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AMP-ACTIVATED PROTEIN KINASE ACTIVATION AS A STRATEGY FOR PROTECTING VASCULAR ENDOTHELIAL FUNCTION

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SUMMARY

1. AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase involved in the regulation of cellular and organismal metabolism. AMPK has a heterotrimeric structure, consisting of a catalytic α -subunit and regulatory β - and γ -subunits, each of which has two or more isoforms that are differentially expressed in various tissues and that arise from distinct genes. The AMPK system acts as a sensor of cellular energy status that is conserved in all eukaryotic cells. In addition, AMPK is activated by physiological stimuli and oxidants.

2. The importance of AMPK in cardiovascular functions is best demonstrated by recent studies showing that widely used drugs, including statins, metformin and rosiglitazone, execute cardiovascular protective effects at least partly through the activation of AMPK. As a consequence, AMPK has been proposed as a candidate target for therapeutic intervention in the treatment of both Type 2 diabetes and metabolic syndrome owing to its central role in the regulation of energy balance; it may also have a role in weight control.

3. In the present brief review, we summarize the recent progress of AMPK signalling and regulation focusing on vascular endothelial cells. We further hypothesize that AMPK is a dual sensor for energy and redox status within a cell and AMPK may be a therapeutic target for protecting vascular endothelial function.

Keywords

AMP-activated kinase; atherosclerosis; diabetes mellitus; endothelium; energy metabolism; hypertension; nitric oxide; oxidative stress; peroxynitrite; superoxide anions

OVERALL REVIEW OF AMP-ACTIVATED KINASE

The AMP-activated protein kinase (AMPK) is a serine/threonine kinase and a member of the Snf1/AMPK protein kinase family that is found in all eukaryotes.^{1,2} AMPK has been proposed to act as a cellular energy sensor, which switches on catabolic pathways that produce ATP and switches off anabolic pathways that consume ATP. AMPK is a heterotrimer, containing α -, β - and γ -subunits, each of which has at least two isoforms. The α -subunit contains the catalytic site; however, all subunits are necessary for full activity. Increases in the ratio of AMP to ATP activate AMPK by a number of mechanisms, including direct allosteric activation and covalent modification due to activation by an AMP-dependent AMPK kinase (AMPKK), which

phosphorylates the α -subunit on Thr¹⁷². Until recently, it was believed that this occurred as a result of activation of AMPKK by an increase in the AMP/ATP ratio. However, recent studies from Woods *et al.*³ and Carlson *et al.*⁴ failed to demonstrate such an increase in AMPKK activity in response to various AMPK activators. Rather, their results indicate that AMP binds to AMPK and that this makes it more susceptible to phosphorylation by AMPKK. Interestingly, the first AMPKK that has been identified is LKB1, a tumour suppressor that is mutated in humans with Peutz–Jegher syndrome,⁵ a disorder associated with an increased risk of developing carcinomas of the colon, stomach and pancreas. A second AMPKK has been identified as calcium calmodulin-dependent kinase kinase (CaMKK; see below).⁶

AMPK is ubiquitous in distribution and its activity increases in a variety of cells in response to stressors such as hypoxia, oxidant stress, hyperosmolarity and, in muscle, exercise. As noted first by Hardie *et al.*,⁷ AMPK activation appears to be a fundamental component of the response of many cells to stresses that threaten their viability. AMPK can be phosphorylated and activated in various tissues by hormones that act through G_q receptors⁸ and by adiponectin,⁹ leptin,¹⁰ α - and β -adrenoreceptor agonists,^{11,12} pharmacological agents such as metformin^{13,14} and the thiazolidinediones,¹⁵ and oxidants such as ONOO⁻^{16,17} and H₂O₂.¹⁸ In addition, AMPK may be regulated by cellular glycogen content, possibly as a consequence of AMPK binding to glycogen via its β -subunit.¹⁹ Activation of AMPK leads to the phosphorylation of a number of target molecules that result in, among other things, increases in fatty acid oxidation and muscle glucose transport (to generate more ATP), as well as inhibition of various synthetic processes (to conserve ATP). Acetyl CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase²⁰ were the first molecules shown to be AMPK targets; however, other molecules are being identified at a rapid rate. For example, AMPK phosphorylates and activates malonyl CoA decarboxylase,²¹ an enzyme involved in malonyl CoA turnover in many tissues, and it phosphorylates and inhibits glycerophosphate acyltransferase,²² the first committed enzyme in glycerolipid synthesis. Likewise, both endothelial and neuronal nitric oxide synthase (eNOS and nNOS, respectively) have been shown to be targets for AMPK in endothelium and muscle by Chen *et al.*²³ and Winder *et al.*²⁴ have shown that treatment of intact rats with 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) increases the expression of a variety of proteins in muscle, including the glucose transporter GLUT-4 and several mitochondrial oxidative enzymes. In addition, AMPK activation has been shown to alter the expression of a variety of genes, including those for uncoupling protein (UCP)-3 and GLUT-4 in muscle,²⁵ and fatty acid synthase (FAS)²⁶ and phosphoenolpyruvate carboxykinase²⁷ in hepatocytes. In addition, one recent study from our group¹³ showed that nicotine, via peroxynitrite (ONOO⁻), activates AMPK, resulting in enhanced threonine phosphorylation and consequent inhibition of FAS in 3T3L1 adipocytes.

