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Regulation of NAD(P)H oxidases by AMPK in cardiovascular systems

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Abstract

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are ubiquitously produced in cardiovascular systems. Under physiological conditions, ROS/RNS function as signaling molecules that are essential in maintaining cardiovascular function. Aberrant concentrations of ROS/RNS have been demonstrated in cardiovascular diseases due to increased production or decreased scavenging, which have been considered as common pathways for the initiation and progression of cardiovascular diseases such as atherosclerosis, hypertension, (re)stenosis, and congestive heart failure. NAD(P)H oxidases are primary sources of ROS and can be induced or activated by all known cardiovascular risk factors. Stresses, hormones, vasoactive agents, and cytokines via different signaling cascades control the expression and activity of these enzymes and of their regulatory subunits. But the molecular mechanisms by which NAD(P)H oxidase is regulated in cardiovascular systems remain poorly characterized. Investigations by us and others suggest that adenosine monophosphate-activated protein kinase (AMPK), as an energy sensor and modulator, is highly sensitive to ROS/RNS. We have also obtained convincing evidence that AMPK is a physiological suppressor of NAD(P)H oxidase in multiple cardiovascular cell systems. In this review, we summarize our current understanding of how AMPK functions as a physiological repressor of NAD(P)H oxidase.

Keywords

NAD(P)H oxidase; endothelial cells; vascular smooth muscle cells; blood cells; AMPK; cardiovascular diseases

Introduction

Overwhelming evidence indicates that reactive oxygen species (ROS) and reactive nitrogen species (RNS) are pathogenic species that cause tissue injury and/or impair tissue repair, mechanisms shared by many cardiovascular disease states including hypertension, atherosclerosis, stroke, (re)stenosis, and cardiac failure [1]. Emerging evidence suggests that ROS/RNS are essential signals in maintaining fundamental biological processes and

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physiological function [2]. ROS/RNS are established signaling molecules which regulate multiple cellular processes such as proliferation, differentiation, migration, and apoptosis in response to stimulation by growth factors, cytokines, and vasoactive agents [3].

Among the various cellular ROS-generating enzymes in the cardiovascular system, including xanthine oxidase, cyclo- and lipo-oxygenases, uncoupled endothelial nitric oxide synthase (eNOS) [4], mitochondrial respiratory enzymes [5], and peroxidases, reduced-form **n**icotinamide **a**denine **d**inucleotide **p**hosphate (**NAD(P)H**) **ox**idases (Nox) appear to be the major contributors of ROS associated with cardiovascular diseases and health [6–11]. Cellular ROS can be scavenged by an additional set of enzymes, which includes superoxide dismutase (SOD), catalase, and glutathione peroxidase. The expression and activation of Nox isoforms and regulatory proteins can be regulated by different stimuli and signaling pathways.

Adenosine monophosphate-activated protein kinase (AMPK) is reported to be a critical energy sensor that regulates oxidative stress [12–14]. Recently, emerging data have indicated that Nox in the cardiovascular system can be regulated by AMPK [15]. In this review article, we will discuss our current understanding of cardiovascular Nox, particularly, their roles and regulation in physiological and pathological conditions. Furthermore, we will address AMPK regulation and function in the cardiovascular system as well as AMPK signaling pathways involved in cardiovascular Nox regulation. We will also emphasize the capacity of AMPK to lower the burden of cardiovascular disease (CVD).

NAD(P)H oxidases are major sources of ROS in cardiovascular systems

The Nox enzymes, also known as respiratory burst oxidases, are a family of multiple-subunit complex enzymes that generate $O_2^{\bullet -}$ via one-electron reduction of oxygen using NAD(P)H as an electron source ($NAD(P)H + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet -}$) [16]. Originally designated as **ph**agocytic **ox**idases (phox) involved in defense and innate immunity, the Nox family is now formally recognized to be widely expressed in different cell types. Importantly, Nox family members are pivotal and “professional” ROS generators in cardiovascular systems including vascular endothelial cells (ECs), vascular smooth muscle cells (VSMCs), adventitial fibroblasts, and cardiomyocytes [7, 9, 17, 18]. Seven Nox isoforms in mammalian cells have been identified: Nox1 to Nox5, and Dual oxidases (Duox1 and Duox2) [17, 19]. All Nox isoforms are transmembrane proteins with six transmembrane domains. Different Nox complexes with unique combinations of different subunits are found to function in various cardiovascular cells (Fig. 1). With regard to physiological responses, Nox-derived ROS have been linked to ECs migration and ischemia-induced angiogenesis [20]. Results from experimental animals [21] (Table 1) indicate that Nox isoforms have been implicated in cardiac fibrosis [22], preconditioning (PC) [23], cardiac remodeling post-myocardial infarction (MI) [24], angiotensin II (AngII)-mediated cardiac hypertrophy [25, 26], atherosclerosis [27–29], aortic aneurysm formation [30], and response to arterial injury [31]. Emerging evidences indicate that moderately elevated ROS have protective effects in multiple cells including in cardiomyocytes [32] and ECs [33, 34]. Thus, there are ample evidences for the role of Nox-derived ROS in modulating cardiovascular physiology and pathophysiology.

NAD(P)H oxidases in the cardiovascular system

All ECs and VSMCs express the catalytic subunits Nox1 [35–39], Nox2 [38, 40–42], Nox4 [20, 39, 41, 43–48], and/or Nox5 [49, 50] (Table 2). Expression of both Nox2 and Nox4 has been described in adventitial fibroblasts [51, 52]. Both Nox2 [53, 54] and Nox4 [32, 55] are expressed in the heart. Additionally, Nox2 is also expressed in neutrophil [56], monocyte/macrophage [57, 58], and platelet [59]. Recently, it is reported that monocyte/macrophage

