK, as depicted by the reduced IL-1-stimulated IKK phosphorylation found in ECs lacking AMPKα$_2$ but not AMPKα$_1$ [79].

The modulatory role of AMPK in vascular endothelium inflammatory response has been related to its interactions with mitochondrial ROS and ER stress [173]. Pharmacological activation of AMPK with salicylate or AICAR ameliorated palmitate-induced endothelial dysfunction by suppression of mitochondrial ROS-associated ER stress and the subsequent TXNIP/NLRP3 inflammasome activation through the reduction of NLRP3 and caspase-1 expression; the latter resulted in inhibition of inflammation and apoptosis, enhancement of eNOS phosphorylation, and improved endothelium-dependent vasodilatation [173]. AMPK has also been reported to suppress the proinflammatory Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway by phosphorylating JAK in ECs [211].

Besides endothelium, AMPK can modulate the inflammatory response in VSM cells through various mechanisms including the phosphatase and tensin homolog, a tumor suppressor gene that acts as a negative regulator of PI3K and regulates cell proliferation, survival, and growth [212]. Moreover, AMPK can also modulate the STAT-1 cascade in VSM [213]. Thus, in human aortic VSMCs, pharmacologic or genetic activation of AMPK inhibited STAT-1 and attenuated the proinflammatory actions induced by STAT-1 activators such as interferon-γ (IFN-γ) and angiotensin II, while inhibition of AMPK had opposite effects. Deletion of AMPKα$_1$ or AMPKα$_2$ resulted in activation of STAT-1 and increased levels of proinflammatory mediators, both of which were attenuated by genetic or pharmacological inhibition of STAT-1. AMPK activation suppressed STAT-1 signaling and inhibited vascular inflammation through upregulation of mitogen-activated protein kinase phosphatase 1 [213].

In cells of the immune system, AMPK activation was early reported to reduce Toll-like receptor 4-induced neutrophil activation and to decrease NF-κB signaling and the release of TNF-α and IL-6 thus ameliorating severity of LPS-induced acute lung injury [214]. The interactions between AMPK and macrophages and T lymphocytes of the immune system have recently been reviewed by Jansen *et al.* [117]. AMPKα$_1$ is involved in proliferation and differentiation of CD8+ cytotoxic T lymphocytes and deletion of AMPKα$_1$ in these cells leads to impaired ability to generate memory CD8+-T-cell responses when activated by infectious stimuli [215]. AMPKα$_1$ has also been demonstrated to be necessary to couple nutrients

availability to T-cell demand during the inflammatory process [216]. AMPK plays a key role in regulating macrophage activation and infiltration through the negative regulation of the proinflammatory JNK signaling. Thus, AMPK-mediated inhibition of JNK phosphorylation has been reported in macrophages [217] and increased JNK phosphorylation has been demonstrated in macrophages and ECs from mice deficient in AMPK subunits [218]. On the other hand, AMPKα_1 deletion in myeloid cells led to dysfunction consisting in maintained polarization of M1 and decreased number of M2 anti-inflammatory macrophages along with impaired release of cytokines and impaired phagocytosis of necrotic and apoptotic cells [219]. AMPKα_1 deletion of myelomonocytic cells in the angiotensin II-induced model of endothelial dysfunction, induced a pro-inflammatory phenotype with severe endothelial dysfunction and oxidative stress, enhanced F4/80 macrophage and GR1+ granulocyte infiltration, and augmented release of IFN-γ and TNF-α [220]

Pathophysiological significance of the AMPK–inflammation axis in vascular disease

Inflammation might be the link between metabolic and cardiovascular disease, and a low-grade proinflammatory state of the vascular wall associated with endothelial dysfunction underlies the vascular complications of obesity, diabetes, and other insulin-resistant states [138]. Metabolic abnormalities such as hyperglycemia, hyperlipidemia, and insulin resistance activate the JAK/STAT signaling pathway, a major intracellular cascade that promotes inflammatory response and insulin resistance and accelerates the development of cardiovascular complications [221,222]. As mentioned above, AMPK is a vascular inflammatory repressor and AMPK deletion or AMPK inhibition in obese humans exacerbate inflammation [161,223]. Reduction of AMPK activity has been associated with chronic inflammation in metabolic syndrome, including obesity and type 2 diabetes, and acceleration of atherosclerosis [224,225], while AMPK activators such as AICAR or antidiabetic drugs like metformin inhibit inflammation, attenuate EC pro-inflammatory atherogenic response by inhibiting monocyte adhesion, and have cardiovascular protective effects [64,226].

