Adenosine Monophosphate-Activated Protein Kinase, Oxidative Stress, and Diabetic Endothelial Dysfunction

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Abstract
Endothelial dysfunction characterized by impaired endothelium-dependent vasorelaxation is one of the earliest detectable pathological events in smoking, diabetes, and many cardiovascular diseases including hypertension, atherosclerosis. Overwhelming data from human and animals demonstrate that the endothelial dysfunction associated with diabetes is due to the local formation of oxidants and free radicals. However, the mechanisms by which diabetes instigates oxidative stress, and those by which oxidative stress perpetuates endothelial dysfunction are the subjects of intensive research in the last 3 decades. The studies from us and others have demonstrated that adenosine monophosphate-activated protein kinase (AMPK), a well-characterized energy sensor and modulator, serves as a highly efficient sensor as AMPK can be activated by very low levels of reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by physiological, pharmacological, and pathologic stimuli (redox sensor). Interestingly, oxidants-activated AMPK feedback lowers the levels of ROS by either suppressing ROS/RNS from reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and mitochondria or by increasing the levels of antioxidant enzymes (redox modulator).

Further, our studies demonstrate that AMPK’s functions as a redox sensor and modulator are vital to maintain endothelial cell function under physiological conditions. Finally, we discovered that under chronic oxidative stress or large influx of ROS, AMPK is particularly susceptible to inhibition by ROS. We conclude that oxidative inactivation of AMPK in diabetes perpetuates oxidative stress and accelerates atherosclerosis in diabetes.

Keywords: Adenosine monophosphate-activated protein kinase; Atherosclerosis; Endothelial cell; Nitric oxide; Oxidative stress

Introduction
The endothelial cell layer is a cell monolayer on the surface of the vascular lumen. It separates circulating blood from the vascular wall and releases active substances to maintain vascular structures and functions. The endothelial cell is an active endocrine and paracrine organ. It releases biologically active substances under both physiological and pathological conditions.

Nitric oxide (NO) generated by endothelial nitric oxide synthetase (eNOS) is the most important factor released by endothelial cells.[1] Most physiological effects of NO in cells occur via activation of guanylyl cyclase-coupled receptors in vascular smooth muscle cells. This process results in cyclic 3’-5’ guanosine monophosphate accumulation and vascular smooth muscle cell relaxation (ie, endothelium-dependent relaxation).[2]

Thus, endothelial function is defined by NO bioactivity modulated by the production of NO from eNOS and/or the inactivation of NO by reactive oxygen species (ROS) such as superoxide anions (O2−).[3] The endothelial cell is also the main site for O2− generation; the reaction of NO with O2− at a diffusion-controlled rate inactivates NO and forms peroxynitrite (ONOO−). Peroxynitrite is a potent oxidant, and study results indicate that O2− generation and the consequent production of ONOO− causes endothelial dysfunction in cardiovascular diseases (CVDs) including hypertension, atherosclerosis, and coronary heart disease.[4]

Diabetes is a disorder characterized by the elevation of blood sugar. The most common and devastating complications of diabetes occur in the cardiovascular system.[3] Adults with diabetes have a two- to three-fold increased risk of heart attacks and strokes.[5] Endothelial dysfunction is one of the earliest detectable pathological events during the early-onset period of diabetes in humans.[6] Clinical trials with large sample sizes found that the hyperglycemia characteristic of diabetes is the most important causative factor for endothelial dysfunction and accelerated CVD in individuals with diabetes.[7] The landmark Diabetes Control and Complications Trial found that intensive control of blood glucose effectively reduces the risk of development of complications in individuals with type 1 diabetes. These benefits remain despite less intensive blood sugar control by many patients years after the Diabetes Control and Complications Trial study ended.[8]

Adenosine monophosphate-activated protein kinase (AMPK) is a three-subunit (α, β, γ) enzyme ubiquitously distributed throughout the mammalian cardiovascular system, including endothelial cells.[9-12] AMPK is a sensor of cellular energy status (energy sensor) and a major regulator of energy metabolism.[9-12] We and others found that AMPK has a broad range of functions in the cardiovascular system. Our research group was the first to...
report that AMPK is activated by low levels of free radicals and oxidants (redox sensor); in return, oxidant-mediated AMPK activation effectively suppresses oxidant production (redox modulator). Metformin, a potent AMPK activator, activates AMPK in a redox-sensitive manner. AMPK activation effectively prevents endothelial dysfunction and atherosclerosis-related CVD in mice and human patients. In this mini-review, we will summarize the roles and etiology of endothelial dysfunction in diabetes. We will then present evidence to support the hypothesis that AMPK is a redox sensor and modulator, and AMPK dysfunction plays a crucial role in the initiation and progression of endothelial dysfunction in patients with diabetes.