express Nox4 [60]. Different Nox isoforms have distinct subcellular localization, regulation, and function (Table 2). Nox4 and Nox2 are abundantly expressed, while Nox1 is less expressed, in ECs [61, 62]. Nox1 and Nox4 are present in higher amounts than Nox2 in VSMCs [39]. Generally, the most abundant vascular Nox isoform is Nox4 [36, 45, 63, 64], based on the gene expression data. Unlike Nox1 and Nox2, Nox4 is constitutively active in cardiovasculature [65, 66], and generates predominantly H_2O_2 rather than $O_2^{\bullet-}$ [67, 68]. Several reports suggest that Nox4 is Rac1 independent [69–72], although some reports imply that Nox4 activation may be partially regulated by Rac1[73, 74]. Recently, two Nox4-binding proteins, polymerase (DNA-directed) δ-interacting protein 2 (Poldip2) in VSMCs [75], and tyrosine kinase substrate with five Src homology 3 domains (Tks5) [76], have been shown to be important (Fig. 1). Nox4 has been associated with CVDs such as hypertension, atherosclerosis, and cardiovascular and renal complications of diabetes, and is involved in the remodeling of pulmonary arteries during hypoxia-induced pulmonary hypertension [48, 77, 78], although the precise mechanisms are unclear. Recent studies suggest that Nox4 may also have protective effects. Specifically, Nox4-null mice develop exaggerated contractile dysfunction, hypertrophy, and cardiac dilatation in response to chronic overload, whereas the cardiomyocyte-targeted expression of Nox4 protects transgenic mice from these effects due to Nox4-enhanced myocardial angiogenesis [32]. In addition, elevated endothelial Nox4 promotes angiogenesis and flow recovery from limb ischemia in an eNOS-dependent manner [20], augments vasodilation, and decreases blood pressure [93]. These results suggest that Nox4 may be a potential therapeutic target for manipulating angiogenesis and tissue repair. Nox1 has been implicated in VSMCs migration, proliferation, and extracellular matrix production; these effects are mediated by cofilin [81]. Studies on transgenic mice suggest a possible role for Nox1 in acute, but not chronic, forms of AngII-dependent hypertension [79, 96–98] as well as in atherosclerosis, neointima formation of post-injury [81], endothelial dysfunction, and stroke [99]. In spite of the extensive experimental data implicating Nox1 in vascular disease (Table 1), there is a paucity of clinical data, although the expression of Nox1 and Nox activator 1 (NoxA1) is induced in human atherosclerotic vessels [100]. The physiological functions of Nox2 in the cardiovasculature remain unclear, but pathological increases in Nox2 contribute to oxidative injury and cardiovascular damage, including endothelial dysfunction [15, 42], vascular inflammation [42], and structural remodeling [54, 92].

Regulation of NAD(P)H oxidases

Different Nox complexes have respective partners (Fig.1). For example, cardiovascular Nox2 activity generally requires the association of a membrane-bound catalytic Nox subunit with a variety of regulatory subunits including Rac1 GTPase, cytosolic phox proteins including $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$, and proteins of the cytoskeleton including $p22^{phox}$. This process is controlled through at least three distinct mechanisms: activation of Rac1 [101], the presence of lipids such as arachidonic acid and phosphatidic acid, as well as phosphorylation, oligomerization, and translocation of cytoplasmic subunits to the plasma membrane [102].

The translocation and oligomerization of the classical cytoplasmic domains ($p47^{phox}$, $p67^{phox}$, and $p40^{phox}$) are initiated by the phosphorylation of $p47^{phox}$ by multiple kinases including protein kinase C (PKC) [112] and p21-activated kinase 1 (PAK1) [113]. $P47^{phox}$ acts as a scaffolding, transport, and molecular compass facilitating the arrival of $p67^{phox}$ at the correct cellular location. At the membrane, $p67^{phox}$ interacts with active Rac (GTP-loaded), which resides at the plasma membrane. Active Rac binds to proteins containing specific domains, such as the tandem tetratricopeptide repeat (TPR) motif in $p67^{phox}$ [114]. Therefore, not only is Rac necessary for Nox-dependent production of ROS, but the translocation of cytoplasmic subunits to the plasma membrane is also required before Rac

can activate Nox. It is well known AngII exert its pathological functions via Nox activation in cardiovascular cells [115, 116]. Moreover, both high glucose level and palmitate enhance ROS production via PKC-mediated Nox activation in cultured vascular cells [117]. We recently found that Nox2 activation due to $p67^{phox}$ membrane translocation mediated by PKCζ contributes to elevated $O_2^{\bullet -}$ formation in ECs in response to thromboxane A2 receptor (TP) activation [118].

Besides the regulation of Nox activity by assembly, changes in the gene expression of Nox subtypes are essential for their function. Tumor necrosis factor α (TNFα) treatment increases the mRNA and protein levels of Nox subunits $p47^{phox}$, $p67^{phox}$, and $gp91^{phox}$, as well as Nox activity in ECs (Wang S and Zou MH, unpublished data), MonoMac1 cells, and monocytes [119], suggesting the transcriptional upregulation of three essential Nox genes via the nuclear factor kappa B (NF-κB) pathway. In human aortic SMCs, NF-κB and activator protein-1 (AP-1) are important regulators of Nox activity and $p22^{phox}$ transcription, and a Janus tyrosine kinase/signal transducers and activators of transcription (JAK/STAT)-dependent mechanism is involved in the modulation of Nox1 and Nox4 expression and Nox-derived superoxide anion production [108, 120, 121]. These data indicate that cooperation among multiple transcription factors, co-activators, and co-repressors is essential for the precise control of Nox transcription and function.

AMPK in cardiovascular systems

AMPK, a critical regulator of energy metabolic homeostasis at the cellular and whole organism levels [122], is a serine/threonine kinase which has been highly conserved during evolution. AMPK exists as a heterotrimeric complex consisting of a catalytic α subunit and regulatory β and γ units. There are two isoforms of α (α1 and α2) and β (β1 and β2) and three γ subunits (γ1, γ2, and γ3), which are differentially expressed in mammalian tissues (Fig. 2). AMPK is activated by allosteric regulation via an increased ratio of adenosine monophosphate (AMP) to adenosine triphosphate (ATP), and its activity is maintained by the inhibition of dephosphorylation through ADP binding [46, 123], and by the phosphorylation of the α subunit (T172) via upstream kinases including liver kinase B1 (LKB1) [124, 125], Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) [126, 127], and transforming growth factor-β-activated kinase 1(TAK1) [128, 129]. Protein phosphatase 2A (PP2A) [130, 131] and PP2C [132] are shown to dephosphorylate AMPK at T172. Moreover, both α1- and α2-containing AMPK complexes are inactivated by PP2C, and the α1 isoform is more resistant than α2 to inactivation by PP2A [133]. Recently, both protein kinase B (PKB) [134] and PKA [135] have been reported to phosphorylate AMPKα1/α2 at S485/491, which inhibits AMPK phosphorylation at T172. More recent evidence has shown that AMPKα1 phosphorylation at T172 can be impeded by AMPKα1 phosphorylation at S173 mediated by PKA in primary mouse adipocytes [136] (Fig.2).