Since its discovery, investigations into the regulation and the effects of AMPK have expanded very rapidly. The AMPK pathway and modulation of energy balance through the AMPK system presents an attractive therapeutic target for intervention in many conditions of disordered energy balance, including obesity, Type 2 diabetes, stroke and atherosclerosis. Among these, research on the protective actions of and the regulation of energy metabolism by AMPK in the endothelium is becoming an important area. Due to page limitation, the scope of the present review will be limited to new developments related to AMPK as a protector and a metabolic sensor in the endothelium. We focus on the effect of AMPK activation on cellular factors contributing to endothelial cell damage and dysfunction and discuss the possibility that AMPK may be a therapeutic target for atherosclerosis.

ENDOTHELIUM: BARRIER AND ATHEROGENESIS INITIATIVE FACTOR

The endothelium is a single layer of cells that covers the inner surface of all vessels in the body, including conduit vessels, resistance vessels, precapillary arterioles and capillaries. The

endothelial layer forms a barrier between the elements of the blood and the tissues by virtue of its direct contact with the circulating blood. The intact function and structure of the endothelium are prerequisite for maintaining normal vascular function and anti-atherogenesis. The arterial wall endothelium has various important physiological functions, including adjusting vascular tone and permeability, antithrombosis and the secretion of diverse active materials. Arterial wall endothelium damage is an initiating factor for atherogenesis. The endothelium is susceptible to changes in blood elements, such as increases in blood glucose or free fatty acids (FFAs), angiotensin II, adrenaline, noradrenaline, bradykinin, as well as hypertension and hypercholesterolaemia. All these factors can cause vascular endothelium damage.

Endothelial dysfunction is characterized by increased superoxide production, impaired nitric oxide (NO) activity, increased endothelial apoptosis and a reduction in endothelium-dependent vasodilation.^{28,29} Chronic dysfunction of the endothelium is implicated in the pathophysiology of several cardiovascular disorders, including atherosclerosis, hypertension, diabetic vasculopathy and heart failure.³⁰ There are many factors that contribute to endothelial damage and dysfunction. The most important factors are hyperglycaemia and FFAs induced by insulin resistance syndrome/diabetes. Hyperglycaemia and FFAs can result in a series of pathophysiological changes at the molecular level, including inflammation,³¹ endothelial cell apoptosis,^{29,32} increases in cell Ca^{2+} , reactive oxygen species (ROS)^{33,34} and nuclear factor (NF)- κB activation,³⁵ and decreases in eNOS activity and NO bioavailability, which contribute to or accelerate endothelial damage and dysfunction.²⁸

AMPK EXPRESSION AND FUNCTION IN ENDOTHELIAL CELLS

AMPK is a heterotrimeric protein consisting of three subunits, α , β and γ , each of which has at least two isoforms that can lead to the formation of 12 different complexes. These combinations render AMPK complexes with different properties and relative tissue specificity.³⁶ The α -subunit contains the catalytic site, whereas the β - and γ -regulatory subunits are important in maintaining the stability of the heterotrimer complex.³⁷ However, all subunits are necessary for full activity.³⁸ Different tissues express distinct α -catalytic subunits that account for the major part of AMPK activity. Muscle cells mainly express AMPK complexes containing the $\alpha 2$ -subunit³⁹ and adipocytes express the $\alpha 1$ isoform.^{12,40} The liver expresses both $\alpha 1$ and $\alpha 2$ isoforms.⁴¹ AMPK is expressed in both endothelial cells and smooth muscle cells. Both $\alpha 1$ - and $\alpha 2$ -catalytic subunits are expressed in arterial smooth muscle cells, although their relative proportion differs between different arteries.⁴² We found that both $\alpha 1$ - and $\alpha 2$ -catalytic subunits are expressed in endothelial cells⁴³ and the predominant isoform is $\alpha 1$.^{16,43} Although the AMPK $\alpha 2$ isoform is barely detectable in endothelial cells, it still has important physiological functions; for example, it is essential for angiogenesis in response to hypoxic stress.⁴⁴ The functional significance of these different combinations remains unclear; however, it is noted that AMPK complexes containing the $\alpha 1$ isoform are less sensitive to AMP.⁴⁵ To date, there are no data concerning the respective expression of other AMPK subunits in endothelial cells.

Several studies have suggested that endothelial AMPK may play important physiological functions, such as modulation of endothelial cell energy supply,⁴⁶ protection from apoptosis,³² mediation of endothelial NOS activation in response to shear stress⁴⁷ and regulation of inflammation, angiogenesis and maintenance of perfusion.^{44,48}

REGULATION OF AMPK IN ENDOTHELIAL CELLS

AMPK kinase

The control of AMPK activity is complex and the classic view is that AMPK is activated allosterically by an increase in the intracellular AMP/ATP ratio and/or by the phosphorylation of Thr¹⁷² within the α -subunit. Several protein kinases responsible for this phosphorylation have been identified, including Peutz-Jeghers syndrome kinase LKB1 (LKB1),⁵ a tumour suppressor kinase in complex with two accessory subunits termed STE20-related adaptor (STRAD) and MO25 and the Ca²⁺/calmodulin-dependent protein kinase kinase (CaMKK).⁶ The major breakthrough in identifying the first AMPKK came from research on the regulation of the AMPK orthologue Snf-1 in *Saccharomyces cerevisiae*.⁷ The T-loop residue of Snf-1 was phosphorylated by a group of three related protein kinases bearing homology to mammalian LKB1, which was subsequently identified by several laboratories as being the major upstream kinase for AMPK.^{5,49} Subsequent studies suggested that AMPK could not be activated in mammalian cells that lacked LKB1 expression or in cells that were treated with heat shock protein (Hsp) 90 inhibitors, which decrease LKB1 expression.⁵⁰ In addition, LKB1 turned out to phosphorylate the T-loop of all 12 human kinases that are phylogenetically related to AMPK (AMPK subfamily).⁵¹