**Etiology of endothelial dysfunction in diabetes**

Cardiovascular complications in patients with diabetes are the most common and devastating manifestations of the disease and are the major cause of hospital admissions. Accelerated atherosclerosis is one striking feature of these complications and is associated with metabolic syndrome, insulin resistance, and oxidative stress. Endothelial cells have crucial roles in the maintenance of cardiovascular homeostasis. They are a physical barrier between the lumen and vessel wall and secrete molecules that regulate coagulation, platelet aggregation, vascular tone, and fibrinolysis. Endothelial dysfunction is characterized by reduced bioavailability of vasodilators (mainly NO) and/or increased numbers and concentrations of endothelium-derived contracting factors. This imbalance impairs endothelium-dependent vasodilation and the impairment manifests as the functional characteristics of endothelial dysfunction. Endothelial dysfunction also includes endothelial activation, which is characterized by a proliferative, proinflammatory, and procoagulatory state that supports the entire progression of atherogenesis. Endothelial dysfunction occurs early ahead of structural vessel wall changes detected using angiography or ultrasound. Endothelial dysfunction is a consistent finding in patients with diabetes; it manifests as impairment of NO production and activity, and endothelial dysfunction is a driving force during the development and progression of cardiovascular complications in diabetes. Indeed, targeting endothelial injury may be an effective approach for the management of diabetes-associated vascular disorders and therapeutic interventions aimed at improving endothelial function in patients with diabetes have been tested in clinical trials.

**Hyperglycemia**

Diabetes Control and Complications Trial and United Kingdom Prospective Diabetes Study findings revealed the efficacy of intensive glucose control for reducing the risk of diabetes-associated microvascular complications (eg, retinopathy, nephropathy, and neuropathy). Findings of Diabetes Control and Complications Trial from long-term follow-up of participants found a decreased incidence in atherosclerosis in those with intensively-controlled blood glucose concentrations. This result suggests that hyperglycemia is important in the development of macrovascular diseases in patients with diabetes. There is an abnormality in endothelium-dependent relaxation in individuals in whom blood glucose and insulin levels are acutely increased using glucose infusion. Multiple pathways can be involved with the dysfunction and activation of endothelial cells induced by hyperglycemia, including enhanced glycolysis, the formation of glycolysis intermediates, and advanced glycation end (AGE) product modifications.

**Free fatty acids**

Hyperglycemia is just one of many metabolic abnormalities associated with diabetes. Another contributing factor for endothelial dysfunction in diabetes is the elevation of circulating free fatty acids (FFAs). Impaired glucose tolerance can occur in young normoglycemic relatives of patients with high FFAs. An increase in FFAs is an important feature of diabetes and is common in type 1 and type 2 forms of the disease. High levels of plasma FFAs caused by infusing a lipid emulsion and heparin impairs endothelium-mediated vasodilatation (and NO production) in human volunteers undergoing the hyperinsulinemic-euglycemic clamp technique. Patients with obesity and type 2 diabetes have elevated plasma FFA levels, which play detrimental roles in the etiology of atherosclerosis and CVD. FFA-driven endothelial dysfunction occurs through mechanisms including impairment of insulin signaling and reduction of NO, inflammation, oxidative stress, endothelial cell apoptosis, and activation of the renin-angiotensin system. Targeting signaling pathways involving FFA-induced endothelial dysfunction can be used to prevent endothelial dysfunction and subsequent complications, such as atherosclerosis.

**Insulin resistance**

Insulin resistance plays a pathophysiological role in type 2 diabetes. Insulin regulates glucose homeostasis by promoting glucose disposal in skeletal muscle and adipose tissue, inhibiting gluconeogenesis in the liver, and regulating the transport of nutrients to target tissues via actions on the microvasculature. Insulin-induced NO production from the vascular endothelium leads to increased blood flow that further enhances glucose uptake in skeletal muscle. Insulin resistance is frequently associated with endothelial dysfunction and has been proposed to have a major role in CVD. The balance between the NO-dependent vasodilator actions and endothelin-1-dependent vasoconstrictor actions of insulin is regulated by phosphatidylinositol 3-kinase-dependent and mitogen-activated protein kinase-dependent signaling in vascular endothelium. During insulin-resistant conditions, phosphatidylinositol 3-kinase-dependent signaling impairment can cause an imbalance between the production of NO and the secretion of endothelin-1 and lead to endothelial dysfunction.

**Inflammation**

Metabolic conditions, such as obesity and type 2 diabetes mellitus (insulin resistance) are associated with inflammation. Adipocytes can constitutively express tumor necrosis factor alpha (TNF-α). Expression of this important proinflammatory cytokine is markedly increased in animal models of obesity. Using soluble TNF-α receptors to neutralize TNF-α receptors can reduce insulin resistance in these animal models. These findings first linked the increased expression of a proinflammatory cytokine to insulin resistance. The results of studies in humans support these findings. Diabetes and obesity (the main risk factor for type 2 diabetes) are inflammatory diseases, as indicated by elevated plasma concentrations of interleukin 6 (IL-6), C-reactive protein,
and plasminogen activator inhibitor 1. Experimental and clinical investigations found that vascular inflammation has an important role in the progression of endothelial dysfunction. In endothelial dysfunction, the endothelium loses its physiological properties, reduces the bioavailability of NO, and changes to a state of vasoconstriction, thrombosis, and inflammation. Inflammation is essential for the development of vascular complications in individuals with obesity and type 2 diabetes. Anti-inflammatory adiponectin can protect endothelial function, and interventions to reduce inflammation (eg, administration of salicylate) can prevent vascular dysfunction and cardiovascular events.