Although AMPK is activated as a consequence of any cellular process, normal or anomalous, that either decreases ATP levels or increases AMP concentrations [137], AMPK is also activated by a variety of stimuli including cytokines (leptin, adiponectin, ghrelin, cannabinoids, IL-6) [138, 139], ciliary neutrotrophic factor (CNTF) [140], certain drugs including metformin [141], thiazolidinediones (TZD), which is PPARγ-independent [142], and may be AMP-dependent [143, 144], and some plant-derived compounds including berberine [145, 146], resveratrol [147], and curcumin [148]. Once AMPK is activated, it switches on catabolic pathways that can generate ATP (e.g., cellular uptake and utilization of glucose), while at the same time, terminates processes that consume ATP (e.g., cellular synthesis pathways). Thus, AMPK orchestrates the regulation of energy-generating and –consuming pathways. The rapid “switching” required to closely and quickly regulate and balance cellular energy resources is achieved by brisk phosphorylation of metabolic

enzymes and of various transcription factors and co-activators that control gene expression [137].

AMPK in the heart

AMPKα2 is the predominant AMPKα isoform expressed in the mouse and rat hearts [150], whereas both α1 and α2 subunits of AMPK equally contribute to total AMPK activity in the human heart [151]. The heart expresses both γ1 and γ2 isoforms but lacks the γ3 isoform. γ1 isoform complexes account for 70% of AMPK activity in the ischemic heart [150], but mutations in the γ2 subunit of AMPK causes familial hypertrophic cardiomyopathy [152]. AMPK can be activated in the heart by several factors, such as adiponectin [153], macrophage migration inhibitory factor (MIF) [154], follistatin-like 1 (Fstl1) [155], resveratrol, a cardioprotective polyphenol from red wine [156], and cardiac ischemia [157, 158]. Conversely, insulin inhibits AMPK activation [159, 160].

Animal experiments indicate that AMPK has distinct functions in the heart (Table 3). AMPK carries out important physiological roles such as regulating fatty acid and glucose metabolism in the heart [161, 162]. AMPK activation has been shown to reduce myocardial ischemic injury [163, 164]. AMPK activation may inhibit myocardial hypertrophy [156, 165, 166] and impede the transition from cardiac hypertrophy to heart failure [151]. In addition, the cardiac hypertrophy induced by isoproterenol or pressure-overload in AMPKα2$^{-/-}$ mice is significantly higher than in wild-type (WT) animals and is associated with p70S6K activation [167, 168]. AMPKα2 has been implicated in the positive regulation of myocardial estrogen-related receptor-α (ERRα) expression which is negatively associated with congestive heart failure [169]. Further, AMPK plays an important role in ischemia-induced autophagy in cardiomyocytes [170]. More recently, our research indicates that AMPKα2 inhibition impedes myocardium autophagy, accentuates cardiac dysfunction, and increases mortality in diabetic mice [171].

AMPK in endothelial cells

Both α subunits of AMPK are expressed in ECs [172, 173]. No compensatory upregulation of AMPKα2 or AMPKα1 is observed in either AMPKα1$^{-/-}$ or AMPKα2$^{-/-}$ mouse aortic ECs, respectively, although AMPKα1 is the predominant isoform of AMPKα in human and mouse ECs and aortae [42, 172, 174]. AMPKα2 however, has more important physiological functions such as anti-atherogenic effects [172] and pro-angiogenic effects in response to hypoxia [175, 176].

In ECs, stimuli such as peroxynitrite [177–179], the anti-diabetic drug metformin [178], and the lipid-lowing drug statin [180] have been reported to activate AMPK via LKB1 phosphorylation and activation. Furthermore, flow shear stress activates either AMPK signaling event for NO bioavailability [181] or extracellular signal-regulated kinase 5 (ERK5)-mediated PPARγ1 signaling pathway to facilitate antiinflammatory response in ECs [182]. However, AMPK can be inactivated in ECs by free fatty acids such as palmitate [131] as well as by high glucose [183]. Moreover, AMPK activity was lower in the aortic endothelium of obese rats compared to control rats [184].

AMPK plays an important role in maintaining vascular endothelial function [185]. First, AMPK signaling contributes to vasodilation of conduit and resistance vessels via the upregulation of eNOS phosphorylation at Ser1177 and Ser633 [186] and the subsequent association of phosphorylated eNOS with heat shock protein 90 (Hsp90) [187, 188]. In addition, AMPK activation by metformin or 5′-aminoimidazole-4-carboxamide ribonucleoside (AICAR) impairs high glucose/diabetes-induced 26S proteasome activity, which mediates the degradation of GTP cyclohydrolase 1 (GTPCH1), the rate-limiting

enzyme in the de novo synthesis of tetrahydrobiopterin (BH4), an essential cofactor for eNOS. Thus, in vivo activation of AMPK attenuates diabetes-enhanced GTPCH1 degradation and restores the impaired endothelium-dependent relaxation in diabetic mice [183]. However, aortae from AMPKα2$^{-/-}$ mice, which exhibit elevated 26S proteasome activity, have reduced levels of GTPCH I and BH4, and consequent endothelial dysfunction [183]. Second, AMPK performs its role of counteracting oxidative stress in ECs via the upregulation of both manganese superoxide dismutase (MnSOD) and uncoupling protein 2 (UCP2) and the downregulation of Nox. Third, AMPK exerts a pivotal role in angiogenesis. Treatment of endothelial cell progenitor cells with AICAR increases their ability to form capillary-like tubes in vitro through an NO-dependent mechanism [189]. Inhibition of AMPK signaling suppresses both human umbilical-vein endothelial cell (HUVEC) migration and in vitro differentiation into tube-like structures in hypoxic, but not normoxic cultures. Dominant-negative AMPK also impairs in vivo angiogenesis in Matrigel plugs implanted subcutaneously in mice [175]. The AMPK-p38 MAPK signaling cascade elevates VEGF production in muscle and promotes angiogenesis in skeletal muscle in response to ischemic injury [190]. More recently, our research indicates that AMPK inhibition with either a pharmacological inhibitor (compound C) or by genetic means (transfection of AMPKα-specific siRNA or either AMPKα1 or AMPKα2 deletion) significantly lowers tube formation in HUVEC or mouse aortic ECs, associated with impaired UCP2 expression [191]. Finally, AMPK activation ameliorates hypoxia and glucose deprivation-induced ECs apoptosis by increasing the expression of anti-apoptotic proteins Bcl-2 and survivin [192].