Several cellular stress conditions, such as glucose deprivation, hypoxia, ischaemia and heat shock, are responsible for the depletion of cellular ATP and elevation of AMP levels that are associated with an increased AMP/ATP ratio.¹ The γ -subunit of AMPK has been identified as the protein that binds AMP. When the γ -subunit binds AMP, AMPK is allosterically activated and becomes a better substrate of AMPKK LKB1.⁵² Then, the AMPK α -subunit undergoes phosphorylation by LKB1 on Thr¹⁷² and its activity increases significantly.⁵² *In vitro* studies have suggested that binding of AMP to AMPK is likely to be the principal regulatory mechanism stimulating phosphorylation of AMPK by LKB1.⁵ However, several lines of evidence point to the presence of non-LKB1 AMPKKs.⁶ Under some conditions (e.g. hyperosmotic stress or antidiabetic drug metformin), AMPK is activated without a detectable change in the AMP/ATP ratio.⁵³ In yeast, three protein kinases, namely Elm1, Pak1 and Tos3, have been identified to phosphorylate and activate SNF1, the yeast homolog of AMPK.^{54,55} The most closely related mammalian protein kinases to these yeast kinases are members of the CaMKK family.^{54,55} The finding that CaMKK β acts upstream of AMPK suggests that, in addition to changes in the AMP/ATP ratio, an increase in intracellular Ca²⁺ may act as a second pathway to activate AMPK.⁵⁶

In endothelial cells, AMPK is activated by two AMPKK pathways, LKB1 and CaMKK β .⁵⁶ Several direct and indirect arguments suggest that LKB1 is involved in AMPK activation in endothelial cells. Treatment of endothelial cells with AICAR, a drug that is transformed in the cell into 5-amino-4-imidazolecarboxamide (AICA)-riboside (also termed 'ZMP'), an analogue of AMP, activates AMPK in endothelial cells.^{16,57} Studies by our group indicate that ONOO⁻ and hypoxia-reoxygenation activate AMPK by stimulating LKB1 phosphorylation at Ser⁴²⁸.¹⁷ Furthermore, direct mutation of Ser⁴²⁸ of LKB1 into alanine and the kinase-inactive LKB1 mutant abolished ONOO⁻-induced AMPK activation.¹⁷ A study of human endothelial cells by Stahmann *et al.*⁵⁶ showed that AMPK is activated by thrombin through a Ca²⁺-dependent mechanism. Inhibition of CaMKK with STO-609 or downregulation of CaMKK β using RNA interference decreased thrombin-induced AMPK activation significantly, indicating that CaMKK β was the responsible AMPK kinase. Endothelial AMPK represents an important protective and adaptive mechanism in response to various cellular stresses, including increased FFA,⁵⁷ hypoxic stress⁴⁴ and peroxynitrite,^{58,59} and mediates the actions of hormones, such as adiponectin⁶² and oestradiol,⁶¹ metformin¹⁶ and vascular mediators, such as bradykinin⁶⁴ and histamine or thrombin.⁶³

AMPK kinase kinase

As mentioned above, LKB1 is an important upstream kinase for AMPK. However, paradoxically, neither the activity of LKB1 itself nor that of AMPK-related kinases was regulated directly by the stimuli known to activate AMPK, such as AMP, AICAR, muscle contraction, hypoxia–reoxygenation and metformin.^{43,58,64} Thus, how these stimuli lead to LKB1-dependent AMPK activation and whether an upstream kinase for LKB1 exists remain unclear.