Vascular AMPK and metabolic disease

Endothelial dysfunction is an early pathogenic event in vascular dysfunction and represents a maladapted endothelial phenotype produced by enhanced exposure to mechanical, metabolic, oxidative, or hypoxic stimuli that lead to impaired vasodilatation, angiogenesis, and barrier function along with elevated expression of proinflammatory and pro-thrombotic factors [138,227]. In diabetes, obesity and other insulin-resistant states, hyperglycemia, hyperlipidemia, or hypertension induce oxidative stress, inflammation and endothelial dysfunction, first step and main predictor of vascular atherosclerotic processes and cardiovascular complications, including myocardial infarction, stroke, and heart and renal failure [227,228]. Dysregulation of vascular AMPK might link these cardiovascular complications to metabolic disease [229–234].

AMPK dysregulation has been reported under conditions of metabolic stress such as obesity and diabetes; as a master energy sensor activated during energy stress conditions and ATP depletion, is not surprising that high levels of glucose, triglycerides, or FAs in obesity and insulin resistance lead to inhibition of vascular AMPK activity [235,236]. The mechanisms of this inhibition are unclear, but it has been proposed that PKC activation observed in metabolic disorders can phosphorylate AMPKα_1 at inhibitory site Ser^{487} thus reducing its activity [237]. Moreover, PKC activation induced by high glucose and FAs causes ROS-derived NADPH oxidase production in both EC and VSM cells [238]. Furthermore, PP2A protein expression was upregulated in aortas of mice fed a high fat diet, and decreased AMPK phosphorylation [129,130]. High glucose levels and hyperinsulinemia in diabetes suppress AMPK activation in VSM through Akt-dependent-mediated inhibitory phosphorylation at Ser^{485} on AMPKα1 subunit [239].

As discussed above, AMPK exerts beneficial actions on the vasculature such as the increase in endothelial NO production, activation of VSM relaxation, suppression of inflammation, downregulation of ROS production, and increase of antioxidant defenses. Therefore, inhibition of AMPK activity has detrimental consequences for the cardiovascular system. It has been reported that downregulation of AMPK/PI3K/Akt/eNOS pathway by high fat diet induces endothelial dysfunction in a rat model of diet-induced obesity [234]. Moreover, high levels of FAs have been reported to lead to lipid accumulation in ECs and endothelial dysfunction in obesity due to impaired AMPK activity and downstream ACC, thus inhibiting FA oxidation and promoting lipotoxicity [240]. Other studies have shown that loss of AMPKα_1 in VSMCs promotes atherosclerotic calcification *in vivo* [136] and mouse aortic ECs from AMPKα_2 knockout mice exhibit aberrant ER stress [122].

The potential therapeutic benefits of AMPK activation for treatment of metabolic and cardiovascular disease can be inferred from the pathophysiological consequences of AMPK vascular dysfunction, as discussed above. In recent years, there has been a remarkable research effort to develop new AMPK activators, and beneficial actions on vascular function of drugs such as AICAR or others widely used in clinical practice such as metformin, thiazolidinediones, or statins have been shown in experimental assays. Thus, these compounds improved endothelial function by enhancing NO production and reducing oxidative and ER stress, inflammation and apoptosis in ECs, and calcification in VSM induced by hyperlipidemia and hyperglycemia under experimental conditions [64,91,122,123,137,235,241,242]. Nevertheless, some of these drugs have shown off-side target effects and low potency and specificity which make them unsuitable for clinical use [97,101]. Therefore, the search for new AMPK activators with therapeutic potential for the treatment of metabolic disease vascular complications, having both vascular and metabolic beneficial effects, is needed.

Conclusions

The information provided in this review summarizes the mechanisms by which the master energy sensor AMPK regulates blood flow in response to tissue metabolic demands, explains its contribution to maintaining a healthy endothelium and also the molecular mechanisms underlying the vasodilator, antioxidant, and anti-inflammatory protective actions of AMPK in the vasculature. The physiological bases of AMPK vascular actions are relevant for understanding the pathogenesis of vascular injury associated with metabolic disease in diabetes and obesity, wherein impaired AMPK activity associated with chronic inflammation and oxidative stress is paralleled by dysfunctional vascular AMPK which contributes to endothelial dysfunction and to the proatherogenic inflammatory response. The beneficial cardioprotective effects of AMPK activators, including antidiabetic drugs currently used in the treatment of diabetes and insulin resistance, highlight the potential therapeutic benefits of AMPK activation for the treatment of metabolic and cardiovascular disease.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

DP conceived the idea and drafted the manuscript. CR, MM, CC and DP wrote and discussed the manuscript. DP revised the final version.

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