AMPK in vascular smooth muscle cells

We and another group have found that both catalytic α subunits of AMPK are expressed in VSMCs. No compensatory upregulation of AMPKα2 or AMPKα1 is observed in AMPKα1$^{-/-}$ or AMPKα2$^{-/-}$ VSMCs, respectively, although AMPKα1 is the predominant isoform contributing to total AMPKα and its activation in VSMCs [198, 206]. AMPK in cultured VSMCs can be activated by the TP agonists IBOP and U46619, in a H_2O_2-dependent manner, whereas SQ29548, a selective TP antagonist, significantly attenuates TP-enhanced AMPK activation [207].

AMPK activation exerts its anti-proliferative role in VSMCs. Igata et al. [208] reported that the AMPK activator AICAR significantly inhibits proliferation of human aortic SMCs induced by both platelet-derived growth factor-BB (PDGF-BB) and fetal calf serum (FCS). Consistent with this finding, AICAR treatment inhibits the expression of p21 but not p27. AICAR increases p53 protein levels and p53-S15 phosphorylation in human aortic SMCs, with both of these effects being blocked by AMPK inhibition. These data suggest that AMPK suppresses VSMCs proliferation by blocking cell cycle progression through p53 up-regulation. Another study has shown that subcutaneous injection of AICAR for 2 weeks suppresses neointimal formation after transluminal mechanical injury of the rat femoral artery by blocking the phosphorylation of extracellular signal-regulated kinase 1/2 [209]. Recently, a potent anti-tumor agent, β-lapachone (3,4-dihydro-2,2- dimethyl-2H-naphtho[1,2-b]pyran-5,6-dione), was shown to inhibit FCS- or PDGF-induced proliferation of VSMCs and to reduce neointimal formation in balloon-injured rat carotid arteries through the LKB1-AMPK-p53-p21 pathway [210]. We have also found that AMPK activation inhibits VSMCs hypertrophy induced by thromboxane receptor activation [207]. Interestingly, our recent work revealed that AMPKα2 deletion exacerbates neointima formation through the upregulation of E3 ligase S-phase kinase-associated protein 2 (Skp2) mediated by the activation of p52 NF-κB-2, leading to p27 downregulation and a resultant accelerated cell cycle in VSMCs. AMPK activation by metformin blunts neointimal hyperplasia after carotid artery injury in WT mice [198]. In addition, AMPK activation in VSMCs has been implicated in attenuated smooth muscle contraction via direct

phosphorylation of myosin light chain kinase [211] and consequent vasorelaxation [193]. Moreover, we reported that AMPKα2 deletion accentuates agonist-induced vascular smooth muscle contraction and high blood pressure in mice [197].

AMPK in blood cells

AMPK is also expressed and exerts critical effects in blood cells. It is well known that AMPKα1 is the predominant AMPKα isoform expressed in both human and mouse monocytes/macrophages; however, AMPKα2 is almost undetectable in macrophages [212, 213]. Either anti-inflammatory or proinflammatory stimuli rapidly activate or inactivate AMPK in macrophages, respectively. Activated AMPK supresses the production of proinflammatory cytokines and promotes macrophage polarization to an anti-inflammatory functional phenotype in a manner associated with NF-κB inhibition [212, 213], which results from SIRT1-mediated p65 deacetylation [213]. It has been reported that the stimulation of glycolysis by hypoxia in activated monocytes requires the phosphorylation and activation of inducible 6-phosphofructo-2-kinase (iPFK-2) by AMPK [214]. Interestingly, AMPK inactivation within monocytic MM6 cells has been implicated in the exercise-induced immunosuppression [215]. Further, it is reported that berberine represses proinflammatory responses through AMPK activation in macrophages [216]. AMPK activation also blunts proinflammatory cytokine production in neutrophils [217]. Recently, Bae et al. found that AMPK activation elevates the ability of both neutrophils and macrophages to ingest bacteria (*Escherichia coli*), and the capacity of macrophages to ingest apoptotic cells, due to cytoskeletal reorganization mediated by Rac1 activation. *In vivo*, AMPK activation leads to augmented phagocytosis of bacteria in the lungs [218]. These data imply that AMPKα1 may be a promising target for immunoregulation including the innate immune and adaptive immune response.

We and other groups have found that AMPKα1 is the predominant isoform of AMPKα expressed in murine erythrocytes [219-221]; however, AMPKα2 is nearly undetectable in erythrocytes [221]. The life span of erythrocytes from AMPKα1$^{-/-}$ mice is shorter than that in their WT littermates, possibly due to the increased osmotic fragility associated with elevated ROS levels and oxidized proteins [219]. AMPKα1$^{-/-}$ erythrocytes are significantly more susceptible to the eryptotic effect of energy depletion due to increased cytosolic Ca^{2+} [220]. Further, AMPKα1$^{-/-}$ erythrocytes have less deformability in response to shear stress, limiting their membrane flexibility [221]. Finally, AMPKα1$^{-/-}$, but not AMPKα2$^{-/-}$, mice are anemic despite excessive reticulocytosis, and exhibit severe splenomegaly [219, 220]. Interestingly, treatment of AMPKα1$^{-/-}$ mice with the antioxidant tempol results in reduced reticulocyte amounts and improved erythrocyte survival [219].

Both AMPKα1 and AMPKα2 isoforms are expressed in human and murine platelets [222]. Either thrombin or insulin activates AMPK in platelets [222, 223]. AMPK inhibition significantly impairs thrombin-induced platelet aggregation and clot retraction. Furthermore, compared with WT mice, AMPKα2$^{-/-}$, but not AMPKα1$^{-/-}$, mice have disrupted clot retraction, higher frequency of rebleeding, and unstable thrombi formation induced by $FeCl_3$, which is attributed to the blunted Thr12 phosphorylation of Fyn, a Src-family kinase, and the consequently impaired Tyr747 phosphorylation of αIIbβ3 integrin [222]. These data suggest that AMPKα2, rather than AMPKα1, positively regulates platelet function.