Our recent studies in endothelial cells¹⁷ found that atypical protein kinase C ξ (PKC ξ), a protein kinase of the AGC family, regulates AMPKK LKB1 acting as AMPK kinase kinase (AMPKKK). The human LKB1 is a serine–threonine kinase of 433 amino acids, which contains both a kinase domain and a nuclear localization signal in its N-terminal region.⁵⁰ The C-terminal region of LKB1 consists of 124 residues and contains several post-translational modifications. Five phosphorylation sites have been identified; two residues are autophosphorylation sites (Thr³³⁶ and Thr⁴⁰²) and three others (Ser³²⁵, Thr³⁶³ and Ser⁴²⁸) are phosphorylated by upstream kinases.⁵⁰ Possibly, the LKB1 C-terminal region serves as a regulatory domain mediating dynamic interactions with several classes of proteins and promotes subcellular targeting.⁶⁵ We found that stimulation of bovine aortic endothelial cells (BAEC) with phorbol myristate acetate activated AMPK in parallel with increased LKB1 phosphorylation at Ser⁴²⁸.¹⁷ Mutation of Ser⁴²⁸ into alanine abolished ONOO[−]-enhanced AMPK activation. These results suggest that Ser⁴²⁸ located in the C-terminus of LKB1 may play a crucial role in regulating AMPK activation. Indeed, a recent study by Forcet *et al.*⁶⁶ suggests that naturally occurring C-terminal mutations, which neither disrupt LKB1 kinase activity nor interfere with LKB1-induced growth arrest, reduce LKB1-mediated activation of AMPK and impair downstream signalling. We have provided evidence that PKC ξ regulates AMPK activation by phosphorylating LKB1, which results in increased association of LKB1 with AMPK.¹⁷ The key evidence stems from studies in endothelial cells that show that ONOO[−], a potent oxidant formed by the combination of superoxide anions ($\cdot\text{O}_2^-$) and NO at a diffusion-controlled rate, activated AMPK independent of cellular AMP/ATP ratios.^{16,43,58} This AMPK activation pathway was LKB1 dependent, involved LKB1 phosphorylation at Ser⁴²⁸ within the C-terminal part of LKB1 and led to the association of LKB1 with AMPK. AMPK is not activated by ONOO[−] in cells lacking LKB1 (HeLa-S3 cells) but expressing normal levels of CaMKK.⁶ In addition, ONOO[−] increased the coimmunoprecipitation of LKB1 with AMPK, which was sensitive to inhibition of PKC ξ . Furthermore, inhibition of PKC ξ attenuated ONOO[−]-enhanced LKB1-Ser⁴²⁸ phosphorylation, as well as the association of LKB1 with AMPK. More importantly, recombinant PKC ξ , in a concentration-dependent manner, caused the phosphorylation of LKB1-Ser⁴²⁸ *in vitro*. Inhibition of PKC ξ abolished ONOO[−]-enhanced association of LKB1 with AMPK, but not with microtubule-associated protein/microtubule affinity-regulating kinase-3 (MARK-3), another member of the AMPK family, suggesting that PKC ξ –LKB1 signalling is exclusively active towards the AMPK pathway.¹⁷ These results are in line with previous findings^{43,64} that AMPK stimuli, such as AICAR or exercise, activate AMPK without altering the activities of LKB1 and other members of the AMPK family. This selective effect of PKC ξ –LKB1 on AMPK may involve the PKC ξ -dependent phosphorylation of LKB1-Ser⁴²⁸ within the C terminus of LKB1. Because phosphorylation of none of the other known regulatory sites on LKB1 was affected by PKC ξ and a low level of LKB1/MARK-3 interaction was independent of PKC ξ inhibition, LKB1-Ser⁴²⁸ phosphorylation by PKC ξ may be the specific step leading to LKB1-dependent AMPK activation.

We have used endothelial cells to establish a novel PKC ξ –LKB1–AMPK signalling axis. In addition, we have also replicated these findings in a variety of tissues, including human retinal pericytes, rat smooth muscle cells and mouse 3T3-L1 preadipocytes (M-H Zou and Y Wu,

unpubl. obs., 2007).¹⁷ Furthermore, PKC ξ inhibition attenuates AMPK activation by a variety of known AMPK stimuli, including oxidant (ONOO⁻), pharmacological reagent (metformin) and hypoxia–reoxygenation, implying that the PKC ξ –LKB1–AMPK pathway is not unique to the endothelium and may be a common pathway shared by other physiological stimuli in a variety of tissues. Thus, we can expect broader implications of this signalling pathway in cell biology. A number of reports has shown that PKC ξ plays critical roles in signalling pathways that control cell growth, differentiation and survival.^{50,67} Thus, the interactions of PKC ξ with LKB1 and AMPK that we have described above may affect our understanding of not only vascular biology, but also cancer development. LKB1 is an upstream activator of AMPK and regulation of the AMPK pathway is believed to be directly involved in LKB1 tumour suppressor function.⁶⁸ Consistent with this model, LKB1 has recently been identified as a negative regulator of mammalian targets of rapamycin (mTOR) signalling through the sequential stimulation of AMPK and of the tuberous sclerosis (TSC) 1/TSC2 tumour suppressor complex.⁶⁹ Furthermore, ACC, a substrate of AMPK, controls fatty acid synthetic metabolism, which is frequently dysregulated in tumours.⁷⁰ We found that phosphorylation of LKB1-Ser⁴²⁸ in its C-terminal part increased association of LKB1 with AMPK, as well as activation of its downstream pathways, thus reinforcing the idea that AMPK plays a role in the control of cell transformation. Indeed, C-terminal mutations in LKB1, which decrease LKB1-mediated AMPK activation, compromise the ability of LKB1 to establish and maintain polarity of both intestinal epithelial cells and migrating astrocytes. Our findings further support the notion that LKB1 tumour suppressor activity depends, at least in part, on the regulation of AMPK signalling and downstream effects on cell polarization.

Serine/threonine phosphatases

Reversible protein phosphorylation is a major mechanism that is used in eukaryotic organisms to regulate all kinds of cellular functions, from metabolic pathways to cell death. At least one-third of human proteins contain covalently bound phosphate. More than 98% of protein phosphorylation occurs on serine and threonine residues. The opposing actions of protein kinases and protein phosphatases control the degree of protein phosphorylation within the cell. Similarly, the delicate AMPK phosphorylation is maintained by upstream protein kinase AMPKK and serine/threonine phosphatase. Two serine/threonine phosphatases, namely protein phosphatase 2A (PP2A) and protein phosphatase 2C (PP2C), were identified to inactivate the active and phosphorylated form of AMPK in cell-free assays.⁷¹