**Oxidative stress and diabetes-associated endothelial dysfunction**

Hyperglycemia is the fundamental abnormality in diabetes and is associated with oxidative stress. Hyperglycemia perpetuates oxidative stress by releasing $O_2^-$ from multiple sources from endothelial cells (Figure 1). Although $O_2^-$ has been previously recognized as an important cytotoxic factor contributing to tissue damage, $O_2^-$ alone does not exhibit high reactivity towards most types of biological molecules. $O_2^-$ can be highly reactive after its reaction of NO to yield a potent oxidizing species ONOO$^-$ by a direct bimolecular reaction of NO with $O_2^-$ at near diffusion-limited rates ($6.7 \times 10^9 \text{ mol}^{-1}\text{ s}^{-1}$) [Figure 1]. This rate constant of NO with $O_2^-$ is 3 times faster than the enzymatic dismutation of $O_2^-$ catalyzed by superoxide dismutase (SOD) at neutral pH ($K_{\text{cat}} = 2.0 \times 10^9 \text{ mol}^{-1}\text{ s}^{-1}$). Thus, depending upon local rates of production of both NO and $O_2^-$, ONOO$^-$ formation represents a major potential pathway of NO reactivity.

Overwhelming evidence supports a causative role of oxidative stress in the development and progression of endothelial dysfunction in diabetes. In patients with diabetes, increased plasma levels of ROS markers like thiobarbituric acid-reactive substances, 8 alpha-isoprostanes, oxidized low-density lipoprotein (oxLDL), protein oxidation products, 8-oxo-deoxyguanosine, and 8-oxoguanosine have been found along with decreased antioxidant defenses (eg, total antioxidant capacity, bilirubin, SOD, and antioxidant vitamins). The sources of oxidative stress in diabetes include non-enzymatic, enzymatic, and mitochondrial pathways. The non-enzymatic mechanisms include generation of hydroxyl ($OH^-$) via autoxidation of glucose and formation of AGEs. Enhanced metabolism of glucose via the polyol pathway produces $O_2^-$; stimulation of the renin-angiotensin system in diabetes also promotes ROS formation. The enzymatic sources of ROS generation in diabetes include NOS, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), and xanthine oxidase. Xanthine oxidase mediates the final 2 reactions of the purine metabolism pathway and generates significant amounts of ROS. The $O_2^-$ and other ROS produced can then stimulate signaling pathways associated with cell growth and the inflammation, apoptosis, and fibrosis that eventually lead to the development of the complications of diabetes.

With respect to oxygen-derived free radicals and oxidants, study results indicate that oxidative stress has a vital role in the pathogenesis of complications associated with diabetes. Hyperglycemia-induced oxidative stress may be the earliest event associated with progression of inflammation. Although insulin resistance has been attributed to the inflammatory processes starting in adipose tissue, study results suggest that endothelial dysfunction represents the upstream event that precedes peripheral impairment of insulin sensitivity. Suppression of ROS-dependent pathways in the endothelium restores insulin delivery to peripheral organs by preserving NO availability. Such a disruptive effect of ROS in diabetes is supported by the comparable endothelial dysfunction in vitamin E-deprived rats. Similarly, defective endothelium-dependent vasodilation in subjects with type 2 diabetes can be reversed using dietary ascorbic acid. This effect of ascorbic

![Figure 1: The reaction of reactive oxygen species and reactive nitrogen species in diabetic endothelial cells. H$_2$O$_2$: Hydrogen peroxide; HOCl: Hypochlorous acid; L-Arg: L arginine; NO: Nitric oxide; O$_2^-$: Superoxide anion; OH$^-$: Oxhydroxyl; ONOO$^-$: Peroxynitrite; SOD: Superoxide dismutase.](image-url)
acids also occur in patients with type 1 diabetes.\textsuperscript{[66,67]} As detailed below, several enzymatic systems in diabetic endothelial cells are activated to become a main source of O$_2^\text{-}$.

**NOX**

PNOX is a membrane-associated, heme-containing enzyme complex consisting of 7 subunits and is a major source of O$_2^\text{-}$ production in diabetes. Vascular NOX consists of multiple subunits, including p22$\text{phox}$, p47$\text{phox}$, p67$\text{phox}$, Nox organizer 1 (NoxO1), Nox activator 1 (NoxA1), Rac1, and unique NOX isoforms (based on gp91$\text{phox}$).\textsuperscript{[68]} NOX isoforms are catalytic subunits for ROS generation. They are differentially expressed and regulated in various cell types and pathological conditions but remain to be fully characterized. Seven homologues of the NOX family have been identified: Nox1-5, DUOX1, and DUOX2.\textsuperscript{[68]} Endothelial cells and endothelial progenitor cells predominantly express Nox2, Nox4, and Nox1 isoforms.\textsuperscript{[69]}

Activation of NOX is implicated in oxidative stress associated with hyperglycemia. Protein kinase C beta-dependent NOX activation has been suggested as the major source of oxidants in diabetes.\textsuperscript{[70]} Treatment of human umbilical vein endothelial cells with high glucose increases NOX expression, apoptosis, and levels of oxidative stress markers.\textsuperscript{[71]} ROS production and expression of p22$\text{phox}$ increase in mouse microvascular endothelial cells treated with high glucose.\textsuperscript{[72]} A human clinical study found that NOX expression and activity was augmented in the metabolic syndrome group compared with the cardiovascular risk factor and control groups.\textsuperscript{[73]} Oxidized LDL and 3-nitrotyrosine levels and carotid intima-media thickness increased in the metabolic syndrome group compared with the cardiovascular risk factor and control groups; these changes were related to the NOX oxidase activity in the overall population.\textsuperscript{[74]} These findings suggest that abnormal activation of NOX is involved in oxidative stress and atherosclerosis in patients with metabolic syndrome.