Redox regulation of AMPK

Recent studies by our laboratory and others demonstrate that AMPK activity can be regulated by oxidative stress. Interestingly, AMPKα1 is more sensitive to oxidative stress [224], while AMPKα2 is much more sensitive to an increase in AMP concentration [133, 224]. Different oxidative stress mediates AMPK activity via a distinct mechanism, including

either AMP/ATP ratio-dependent, upstream kinase-mediated, or oxidative modification manner. Our laboratory was the first to demonstrate that low concentrations of $ONOO^-$ (<10 μM) increase the activity of AMPK and its downstream enzymes such as eNOS and ACC in ECs [225, 226]. We have shown that $ONOO^-$-dependent AMPK activation occurs after hypoxia-reoxygenation [227] and in metformin- [226], simvastatin- [180], or berberine- [146] stimulated ECs, and $ONOO^-$ is capable of activating AMPK independently of changes in AMP/ATP ratio [226, 227]. AMPK is also activated by hypoxia via mitochondrial ROS through an unknown mechanism [228] or the upstream kinase LKB1 [229]. Recently, Mungai et al. reported that hypoxia triggers AMPK activation even when there is an apparent lack of AMP elevation, through ROS-dependent calcium release-activated calcium (CRAC) channel activation, leading to an elevation of cytosolic Ca^{2+} that activates the AMPK upstream kinase CaMKKβ [230]. Moreover, we found that thromboxane receptor activates AMPK in VSMCs via LKB1 in an H_2O_2-dependent manner [207]. H_2O_2 transiently activates AMPK in a dose-dependent manner, which is associated with an increased AMP/ATP ratio [231] or via an unknown mechanism without altering the ratios of AMP/ATP [224]. Interestingly, H_2O_2 directly causes the oxidative modification of AMPKα including S-glutathionylation of C299/304 without depletion of cellular ATP, which contributes to AMPK activation [232]. On the other hand, AMPK becomes inactivated under disease conditions including diabetes and obesity [183], which is reportedly associated with elevated oxidative stress [233]. Collectively, several ROS/RNS acutely activate AMPK and cellular functions mediated by this molecule, including the maintenance of redox homeostasis, such that AMPK might function as an “early warning system” in response to oxidants to attenuate oxidative injury [234].

AMPK activation suppresses oxidative stress

Either AMPKα1 or AMPKα2 deletion/inhibition elevates oxidative stress in different cell types/tissues through distinct mechanisms [15, 42, 219]. For example, AMPKα1 silencing in HUVEC downregulates the expression of genes involved in antioxidant defense, including MnSOD, catalase, γ-glutamylcysteine synthase, and thioredoxin [174]. Furthermore, AMPKα1 deletion significantly downregulates Foxo3 and ROS scavenging enzymes including catalase, MnSOD, and glutathione peroxidase (GPx-1) in mice erythroblasts [219]. In contrast, Ruderman et al have reported [235] that AMPK activation diminishes oxidative stress in ECs. There is emerging evidence that AMPK signaling pathways can ameliorate the oxidative stress associated with cardiovascular disease by upregulating the expression of endogenous antioxidant genes. For example, AMPK activation by metformin or AICAR normalizes hyperglycemia-induced mitochondrial ROS production by induction of MnSOD and promotion of mitochondrial biogenesis, via the activation of the AMPK-peroxisome proliferator-activated response-gamma coactivator-1α (PGC-1α) pathway [236]. AMPK activation by AICAR or metformin attenuates either AngII- or lipopolysaccharide-induced oxidative endothelium injury, and c-Jun N-terminal kinase (JNK) activation, via mitochondrion stabilization and increased mitochondrial biogenesis by PGC-1α [237]. Additionally, our work demonstrates that AICAR upregulates the AMPK-dependent mitochondrial uncoupling protein 2 (UCP2) via p38, which is associated with a reduction in both $O_2^{\bullet-}$ and prostacyclin synthase nitration in diabetic WT mice, but not in their $AMPK\alpha 2^{-/-}$ counterparts [238]. Collectively, these results establish that chronic AMPK activation functions as a conductor of the redox orchestra.

Regulation of NAD(P)H oxidases by AMPK

Nox enzymes are generally regulated via either Nox complex assembly or Nox subunits expression. Several lines of research indicate that AMPK activation may function as a Nox inhibitory mechanism. AMPK activators, such as AICAR or 5′-AMP, are reported to

attenuate both phorbol 12-myristate 13-acetate (PMA)- and formyl methionyl leucyl phenylalanine (fMLP)-stimulated $p47^{phox}$ phosphorylation and translocation to the cell membrane in human neutrophils, which is consistent with reduced $O_2^{\bullet-}$ production [12]. AMPK activation by rosiglitazone may inhibit PKC [142], which phosphorylates $p47^{phox}$ at several serine sites [112], mediating p47 membrane translocation associated with Nox activation (Fig. 3). Thus, AMPK activation mitigates glucose-induced oxidative stress via Nox inhibition in ECs [142, 235]. These data suggest that AMPK may restrain Nox activation in the cardiovascular system [239] via assembly inhibition. Additionally, metformin, an AMPK activator, also suppresses Nox activity by an unknown mechanism in podocytes [13].

Our recent work might reveal an alternative mechanism for AMPK-mediated Nox inhibition [15]. In that study, we found that AMPK negatively regulates NF-κB activation by inhibiting the 26S proteasome-dependent IκBα degradation pathway and the consequent expression of Nox4, Nox2 and its partners $p47^{phox}$ and $p67^{phox}$. The finding that either AICAR-induced AMPK activation or constitutively active AMPK increases IκBα protein levels in TNFα-treated ECs, without affecting IκBα mRNA levels, suggests that AMPK regulates IκBα only at the protein level. The ability of pharmacological inhibition of NF-κB to block the endothelial Nox gene as well as protein expression induced by either TNFα or AMPKα deletion suggests that both stimuli regulate Nox gene transcription via NF-κB. In addition, the ability of NF-κB inhibition to decrease components $p67^{phox}$ and $p47^{phox}$ protein levels in the absence of either stimulus suggests that the basal expression of these subunits is also regulated by the NF-κB pathway. These results are consistent with several recent reports indicating that AMPK activation by either metformin, AICAR, or NO inhibits NF-κB activation by decreasing IKK-dependent IκBα phosphorylation in ECs [240, 241]. More recently, Foretz et al. report that Nox2, neither Nox1 nor $p22^{phox}$, is upregulated by unknown means in the aortae of $AMPK\alpha1^{-/-}$ mice chronically infused with AngII at low subpressor doses; this is associated with elevated Nox activity [42]. In addition, AMPK inhibition by high glucose upregulates Nox4 via an unreported mechanism, increases Nox activity, and causes podocyte apoptosis through p53 upregulation [242]. These results indicate that AMPK may exert its anti-oxidant role by inhibiting the expression of Nox subunits. Although these studies are highly suggestive of potential crosstalk between AMPK and Nox, how AMPK suppresses Nox remains to be established. Additionally, it is reported that JAK/STAT inhibitors significantly diminish the interferon γ (IFNγ)-mediated upregulation of Nox1 and Nox4 expression, Nox activity, and resultant $O_2^{\bullet-}$ production in human VSMCs [108]. Interestingly, $AMPK\alpha2^{-/-}$ VSMCs show elevated STAT1/3 activation (Song P and Zou MH, unpublished data). Whether AMPK activation blunts Nox expression via STAT warrants further investigation.