It has been shown that PP2C is responsible for dephosphorylation of AMPK.⁷² Wang and Unger⁷⁵ have demonstrated that AMPK phosphorylation was significantly reduced in both Zucker diabetic rat *fa/fa* rats and *ob/ob* mouse hearts compared with lean, wild-type controls and the reduction in active phosphorylated AMPK α is associated with an increase in PP2C. The PP2C are characterized by the requirement of Mn²⁺ or Mg²⁺ for activity.⁷⁴ Two isoforms of PP2C, α and β , are known from a variety of mammalian tissues.⁷⁵ They are considered monomeric (43–48 kDa), share 75% sequence identity and have the same enzymatic characteristics.⁷⁶ Both isozymes are ubiquitously expressed. PP2C α was expressed mainly in different epithelial cell types in organs and tissues including the skin, lungs, kidney, breast, digestive tract, endocrine glands, testis, prostate, ovary, uterus, brain, lymph nodes and bone marrow. Human endothelial cells, smooth muscle cells and extracellular matrix contained no or very little PP2C α .⁷⁷ In contrast with the other Ser/Thr protein phosphatases, how the activity, localization and substrate specificity of PP2C are governed is not clear.⁷⁶ An alignment of the amino acid sequence of AMPK with that of other serine/threonine protein kinases around the regulatory phosphorylation site (subdomains VII–VIII) revealed a high degree of conservation.⁷⁸ Phosphopeptides derived from this region of AMPK and containing the almost invariant threonine (Thr¹⁷² in AMPK) were found to be good substrates for PP2C α .⁷⁸ PP2C plays an important role in regulating AMPK phosphorylation in physiological conditions and the basal

AMPK phosphorylation is largely dependent on PP2C activity. Using the inactivation assay as the criterion, Davies *et al.*⁷¹ found that dephosphorylation of AMPK by PP2C α is inhibited by 5'-AMP. Therefore, the AMP activation of AMPK has two aspects: (i) binding of AMP to the AMPK γ -subunit leads to allosterical changes in AMPK and renders AMPK a better substrate for AMPKK LKB1; and (ii) binding of AMP to the phosphoform shields the phosphorylation site against the action of protein phosphatase, thus protecting the phosphorylation status.

It is known that PP2A is a major protein phosphatase in all eukaryotic cells that has a wide range of biological functions, including control of the cell cycle, organization of the cytoskeleton, transcription of immediate early genes, and cholesterol and protein biosynthesis. PP2A is a multimeric ubiquitously expressed serine/threonine phosphatase consisting of scaffolding A, regulatory B and catalytic C subunits. PP2A has been shown to be ceramide responsive *in vitro* and *in vivo*^{59,79} and is also termed 'ceramide-activated protein phosphatase' (CAPP).⁷⁹ In contrast with PP2C, endothelial cells have abundant PP2A expression.^{57,80} Increasing evidence indicates that PP2A plays important roles in maintaining endothelial cell physiological functions, including regulation of endothelial cell cytoskeletal structure, protection of the endothelial cell barrier,⁸¹ maintenance of endothelial cells in a resting state and limiting the motility that is needed for the morphogenic process of angiogenesis.⁸⁰ Moreover, PP2A can regulate eNOS phosphorylation status directly by dephosphorylating serine 1177/1179.⁸² However, excessive PP2A activation in pathological conditions results in endothelial cell damage or dysfunction by inhibition of AMPK activity. Our recent studies⁵⁹ have demonstrated that prolonged exposure of endothelial cells to palmitate significantly suppresses the phosphorylation of AMPK and its downstream enzyme ACC. We have provided compelling evidence obtained *in vitro* and *in vivo* that palmitate inhibits both AMPK and eNOS phosphorylation by ceramide-dependent PP2A activation. This observation provides further support for the concept that PP2A is an important component for the dephosphorylation and inactivation of AMPK and may directly modulate AMPK function. In support of this idea, it has been reported that the PP2A complex is involved in regulating the interaction between AMPK α 2 and γ 1⁸⁵ and inactivation of AMPK in pancreatic β -cells,⁸⁴ and that the active phosphorylated form of AMPK can be inactivated in cell-free assays by PP2A.⁷¹ Thus, PP2A may be a physiologically relevant AMPK phosphatase.

AMPK AS A SENSOR FOR REACTIVE OXYGEN AND NITROGEN SPECIES IN ENDOTHELIAL CELLS

Reactive nitrogen species (RNS) are derived from NO. Of these, peroxynitrite (ONOO⁻) is the best characterized and appears to have the most biological activity. Peroxynitrite is formed by the biradical reaction of NO and O₂⁻. Many oxidation and nitration products are produced from the reaction of ONOO⁻ with cellular macromolecules. Reactive nitrogen species comprise a class of products formed by cells in response to various stress stimuli. There is evidence that cells exposed to oxidants such as ONOO^{-16,17} and H₂O₂¹⁸ activate AMPK, although the mechanism remains to be determined. In our exciting preliminary studies, we found that ONOO⁻ activated AMPK in endothelial cells and increased the phosphorylation of its downstream enzymes eNOS-Ser^{13,79} and ACC-Ser.⁷⁹ In order to determine whether activation of AMPK by ONOO⁻ was due to AMP accumulation, we examined cellular adenosine nucleotide levels in BAEC exposed to bolus additions of 50 μ mol/L ONOO⁻. We found that bolus addition of ONOO⁻ did not result in a drop in the ATP/AMP ratio.⁵⁸ Using pharmacological and molecular biological approaches, we identified that ONOO⁻ may activate AMPK via an AMP-independent process and involves c-Src/phosphatidylinositol 3-kinase (PI3-K)/3-phosphoinositide-dependent kinase 1 leading to PKC ξ phosphorylation and activation, which activates LKB1 and increases LKB1-AMPK association. This activation is LKB1 dependent. In the early phase (less than 5 min) of hypoxic endothelial cells, inhibition