NOX-dependent superoxide production links palmitate-stimulated toll-like receptor 4 activations to nuclear factor-$\kappa$B (NF-$\kappa$B) signaling in endothelial cells.\textsuperscript{[75]} FFA-induced NOX subunit overexpression and ROS production could be involved in the endothelial dysfunction in obese Zucker diabetic fatty rats. Study results suggest that pitavastatin or NOX inhibitors protect against the effects of these changes.\textsuperscript{[76]} Higher levels of superoxide in insulin-resistant endothelial cells can be pharmacologically inhibited (both acutely and chronically) using the NOX inhibitor, gp91ds-tat. Similarly, insulin resistance-induced impairment of endothelial-mediated vasorelaxation can be reversed using gp91ds-tat. Knockdown of Nox2, which is specifically elevated in insulin-resistant endothelial cells, can significantly reduce superoxide levels. Double transgenic mice with endothelial-specific insulin resistance and deletion of Nox2 have reduced superoxide production and improved vascular function. This study identified Nox2 as the central molecule in insulin resistance-mediated oxidative stress and vascular dysfunction.\textsuperscript{[77]} It also established pharmacological inhibition of Nox2 as a novel therapeutic target in insulin resistance-related vascular disease.

**Electron transport chain**

The electron transport chain can generate ROS under physiological and pathological conditions. In diabetes, persistent hyperglycemia elevates FFA levels. FFAs are metabolized to produce increased levels of reduced coenzymes (nicotinamide adenine dinucleotide and flavin adenine dinucleotide). These reduced coenzymes enter the respiratory chain, and consequently, increase the production of O$_2^\text{-}$ to combat the flux of electrons.\textsuperscript{[78]} In diabetes, this mitochondrial O$_2^\text{-}$ stimulates the production of more ROS and reactive nitrogen species via activation of NF-$\kappa$B-mediated cytokines, protein kinase C, and NOX.\textsuperscript{[79,80]}

In diabetes, excessive mitochondrial ROS production has been implicated as a “master switch” for activation of discrete pathologic signaling pathways leading to subsequent endothelial dysfunction. This phenomenon is primarily related to the increased mitochondrial ROS because bluntng of the effect occurs only with pharmacologic inhibition of complex II, overexpression of mitochondrial uncoupling protein 1, or exposure to manganese SOD. Indicative of the cell-signaling capacity of mitochondrial ROS, mitochondrial ROS inhibitors also reduce NF-$\kappa$B and protein kinase C activation and reduce the production of toxic AGE products and sorbitol.\textsuperscript{[79,81,82]} Expression of cell-adhesion molecules on the endothelial cell surface depends on mitochondrial ROS when high glucose concentrations are present.\textsuperscript{[79,84,85]} Exposure of endothelial cells to high concentrations of FFAs increases mitochondrial membrane potential, mitochondrial ROS, and NF-$\kappa$B in human aortic endothelial cells.\textsuperscript{[86]} Each of these responses is inhibited by overexpression of uncoupling protein 2 (UCP2). Therefore, reductions in mitochondrial ROS, together with the reductions in mitochondrial membrane potential, are responsible for the observed improvement in endothelial function. Similarly, in intact vessels, overexpression of UCP2 in the rat aorta reverses endothelial dysfunction induced by lysophosphatidylcholine.\textsuperscript{[82,86]}
Mitochondrial fission could be an effective target to improve diabetic endothelial function. Metformin reduces diabetes-accelerated development of atherosclerosis by inhibiting mitochondrial fission in endothelial cells. Metformin treatment significantly reduces streptozotocin-induced mitochondrial fragmentation, suppresses mitochondrial superoxide production, inhibits vascular inflammation, improves endothelium-dependent vasodilation, and inhibits atherosclerotic lesions in diabetic apolipoprotein E knockout (ApoE−/−) mice. In endothelial cells exposed to high glucose concentrations, metformin treatment suppresses mitochondrial superoxide production, reduces levels of dynamin-related protein 1 (DRP1) and its transport to mitochondria, and prevents mitochondrial fission.[87]

Antioxidants

Defense mechanisms in the form of antioxidants are present in the body to counteract oxidative stress. They include enzymes like SOD, catalase, glutathione peroxidase, and the fat- (biliurbin, coenzyme Q, vitamin A, and vitamin E) and water-soluble (vitamin C and uric acid) antioxidants.[82] A general reduction in antioxidant defense mechanisms occurs in diabetes.[51,53,56] Low levels of serum carotenoid also play a role in the development of insulin resistance and diabetes.[67] Antioxidant therapy achieved using supplementation with pharmaceutical preparations of antioxidant nutrients or non-nutrients,[93,94] or both, may confer cardiovascular and metabolic benefits in diabetes. Experimental findings indicate that antioxidants improve endothelium-dependent vasodilation and insulin sensitivity. Results of epidemiological studies indicate there are strong associations between dietary intake of antioxidant nutrients and protection against CVD.[95–97]