Taken together, AMPK activation suppresses Nox activity may via either blocking Nox assembly by inhibiting $p47^{phox}$ phosphorylation and translocation to cell membrane or inactivation of transcription factors including NF-κB and STAT for Nox or the expression of its partners (Fig.3). In contrast to a direct phosphorylation of IKK at Ser-177/181 by AMPKα2 *in vitro* [241], currently there is no evidence suggesting that AMPK can phosphorylate $p47^{phox}$, IκBα, NFκB components, or STATs. Thus, how AMPK regulates Nox in monocytes/macrophages and platelet remains to be established.

AMPK and NAD(P)H oxidases in cardiovascular diseases

Numerous cellular and animal experiments (Table 3) report cardiovascular-protective effects of AMPK [234, 243–246]. Many therapeutic agents used for the treatment of diabetes and atherosclerosis, including metformin [141, 226], thiazolidinediones [142], and statins [180, 247] may exert their cardiovascular protective effects by the activation of AMPK. AMPK

activation has a number of potentially beneficial anti-atherosclerotic effects including reducing the adhesion of inflammatory cells to the blood vessel endothelium, reducing lipid accumulation and the proliferation of inflammatory cells caused by oxidised lipids, stimulation of gene expression responsible for cellular antioxidant defenses [248], and stimulation of enzymes responsible for NO formation [181, 183, 249].

Recently, we showed that AMPKα2 deletion upregulates Nox2/4 and its partners $p67^{phox}$ and $p47^{phox}$ via NF-κB activation. Increased Nox activity results in elevated $O_2^{\bullet-}$ production in ECs, which leads to endothelial dysfunction contributing to exacerbated atherosclerosis in low-density lipoprotein receptor knockout ($LDLr^{-/-}$) mice given a high-fat diet [15]. AMPKα1 deletion also upregulates Nox2, associated with elevated Nox activity in response to AngII. The increased Nox activity contributes to augmented $O_2^{\bullet-}$ production and the resultant endothelial dysfunction [42]. In addition, we found that oxidized and glycated LDL (HOG-LDL) enhances the $p47^{phox}$ membrane translocation associated with Nox activation [196]. Augmented Nox activity causes ROS elevation, which oxidizes the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA), and subsequently increases cytosolic Ca^{2+}, which is associated with endoplasmic reticulum (ER) stress in ECs. The aberrant ER stress results in impaired endothelium-dependent vasorelaxation in isolated aortae from $ApoE^{-/-}$/AMPKα$2^{-/-}$ mice fed a high-fat diet, which contributes to severe atherosclerosis. However, AMPK activation by AICAR blunts $p47^{phox}$ membrane translocation and ER stress. These data indicate that AMPK activation suppresses HOG-LDL–induced ER stress by inhibiting Nox–derived ROS. More recently, it is reported that early atrial fibrillation (AF) causes to the upregulation of Nox2 expression and activity. Ex vivo atorvastatin inhibits atrial Rac1 and Nox2 activity by unknown method in patients with postoperative AF [54]. Whether the function of statin is mediated by AMPK warrants further investigation. Overall, AMPK activation attempts to suppress oxidative injury by suppressing Nox-derived ROS and associated ER and mitochondria dysfunction. This feedback mechanism might be essential for maintaining cardiovascular homeostasis, therefore AMPK exerts its critical role in preventing cardiovascular disease including heart disease [151], atherogenesis [15, 196, 250], neointima formation [198, 209], and hypertension [197, 251].

Conclusions and perspectives

Several reported cellular and animal experiments indicate that either the expression of Nox and its partners or the assembly and activation of Nox complex are regulated by AMPK via different mechanisms (Fig.3). AMPK activators such as metformin may exert their cardiovascular protective function through Nox inhibition by AMPK activation. It is not clear whether other clinical AMPK activators including TZD and statin elicit their cardiovascular protective function via Nox inhibition mediated by AMPK. Treatment of Nox isoform knock out animals or Nox/AMPK double-knock out animals with these drugs will be beneficial for answering the question.

The AMPKα1 and α2 isoforms have ~90% homology in their N-terminal catalytic domains and ~60% homology in their C-terminal domains [252], suggesting that they likely have distinct upstream and downstream signaling pathways [133], although both AMPKα isoforms have common downstream pathways. It is therefore important to develop activators which specifically target different AMPKα isoforms. Different AMPK isoforms are predominantly expressed in different cardiovascular cell types and tissues. Nox subcellular localization and its components may be differentially regulated by distinct AMPKα isoforms via its respective molecular mechanisms. Tissue-specific conditional knockout of α isoforms in mouse/rat would afford the ability to assign downstream signaling to individual α subunits of AMPK for Nox regulation. It is also critical to re-evaluate the distinct

functions of the different catalytic AMPKα subunits in both normal and pathological settings.

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Abbreviations

7-KC	7-ketocholesterol
AAA	abdominal aortic aneurysm
AICAR	5′-aminoimidazole-4-carboxamide ribonucleoside
AMPK	adenosine monophosphate-activated protein kinase
AngII	angiotensin II
ApoE	apolipoprotein E
BH_4	5,6,7,8-tetrahydrobiopterin
CAD	coronary artery disease
CaMKKβ	Ca^{2+}/calmodulin-dependent protein kinase kinase β
CRAC	calcium release-activated calcium
CVD	cardiovascular disease
ECs	endothelial cells
EGF	epidermal growth factor
eNOS	endothelial nitric oxide synthase
ER	endoplasmic reticulum
ERRα	estrogen-related receptor-α
ET-1	Endothelin 1
GPx-1	glutathione peroxidase
GTPCH1	GTP-cyclohydrolase 1
H_2O_2	hydrogen peroxide
HF	heart failure
HUVEC	human umbilical vein endothelial cells
LKB1	liver kinase B1
MI	myocardial infarction
MPO	myeloperoxidase
NAD(P)H	nicotinamide adenine dinucleotide phosphate reduced form
NF-κB	nuclear factor kappa B