of ONOO⁻ formation by overexpression of superoxide dismutase (SOD; to scavenge O₂⁻) or NOS inhibition with N^G-nitro-L-arginine methyl ester (L-NAME; to prevent the formation of NO) attenuated hypoxia–reoxygenation-enhanced phosphorylation of both AMPK and ACC. Because ·O₂⁻ alone has no effect on AMPK activation and the addition of authentic ONOO⁻ did not affect the AMP/ATP ratio, the attenuation of AMPK and ACC phosphorylation by L-NAME and SOD in cells exposed to hypoxia–reoxygenation suggests the involvement of ONOO⁻ and cannot be explained solely by the AMP/ATP ratio. Inhibition of c-Src activity or PI3-K with a dominant negative mutant also blocked both AMPK and ACC phosphorylation. Thus, our results suggest that a signalling pathway initiated by ONOO⁻ is capable of activating AMPK independent of changes in the AMP/ATP ratio.

Interestingly, our recent studies indicate NO can also activate AMPK (M-H Zou and J-H Zhang, unpub. obs., 2007). However, NO-induced AMPK activation is LKB1 independent. Nitric oxide can increase intracellular Ca²⁺ content and activate CaMKKβ, an AMPKK. It is noted that the structure-similar isogenous derivatives (NO and ONOO⁻) activate AMPK through an entirely distinct pathway. The probable explanation is that ONOO⁻-induced AMPK activation is a response to cellular stress under non-physiological conditions, such as oxidative stress or inhibition of the mitochondrial respiration chain, whereas NO, which exists in normal endothelial cells, activates AMPK and then regulates endothelial metabolism and function under physiological conditions.

NITRIC OXIDE, AMPK AND ENDOTHELIAL FUNCTION

There are roughly 10¹⁴ endothelial cells in our vasculature to protect us against atherosclerosis and thrombosis.⁸⁵ A major weapon of endothelial cells to fight vascular diseases is eNOS, an enzyme that generates the vasoprotective molecule NO·,⁸⁵ which requires Ca²⁺/calmodulin, flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin (BH₄) as cofactors.⁸⁶ Vascular NO· has a variety of functions, but its most important action is to dilate all types of blood vessels and maintain vascular homeostasis by stimulating soluble guanylyl cyclase and increasing cGMP in smooth muscle cells. Impairment of endothelium-dependent relaxations (EDR) represents reduced eNOS-derived NO bioavailability and is present in atherosclerotic vessels even before vascular structural changes occur.⁸⁷ Endothelial dysfunction, as characterized by an impairment of EDR and reduced NO bioactivity, is the critical step for atherogenesis. In addition, vascular NO· is a potent inhibitor of platelet aggregation and adhesion and can also inhibit leucocyte adhesion to the vessel wall. White cell adherence is an early event in the development of atherosclerosis; therefore, NO· may protect against the onset of atherogenesis.⁸⁵ Furthermore, NO· has been shown to inhibit DNA synthesis, mitogenesis and proliferation of vascular smooth muscle cells, which are important components of vessel wall remodelling during atherosclerosis formation.⁸⁸ In addition, NO· suppresses smooth muscle exposure to platelet-derived growth factor(s) by inhibiting platelet aggregation and adhesion. Thus, NO· also prevents the formation of fibrous plaque, a later step in atherogenesis. Based on these effects, endothelial NO· probably represents the most important anti-atherogenic defence in the vasculature.⁸⁵ It is therefore not surprising that loss of endothelial NO function is associated with several cardiovascular disorders, including atherosclerosis, which is due either to decreased production or to increased degradation of NO.

AMPK is an important activator of eNOS and also the focus of considerable interest as a therapeutic target for the treatment of Type 2 diabetes and the metabolic syndrome, which are both associated with endothelial dysfunction and cardiovascular disease, yet the role of AMPK in the endothelium is poorly characterized. Ongoing studies within our group aim at understanding the role of AMPK in the endothelium. We⁶¹ and others²³ have demonstrated that AMPK phosphorylates and activates eNOS in cultured endothelial cells. Recent studies in our laboratory have indicated that AMPK mediates the stimulation of NO synthesis in

response to several agonists (M-H Zou and Y Wu, accepted for publication, 2008). In addition, we have studied the functional effects of AMPK activation in the endothelium and have demonstrated that AMPK activation increases endothelium-dependent vasodilatation in animal model (M-H Zou and Y Wu, accepted for publication, 2008). Such data indicate an anti-atherogenic role for the AMPK cascade.