Hyperglycemia inhibits endothelial function in the forearm microvasculature via suppression of NO bioavailability.[52] This reduced dilation during acute hyperglycemia is also found in patients with diabetes and can be improved using antioxidant therapy.[63,66,98] NO has important antioxidant activities in the patients with diabetes and can be improved using antioxidant defense mechanisms occurs in diabetes.[51,53,56] Low antioxidant role can make basal endothelium-dependent, NO-mediated vasodilator function especially vulnerable to inhibition during an oxidative insult. Conversely, loss of basal endothelium-derived NO in this manner can also help preserve vasodilator responses to endothelial agonists and NO donors. However, any lesion in the endogenous NO signaling pathway has major pathological implications for microangiographic disease progression in diabetes. Disregulation of vascular tone, the proliferation of vascular smooth muscle cells, platelet aggregation, coagulation, fibrinolysis, leukocyte adhesion, vascular permeability, and lipoprotein oxidation contribute to the progression of the disease.[98] Many of these pro-atherogenic sequelae can be triggered by hyperglycemia via formation of AGE, which inactivates NO to generate endothelial intracellular oxidant stress.[79,98,102,103]

eNOS

Study results suggest that diminution of endothelial synthesis of NO contributes to the pathogenesis of type 2 diabetes.[64] In addition to detracting from the vascular antioxidant defense, this change compounds any defect in the anti-atherogenic signaling role of NO.[67,104] The 3 isoforms of NOS (eNOS, neuronal NOS, and inducible NOS) require 5 cofactors/prosthetic groups (flavin adenine dinucleotide, flavin mononucleotide, heme, 5,6,7,8-tetrahydrobiopterin, and Ca-calmodulin) to convert l-arginine to NO. If NOS lacks any of the cofactors or substrates, it will switch from the coupled state of NO production to the uncoupled state of O2− production.[104] The resulting O2− reacts with the preformed NO to form ONOO−, which is an extremely potent oxidizing agent. Peroxynitrite, in turn, can oxidize the cofactor of eNOS and 5,6,7,8-tetrahydrobiopterin to 7,8-tetrahydrobiopterin, thus further uncouple NO generation.[104] The increased pro-oxidant activity can facilitate oxidation of LDL to oxLDL particles, which can limit the availability of l-arginine, enhance the uncoupling of NOS, and contribute to increased ROS generation and decreased availability of NO in diabetes.[54,56] ONOO−-mediated tyrosine nitration of prostacyclin synthase contributes to vascular complications in diabetes mellitus. In diabetes, hyperglycemia or hyperlipidemia increases O2− and ONOO− generation, which results in prostacyclin synthase nitration and subsequent thromboxane receptor stimulation. ONOO− also promotes endothelium-derived cyclooxygenase-dependent vasoconstriction factor release via accumulation of non-metabolized prostaglandin H2. Thus, ONOO−-dependent prostacyclin synthase nitration shifts the balance toward platelet aggregation, atheroma, and thrombus formation.[13]

Results of studies performed by us[105,106] and others[107] suggest that 3 correlated mechanisms are involved in the regulation of eNOS uncoupling via O2− and then ONOO− in diabetes. They include (1) oxidative modification of tetrahydrobiopterin (BH4) into dihydrobiopterin; (2) regulation of the zinc-thiolate center of eNOS, leading to zinc-depleted eNOS dimers and reduction of affinity to BH4 and l-arginine; and (3) ubiquitination and proteasomal degradation of guanosine triphosphate cyclohydrolase 1, a rate-limiting enzyme in BH4 synthesis.[113] We found that O2− generated from hyperglycemia can quench NO to form ONOO−, a powerful oxidant that not only causes prostacyclin synthase nitration and thromboxane receptor activation but also uncouples eNOS to produce oxidants.[103,108]

Adenosine monophosphate-activated protein kinase and diabetic endothelial dysfunction

The heterotrimer AMPK comprises α, β, and γ subunits. Each subunit has at least 2 isoforms.[9–12] The α subunit contains the catalytic site. Increases in the Adenosine monophosphate (AMP)/adenosine triphosphate (ATP) ratio activate AMPK via mechanisms including direct allosteric activation and phosphorylation of the α subunit at Thr-172 by an AMP-dependent AMPK kinase (AMPKK).[109] Until 2013, AMPKK was thought to be activated by an increase in the AMP/ATP ratio. However, study results indicate that AMP binding to AMPK makes it more susceptible to
phosphorylation by AMPK.Liver kinase B1 (LKB1) was the first AMPK identified. A gene mutation in this tumor suppressor increases the risk of colon, stomach, and pancreatic cancer (Peutz-Jegher syndrome). Calcium calmodulin-dependent kinase was the second AMPK identified.