NO	nitric oxide
Nox	NAD(P)H oxidase
NoxA1	Nox activator1
NoxO1	Nox organizer 1
$O_2^{\bullet-}$	superoxide anion
$ONOO^-$	peroxynitrite
PC	preconditioning
PDGF	platelet-derived growth factor
PDI	protein disulfide isomerase
PGC-1α	peroxisome proliferator-activated response-gamma coactivator-1α
PKA	protein kinase A
PKC	protein kinase C
PM	plasma membrane
RAS	renin-angiotensin system
RNS	reactive nitrogen species
ROS	reactive oxygen species
SERCA	sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase
Skp2	S-phase kinase-associated protein 2
SOD	superoxide dismutase
TAC	transverse aortic constriction
TAK1	transforming growth factor-β-activated kinase 1
TGF-β1	transforming growth factor β1
TNFα	Tumor necrosis factor α
TP	thromboxane A2 receptor
TPR	tetratricopeptide repeat
TZD	thiazolidinediones
SMC	smooth muscle cell
VSMCs	vascular smooth muscle cells
WT	wild-type

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Highlights

- AMPK is activated by oxidants;
- AMPK suppresses NAD(P)H oxidase;
- Dysfunctional AMPK results in oxidative stress.

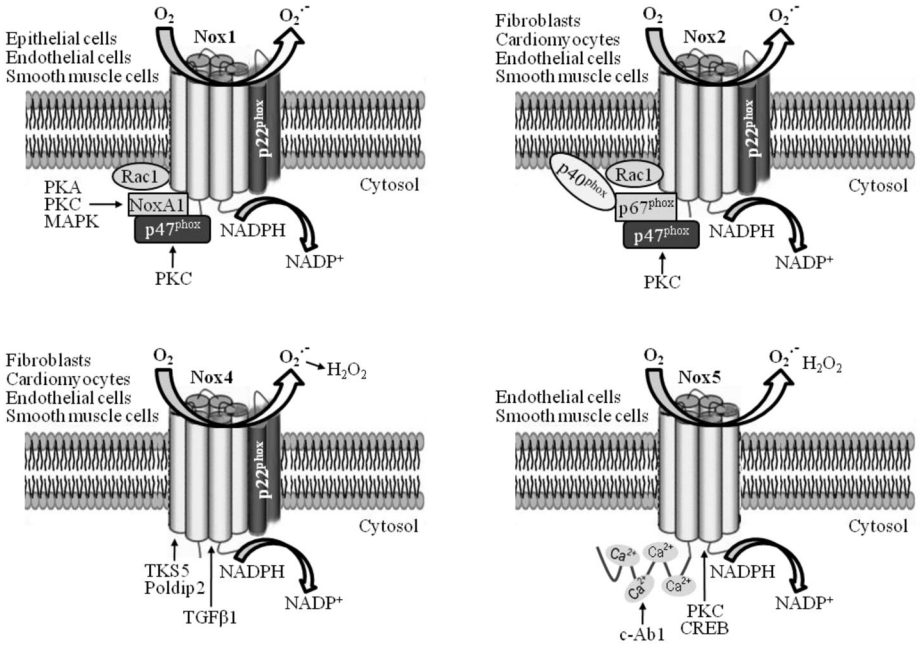

Fig. 1.

Distribution and regulation of NAD(P)H oxidases in cardiovascular systems. With the exception of Nox4, the Nox homologues are basally inactive. Regulation of the cardiovascular Nox complex is mediated by variations of Nox isoform structure as well as interactions with various partners. See text for details.

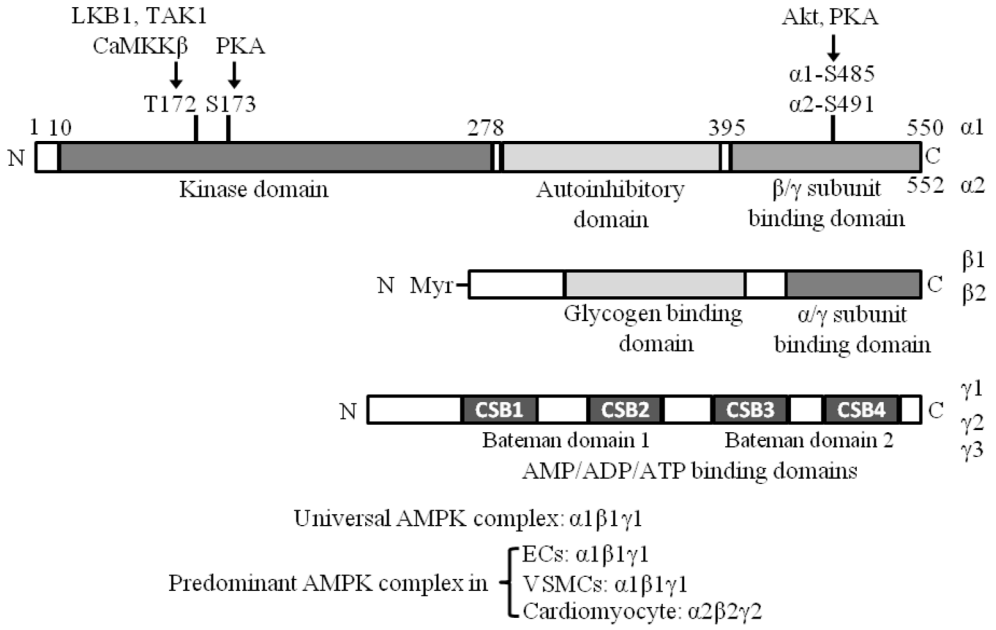

Fig. 2.

Structure features of the catalytic α and regulatory β and γ subunits of AMPK. Residues phosphorylated by AMPKK (LKB1, CaMKKβ, TAK1), PKA, and Akt are shown within the α subunit. This figure is modified from Viollet et al [149]. Refer to the text for expanded forms of abbreviations.

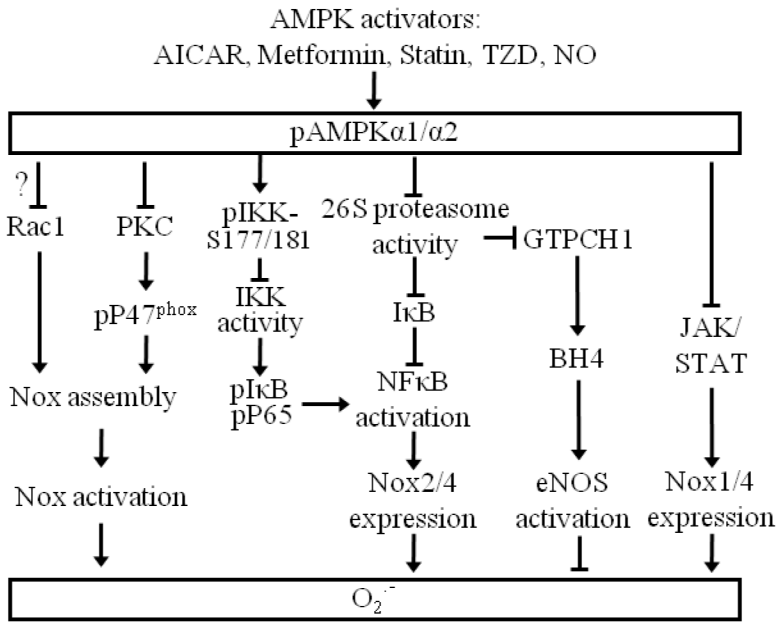

Fig. 3.