OXIDATIVE STRESS, AMPK AND ENDOTHELIAL FUNCTION

Oxidative stress in the vascular wall is an important feature of vascular disease. Reactive oxygen species (ROS) are increasingly recognized as the major cause of compromised endothelial cell function by oxidative modification of cellular components, such as proteins, lipids, carbohydrates and DNA, but also by interfering with signal transduction pathways and the action of endothelial products necessary for the regulation of vascular homeostasis.⁸⁹ In addition, ROS influence endothelial barrier function and leucocyte adhesion and transmigration.⁹⁰ Many *in vitro* and *in vivo* studies suggest that oxidative stress is increased in patients or animal models with diabetes or dyslipidaemia.^{91,92} Oxidative stress may be crucial for the pathogenesis of diabetic mellitus and its complications. Mitochondria have been reported to be the major source of ROS. Therefore, mitochondrial ROS production seems to be one of the important targets to prevent a progression of diabetic complications. The most universal and critical mitochondrial function is oxidative phosphorylation. The overall system of oxidative phosphorylation includes five large multi-enzyme complexes, designated complexes I, II, III, IV and ATP synthase. This mitochondrial respiratory system is thought to be the major source of ROS under normal physiological conditions. Two main sites of superoxide generation exist in this system: NADH dehydrogenase at complex I and the interface between coenzyme Q (CoQ) and complex III. It has been shown that hyperglycaemia could increase the production of ROS from the mitochondrial electron transport chain.³³

The endothelium is the first organ that comes in contact with and perceives high glucose or high lipid concentrations and, therefore, is very susceptible to these changes. Although endothelial dysfunction encompasses a broad range of abnormalities, a reduced bioavailability of endothelium-derived NO is the most widely studied aspect. A reduction in NO bioavailability in the vessel wall impairs endothelium-dependent vasorelaxation and reduces other beneficial effects of NO, such as its inhibition of platelet and leucocyte adhesion and its antiproliferative effects.³⁰ A decline in NO bioavailability may be caused by: (i) reduced activity or expression of eNOS; (ii) a deficiency of eNOS substrate (L-arginine) or cofactors (BH₄),^{93,94} and/or (iii) increased inactivation of NO by O₂⁻. The latter is now recognized as a fundamentally important underlying mechanism in most settings. In addition, recent studies suggest that ROS or ONOO⁻, the reaction product of NO and ·O₂⁻, oxidize and degrade BH₄, leading to eNOS uncoupling and the promotion of endothelial dysfunction.⁹³ Our studies also suggest that high glucose via the mitochondrial respiration chain leads to ROS overproduction, which decreases NO production either directly by reacting with NO and then converting into ONOO⁻ or ROS or indirectly via ONOO⁻-induced degradation of BH₄, which leads to eNOS uncoupling and then decreases in NO levels.

Our previous investigations have suggested that increased ROS production promotes AMPK activation by the LKB1 complex in cultured BAEC.¹⁶ Thus, AMPK activation in response to oxidative stress may be implicated in an anti-oxidative or protective role of AMPK in endothelial cells. Studies from Ido *et al.*³² have shown that incubation of human umbilical vein endothelial cells (HUVEC) with 30 compared with 5 mmol/L glucose for 72 h causes a significant increase in apoptosis related to an increase in oxidative stress. However, incubation with the AMPK activator AICAR increased AMPK activity and completely prevented this change, suggesting that AMPK could play an important role in protecting the endothelial cell against the adverse effects of sustained hyperglycaemia. Interestingly, Ouslimani *et al.* reported

that metformin, an AMPK activator, decreased intracellular production of ROS in aortic endothelial cells mainly by NAD(P)H oxidase and also, to a lesser extent, by the respiratory mitochondrial chain.⁹⁵ Kukidome *et al.* provided more direct evidence that activation of AMPK reduces hyperglycaemia-induced mitochondrial ROS production by induction of Mn-SOD and promotion of mitochondrial biogenesis through activation of the AMPK–peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) pathway in HUVEC.⁹⁶ These studies support the notion that AMPK has a beneficial effect on endothelial function by suppressing oxidative stress in endothelial cells.

AMPK AND LIPOTOXICITY IN ENDOTHELIAL CELLS

Fatty acids can be a major fuel of the endothelium and, over a brief period of time, they can be derived predominantly from intracellular stores.⁹⁷ In fact, high rates of fatty acid oxidation are demonstrable in HUVEC when the cells are provided with carnitine.⁹⁷ Endothelial cells can maintain their energy state when fatty acids are essentially their sole fuel.⁹⁸ The AMPK–ACC–malonyl CoA–carnitine palmitoyl-transferase 1 mechanism for regulating long-chain fatty acid oxidation, similar to that of muscle, operates in the endothelial cell and is regulated by AMPK under physiological conditions.^{97,98} Endothelial cells also have the capacity to store triglycerides (TG), which are hydrolysed continuously to provide an endogenous source of FFAs.⁹⁷

However, excessive exposure of vascular endothelium to FFAs leads to endothelial dysfunction. This overexposure is associated with insulin resistance and diabetes, in which the ability of insulin-resistant adipocytes to store and retain FFAs is compromised, leading to a sustained elevation in serum FFAs. In a similar manner, diets rich in fat and/or calories are considered a risk factor for the development of atherosclerosis. Certain diet-derived lipids and their derivatives can disrupt normal endothelial integrity, thus reducing the ability of the endothelium to act as a selectively permeable barrier to blood components. An excess of FFAs or suppressed fat oxidation in endothelial cells leads to increased *de novo* synthesis of diacylglycerol (DAG),⁹⁹ which activates certain isoforms of protein kinase C (PKC).³⁴ Overactivity of PKC contributes to endothelial damage and vasculopathy by stimulating endothelial superoxide production via NAD(P)H oxidase,³⁴ inhibiting NOS activity,¹⁰⁰ impairing endothelial responsiveness to insulin¹⁰⁰ and promoting activation of NF- κ B.¹⁰¹ High levels of FFAs also lead to endothelial cell lipid accumulation. Lipid accumulation *per se* in vascular endothelial cells may play an important role in the pathogenesis of atherosclerosis in obese subjects. Selective lipid accumulation and fatty acid changes in endothelial cells can modulate membrane fluidity, proteoglycan metabolism and signal transduction mechanisms. Reduced AMPK activation may play an important role in the lipid accumulation and genesis of endothelial dysfunction in obese rats.¹⁰²