AMPK is activated in response to stresses such as hypoxia, oxidant stress, hyperosmolarity, and exercise (in muscle). Other studies indicate that AMPK activation is a fundamental component of cellular responses to stress that threaten viability. AMPK can be phosphorylated and activated in various tissues by hormones acting through Gq receptors, adiponectin, leptin, and β-adrenoceptor agonists, metformin, the thiazolidinediones, and oxidants such as ONOO− and hydrogen peroxide. AMPK activation results in phosphorylation of target molecules to increase fatty acid oxidation and muscle glucose transportation; it also initiates synthetic processes to conserve ATP. Acetyl CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl CoA reductase were the first identified AMPK targets. Other AMPK targets are being identified at a rapid rate. AMPK inhibits proliferation through multiple mechanisms. These mechanisms include cell cycle regulation, inhibition of protein synthesis, and de novo synthesis of fatty acids, particularly mevalonate and its downstream products during cholesterol synthesis. Dual deficiency of AMPK α1 and α2, 2 catalytic subunits of AMPK, is lethal to mouse embryos, which indicates the importance of AMPK.

**AMPK in endothelial cells**

Compared with AMPKα2, AMPKα1 is predominantly expressed in endothelial cells. However, both isoforms might have equal roles in the maintenance of endothelial function. In endothelial cells, the activity of AMPK can be regulated by stresses such as shear stress, Ca2+ -elevating agonists, and hormones such as adiponectin. The AMPK in endothelial cells is implicated in the regulation of fatty acid oxidation, small G protein activity, NO production, inflammation, and angiogenesis. AMPKα1 and AMPKα2 increase NO release by phosphorylating both Ser1177 and Ser635 of eNOS in endothelial cells. Selective deletion of endothelial PKKA1 coding for protein kinase AMPKα1 reduces glycolysis, compromises endothelial cell proliferation, and accelerates the formation of atherosclerotic lesions in hyperlipidemic mice.

Endothelial-specific deletion of the AMPKα1 subunit attenuates phenylephrine-mediated contraction in an eNOS- and endothelium-dependent manner. AMPKα2 mice have impairment of acetylcholine-induced endothelium-dependent relaxation, along with increased oxidant production.

**AMPK activation suppresses oxidative stress**

AMPK is dysregulated in animals and humans with type 2 diabetes or metabolic syndrome. AMPK activation via physiological or pharmacological means can improve the sensitivity of insulin and metabolic hemostasis. Various hormones, natural compounds, and pharmacological agents can activate AMPK. Some, like metformin and thiazolidinediones, are used to treat type 2 diabetes. Metformin is also used off-label to limit the insulin dose requirement during treatment of type 1 diabetes. Results of REducing with Metformin Vascular Adverse Lesions (REMOVAL) trial indicate that metformin treatment in type 1 diabetes can alleviate atherosclerosis progression and reduce LDL-cholesterol levels and body weight. These findings suggest a new perspective on the therapeutic activation of AMPK in type 1 diabetes and indicate a potential role in the reduction of long-term CVD risk. For instance, choric acid is likely to protect against diabetes-induced endothelial dysfunction via the activation of the AMPK signaling pathway. AMPK is an important candidate for the effective treatment of the diabetes-associated vascular endothelial injury.

**AMPK and NO**

Hyperglycemia is the core determinant of long-term complications in diabetes, mainly via induction of oxidative stress. Ido et al. found that incubation with the first direct AMPK activator, 5-aminoimidazole-4-carboxamide riboside (AICAR), prevents hyperglycemia-drive oxidative stress and apoptosis. This result suggests that AMPK has a protective role against damage in endothelial cells resulting from sustained hyperglycemia. NOX is the major source of high glucose-induced oxidative stress. Study results indicate that AMPK activation functions as a NO inhibitory mechanism via regulation of NOX complex assembly or NOX subunit expression. For instance, AMPK inhibits activation of NOX in human neutrophils. Treatment of neutrophils with AICAR or AMPK suppresses the rise in O2− production triggered by phorbol esters or N-formylmethionyl-leucyl-phenylalanine. AMPK activity prevents the serine phosphorylation and membrane translocation of p47phox induced by these agonists. A widely used antidiabetic drug, metformin, inhibits O2− production by stimulated neutrophils and in platelets. Genetic deletion of AMPKα2 in LDL receptor-knockout LDLr−/− mice significantly increases 26S proteasome activity, IκB degradation, NF-κB transactivation, NOX subunit expression, ROS production, and endothelial dysfunction. All these effects are markedly suppressed by long administration of the proteasome inhibitor, MG132. These findings suggest that AMPKα2 is a physiological inhibitor of NOX in endothelial cells. As a result, AMPK is essential in maintaining the non-atherogenic and non-inflammatory conditions in endothelial cells. Rosiglitazone activates AMPK in endothelial cells which, in turn, prevents hyperactivity of NOX induced by high glucose, possibly through protein kinase C inhibition. Taken together, these results suggest that AMPK functions as an “early warning system” in response to oxidant stress and upon activation, AMPK can suppress oxidative injury by suppressing NOX-derived ROS. This feedback mechanism becomes impaired in diabetes, and AMPK inhibition accentuates oxidative stress and vascular injury in diabetes in vivo.