Schematic summary of available information on the regulation of cardiovascular Nox by AMPK. Refer to the text for expanded forms of abbreviations.

Table 1

NAD(P)H oxidase knockout or transgenic mouse models associated with cardiovascular disease.

KO or Tg mouse model	Phenotype or related cardiovascular outcome	Reference(s)
Nox1 null	Attenuated renal-sensitive signaling	[79]
Nox1 null	Lower blood pressure, reduction in aortic media hypertrophy	[80]
Nox1 null	Reduced neointima formation	[81]
Nox2 null	Attenuated cardiac remodeling and PC effect	[22–24]
	Beneficial effects on neovascularization	[82, 83]
	Impaired ischemia-induced flow recovery	[84, 85]
	Decreased neointima formation	[31]
	Attenuated AngII-induced hypertrophy but not TAC	[25, 26, 86]
Nox2 null/ApoE null	No significant effects on atherogenesis	[87]
Nox2 null/ApoE null	Reduced early atherosclerosis	[27]
Nox4 null	Exaggerated contractile dysfunction, hypertrophy, and cardiac dilatation	[32]
$p47^{phox}$ null	Impaired pressor responses to AngII	[88]
$p47^{phox}$ null/ApoE null	Reduced atherosclerosis and AngII-induced AAA	[28–30]
Cardiomyocyte-specific Rac1 null	Attenuated AngII-induced hypertrophy	[89]
Tg-SMC-Nox1	Elevated AngII-induced hypertension and VSMC hypertrophy	[90, 91]
Tg-tie2-Nox2	Elevated AngII-induced EC dysfunction and hypertension	[92]
Tg-VECad-Nox4	Promote EC angiogenesis	[20]
Tg-tie2-Nox4	Lower systemic blood pressure	[93]
Tg-αMHC-Nox4	Protective myocardial angiogenesis to overload stress	[32]
Tg-SMC-$p22^{phox}$	Elevated AngII-induced vascular hypertrophy	[94]
Tg-SMC-$p22^{phox}$	Enhanced carotid arterial lesions	[95]

Refer to the text for expanded forms of abbreviations.

Table 2

Localization, regulation, and (patho)physiological functions of Nox isoforms in cardiovasculature

Cell/tissue type	Nox isoform	Subcellular localization	Regulation	Function	Reference(s)
ECs	Nox2	PM, Perinuclear, ER, Caveolae	AngII, ET-1	Cell proliferation, angiogenesis	[41, 103]
	Nox4	Perinuclear, ER	AngII	Cell proliferation, angiogenesis, and senescence	[20, 41, 104, 105]
	Nox1	Perinuclear,	AngII, shear stress	Angiogenesis	[35]
	Nox5	Perinuclear	AngII, ET-1	Angiogenesis	[49]
VSMCs	Nox1	Caveolae, PM, endosomes	AngII, EGF, PDI	Injury-induced neointima formation	[36, 39, 106, 107] [81, 108]
	Nox4	Focal adhesions, ER, mitochondria, nucleus	AngII, 7-KC, TGF-β1	ER stress, apoptosis, proliferation, migration, differentiation	[39, 106, 109, 110] [64]
	Nox2	Perinuclear	AngII	SM contraction, neointima formation	[31, 111]
	Nox5	PM	PDGF	Proliferation	[50]
Cardiomyocyte	Nox2	Sarcolemmal and ttubule membrane	Physiological stretch	Stretch-dependent ROS and Ca^{2+} release, new-onset AF	[53, 54]
	Nox4	Perinuclear	Pressure overload, hypoxia	Enhance myocardial angiogenesis	[32, 55]

Refer to the text for the expanded forms of abbreviations.

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Table 3

AMPK and transgenic or knockout mouse models related to cardiovascular disease

Animal model	Tissue specificity	Phenotype or related cardiovascular outcome	Reference(s)
AMPKα1 KO	Whole body	Impaired eNOS activation in EC	[187]
	Whole body	Impaired AICAR-enhanced vasorelaxation	[193]
	Whole body	Aggravated endothelial dysfunction and vascular inflammation in response to AngII	[42]
	Whole body	Impaired angiogenesis	[191]
AMPKα2 KO	Whole body	More rapid onset of ischemia-induced contracture, Impaired glucose transport and glycogen metabolism in heart	[194, 195] [194, 195]
	Whole body	Higher cardiac hypertrophy induced by isoproterenol or pressure-overload	[167]
	Whole body	Endothelial dysfunction, elevated atherosclerosis in $LDLr^{-/-}$ mice	[15]
	Whole body	Impaired SERCA activity, aberrant ER stress, Elevated atherosclerosis in $ApoE^{-/-}$ mice	[196]
	Whole body	Impaired angiogenesis	[191]
	Whole body	Hypertension	[197]
	Whole body	Elevated neointima formation after injury.	[198]
TgAMPKγ2(CA-T400N)	Heart	Glycogen storage cardiomyopathy, greater infarct sizes and apoptosis after ischemia-reperfusion	[199]
TgAMPKγ2(CA-N488I)	Heart	Glycogen storage cardiomyopathy	[200, 201]
TgAMPKγ2(DN-R531G)	Heart	Glycogen accumulation and hypertrophy	[202]
TgAMPKγ2(DN-R302Q)	Heart	Glycogen accumulation cardiomyopathy	[203]
TgAMPKα2(DN-K45R)	Heart	Impaired glucose uptake and glycolysis, and elevated injury after ischemia-reperfusion	[163]
TgAMPKα2(DN-D157A)	Heart	Impaired glucose uptake,	[204]
		Increase myocardial infarct size,	[205]
		Impaired autophagy, aggravated cardiomyopathy and mortality in diabetic mice	[171]

Refer to the text for expanded forms of abbreviations.