**AMPK and the electron transport chain**

Mitochondrial ROS levels increase in response to many atherosclerosis-associated stimuli, including hyperglycemia, oxLDL, and triglycerides. Pharmacological activation of AMPK by 5-aminoimidazole-4-carboxamide ribonucleotide or salicylate restores mitochondrial morphology and relieves endothelial dysfunction via suppression of mitochondrial ROS, endoplasmic reticulum stress, and subsequent activation of inflammation. AMPK regulates mitochondrial fission via autophagy-dependent degradation of DRP1 in endothelial cells. Deletion of AMPKα1 or AMPKα2 results in defective autophagy, DRP1 accumulation, and aberrant mitochondrial fragmentation.
Metformin administration markedly inhibits mitochondrial fragmentation, decreases mitochondrial-induced superoxide, improves endothelium-dependent vasodilation, suppresses vascular inflammation, and alleviates atherosclerotic lesions in streptozotocin-induced diabetic mice. Another study found that metformin attenuates the development of atherosclerosis by reducing DRP1-mediated mitochondrial fission and mitochondrial superoxide anion production in an AMPK-dependent manner.\[^{87}\] Taken together, these findings suggest that suppression of mitochondrial fission is a potentially effective therapeutic approach for treating macrovascular complications in patients with diabetes. C-peptide prevents diabetic vasculopathy by suppressing ROS-mediated endothelial cell apoptosis. C-peptide activates AMPK and inhibits high glucose-induced ROS generation, mitochondrial fragmentation, mitochondrial membrane potential decline, and endothelial cell apoptosis. C-peptide replacement therapy restores AMPK phosphorylation, ROS generation, and mitochondrial morphology in aortas of diabetic mice. These findings reveal a novel mechanism that is associated with the use of C-peptide to activate AMPK and alleviate hyperglycemia-induced vasculopathy.\[^{149}\]

Continuously produced ROS can be eliminated by antioxidant systems. AMPK activation can suppress diabetic endothelial dysfunction by upregulating antioxidant potentials. Pharmacological AMPK activator 5-aminimidazole-4-carboxamide riboside can markedly increase UCP-2 expression and decrease $O_2^-$ production and prostacyclin synthase nitration in diabetic wild-type mice but not in AMPKα2-KO counterparts.\[^{150}\] Direct evidence indicates that AMPK activation suppresses hyperglycemia-induced mitochondrial ROS generation via induction of manganese SOD and promotion of mitochondrial biogenesis through the AMPK-peroxisome proliferator-activated receptor gamma coactivator 1 alpha pathway in human umbilical vein endothelial cells.\[^{153}\]

Taken together, we conclude that AMPK functions as oxidant stress suppressor or redox modulator as its activation can trigger physiological responses to suppress the processes that generate oxidants (mitochondria, NOX) and/or to increase antioxidant defense systems [Figure 2].

**Dual effects of oxidative stress on AMPK activity**

Reversible and transient activation of AMPK by acute production of low levels of oxidants and ONOO$^-$. A line of evidence supports the responsiveness of AMPK in control of imbalanced redox status.\[^{126,132–134}\] For instance, the addition of the vitamin E analog and antioxidant, Trolox, to mouse embryonic fibroblasts results in reduced basal AMPK

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**Figure 2:** AMPK activation suppresses oxidative stress and alleviates diabetic endothelial dysfunction. AMPK: Adenosine monophosphate-activated protein kinase; DRP1: Dynamin-1-like protein; eNOS: Endothelial nitric oxide synthase; ETC: Electron transport chain; MnSOD: Manganese superoxide dismutase; NF-κB: Nuclear factor-κB; NO: Nitric oxide; NOX: Nicotinamide adenine dinucleotide phosphate oxidase; $O_2^-$: Superoxide anion; ONOO$^-$: Peroxynitrite; UCP2: Uncoupling protein 2.
Hydrogen peroxide activates AMPK in various cell types. ROS-triggered energy stress strongly activates AMPK and is likely to be the major mechanism of ROS-caused AMPK activation. Alternatively, 1 study found that ROS can directly activate AMPK; this effect is independent of the changes in AMP, adenosine diphosphate, and ATP. Glucose oxidase-generated hydrogen peroxide also contributes to AMPK activation through direct S-glutathionylation on the AMPKα subunit at residues Cys299 and Cys304. Reactive nitrogen species are also implicated in AMPK activation. Subsequent studies from us have further determined that AMPK acts as a proximal “oxidant sensor” within endothelial cells, either through its ability to respond to minimal changes in AMP levels or through its activation by oxidant-initiated signaling events [Figure 3].

Irreversible inhibition of AMPK inhibition by chronic exposure of elevated oxidants such as ONOO⁻

Study findings support a role for ROS in the impaired endothelium-dependent vasodilation found in various vascular preparations isolated from experimental diabetic animals. It is likely that this dysfunction is mainly due to the reduced bioavailability of NO resulting from its rapid inactivation by superoxide radicals. This mechanism, together with the production of superoxide anion by “uncoupled” NO synthases, may generate significant sources of endothelial superoxide anion. AMPK contribution to eNOS bioactivity is supported by in vitro and in vivo work by our group and others. In addition to direct phosphorylation of eNOS to increase NO production, AMPK can promote NO bioactivity by suppressing NO inactivation via reduction of the amount of ROS in the diseased vessel. Non-toxic levels of ROS/ONOO⁻ activate AMPK or AMPK-dependent pathways, while activation of AMPK suppresses ROS/ONOO⁻ generation.

AMPK activity decreases in muscle in multiple animal models with a metabolic syndrome phenotype. Evidence indicates that AMPK activity is diminished in adipose tissue and skeletal muscle of humans with obesity or type 2 diabetes. Decreased AMPK activity and subsequent reduction in cardiac autophagy are important events during the development of diabetic cardiomyopathy. Chronic activation of AMPK by metformin prevents cardiomyopathy by upregulating autophagy activity in diabetic OVE26 mice. This finding suggests that stimulation of AMPK represents a novel approach for treatment of diabetic cardiomyopathy. Hyperglycemia impairs autophagy in human umbilical vein endothelial cells via inhibition of AMPK, which directly results in endothelial cell damage. Diabetes downregulates AMPK and acetyl-CoA carboxylase in mouse aortas. This downregulation can be suppressed by metformin treatment. Similar results are found in cultured human umbilical vein endothelial cells. Endothelial dysfunction caused by a high fat diet is related to a dysfunctional endothelial AMPK-phosphoinositide 3-kinases-protein kinase B-eNOS pathway. The dysfunction correlates with increases in plasma non-esterified fatty acids, triglyceride, and impaired glucose management.

![Figure 3: Signal transduction pathways for AMPK as a redox sensor and modulator in endothelial cells. AMPK: Adenosine monophosphate-activated protein kinase; LKB1: Liver kinase B1; NF-κB: Nuclear factor-κB; NOX: Reduced nicotinamide adenine dinucleotide phosphate oxidase; ONOO⁻: Peroxynitrite; PKC: Protein kinase C.](image-url)
ROS and other reactive molecules can inhibit the LKB-AMPK axis.\textsuperscript{[179,180]} For instance, chemically reactive lipids, such as cyclopentenone prostaglandins (exogenous or generated from cyclooxygenase-2) form a covalent adduct with LKB1 Cys210 in its activation loop and thus inhibit LKB1 kinase activity.\textsuperscript{[179]} Similarly, findings from a study using a spontaneously hypertensive rat model indicate that the development of left ventricular hypertrophy is associated with an increase in the oxidative stress-derived lipid peroxidation byproduct 4-hydroxy-2-nonenal.\textsuperscript{[180]} This increase results in the formation of 4-hydroxy-2-nonenal-LKB1 adducts that inhibit LKB1 and subsequent AMPK activity.\textsuperscript{[180]} However, these two studies did not describe any direct post-translational modification of AMPK by ROS. In conditions of glucose starvation or exogenous hydrogen peroxide treatment, AMPK is negatively regulated by oxidation of cysteine residues Cys130 and Cys174 on its $\alpha$ subunit. The changes cause oxidative aggregation and interfere with the interaction between LKB1 and AMPK.\textsuperscript{[181,182]}

Our unpublished results indicate that in chronic diseases such as diabetes, AMPK becomes a target of excessive oxidative stress. We found that: (1) When given exogenously or when produced in the vasculature from endogenous sources, ONOO$^-$ inhibits AMPK activation likely by increasing tyrosine nitration of AMPK. The catalytic activities of AMPK$\alpha$ are exquisitely and uniquely sensitive to pathologically relevant concentrations of ONOO$^-$; (2) Chronic exposure of endothelial cells to elevated glucose concentrations results in loss of AMPK, aberrant overexpression of NOX activity, and increased $O_2^-$ release similar to that seen with genetic depletion of AMPK. The effects of elevated glucose are prevented by superoxide dismutase-polyethylene glycol or NG-nitro-l-arginine methyl ester (l-NAME); these findings indicate that endogenous generation of ONOO$^-$ is responsible for the AMPK loss; (3) Direct demonstration of the occurrence of AMPK nitration in endothelial cells exposed to hyperglycemia and in tissues from diabetic mice. Overall, our results support the hypothesis that AMPK is inhibited by pathologically relevant concentrations of ONOO$^-$ generated in a diabetic milieu.

**Conclusion**

Endothelial dysfunction is the earliest detectable pathological event in diabetes. Glucose levels elevated via the increased production of free radicals and oxidants are likely to be the main causes of endothelial dysfunction. Our work has firmly established that AMPK in endothelial cells is activated by very low levels of free radicals and oxidants; activation of AMPK by low levels of oxidants can feedback to the suppression of oxidant stress, either via its suppression on multiple sources (NOX, mitochondria, etc) and/or the promotion of antioxidant enzyme levels within endothelial cells. As a result, AMPK reverses or prevents endothelial dysfunction caused by diabetes, and aberrant oxidant stress-induced AMPK inactivation accelerates both endothelial dysfunction and CVD in diabetes. Based on these observations, we propose that besides the traditional ATP/ADP/AMPK regulation pathway and traditional roles of AMPK in energy metabolism, AMPK is the main sensor in redox homeostasis and is critical for maintaining redox homeostasis by suppressing the levels of oxidant stress [Figure 4].

In summary, we conclude that AMPK inactivation caused by aberrant oxidant stress plays a crucial role in endothelial dysfunction in diabetes. Selective AMPK activation can effectively treat endothelial dysfunction in diabetes by normalizing the critical roles of AMPK for maintenance of endothelial cell redox homeostasis.

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**Conflicts of interest**

None.

**References**


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