Recent studies^{105,104} in heart and liver have revealed that AMPK may be sensitive to the ‘lipid status’ of a cell and activation may be influenced by intracellular fatty acid availability independent of cellular AMP levels. Indeed, a reduced fat oxidative capacity has been reported in Type 2 diabetic patients.¹⁰⁵ In addition, feeding of a high-fat diet significantly decreases p-AMPK in the liver and muscles of rodents.^{106,107} AMPK activity is reduced in aortic endothelium or skeletal muscle of obese rats compared with lean animals.¹⁰² These investigations raise the possibility that chronic exposure of cells to fatty acids inhibits AMPK activation. Recently, our studies⁵⁹ indicated that long-term high levels of FFAs trigger a ceramide–PP2A-dependent inhibition of both AMPK and eNOS, which may contribute to vascular endothelial injury and dysfunction. Taken together, impaired endothelial AMPK activation may contribute to lipid accumulation, lipotoxicity and endothelial dysfunction.

One feasible way to alleviate the endothelial lipotoxicity associated with insulin resistance syndrome/diabetes is to lessen the excessive flux of FFAs into the endothelium, which can be achieved by eating very low-fat meals, improving the insulin sensitivity of insulin-resistant adipocytes, achieving appropriate weight loss and/or by administering insulin-sensitizing agents, such as thiazolidinediones or bioactive chromium, which improve adipocyte insulin responsiveness.¹⁰⁸ The other alternative way to deal with endothelial lipotoxicity is to promote FFA oxidation and ameliorate lipid accumulation in endothelial cells, which can be achieved by activating endothelial AMPK.^{46,97} As in muscle cells, AMPK has been shown to phosphorylate and inhibit ACC activity; this leads to a reduction in malonyl-CoA, which, in turn, disinhibits carnitine palmitoyltransferase and accelerates mitochondrial FFA oxidation.¹⁰⁸ Accordingly, Ruderman's group has reported that treatment of endothelial cells with AICAR, a natural activator of AMPK, largely offsets the adverse effects of palmitate on endothelial superoxide production and NF- κ B activation.⁹⁸ The recently characterized hormone adiponectin, which can decrease lipid accumulation in non-adipose tissues, is now known to activate endothelial AMPK via a membrane receptor.¹⁰⁹ The study of Lee *et al.*¹⁰² indicated that reduced AMPK activation may play an important role in lipid accumulation and the genesis of endothelial dysfunction in obese rats and that α -lipoic acid improves vascular dysfunction by normalizing lipid metabolism and activating AMPK in endothelial cells.

OTHER FUNCTIONS OF AMPK IN ENDOTHELIAL CELLS

Apart from activating eNOS and suppressing oxidative stress, AMPK has other potential benefits to endothelial cells. Activated AMPK increases fatty acid oxidation by phosphorylating and inhibiting ACC, leading to a decrease in the concentration of malonyl-CoA.⁹⁸ In addition, AMPK decreases fatty acid incorporation into glycerolipids, either secondary to its effect on fatty acid oxidation or by virtue of the fact that, in some tissues, it phosphorylates and inhibits sn-glycerophosphate acyltransferase, the first committed enzyme in DAG and TG synthesis.¹¹⁰ AMPK is involved in eNOS activation and subsequent NO production in response to shear stress. Hence, AMPK, in addition to serving as an energy sensor, also plays an important role in regulating vascular tone.⁴⁷ An additional benefit of endothelial AMPK activity is that it may inhibit glycerol-3-phosphate acyltransferase, required for *de novo* synthesis of DAG.¹¹⁰ That is, AMPK may lessen endothelial DAG production (and, thus, PKC activation) both by diminishing the availability of the FFA substrate required for synthesis and by directly inhibiting the enzyme that catalyses it. Endothelial AMPK has the further advantage of activating NOS via phosphorylation on Ser1177,²³ thus opposing the adverse impact of PKC on this enzyme. AMPK could play an important role in protecting the endothelial cell against hyperglycaemia-induced alterations in fatty acid metabolism, impaired Akt activation by insulin, increased caspase 3 activity and apoptosis.³² In addition, AMPK mediates the inhibitory effect of the high molecular weight form of adiponectin on endothelial cell apoptosis.¹¹¹ Furthermore, AMPK activation inhibits fatty acid-induced increases in NF- κ B transactivation¹¹⁴ and cytokine-induced NF- κ B activation in vascular endothelial cells,¹¹³ suggesting that it has beneficial effects on endothelial inflammation induced by deleterious stimuli.

In this regard, therapeutic strategies that target the endothelium to stimulate AMPK and fatty acid oxidation may have positive effects on endothelial energy metabolism and may improve endothelial function.

CONCLUSIONS AND PERSPECTIVES

Although the AMPK pathway is traditionally thought of as a sensor of cellular energy status and a regulator of metabolism, recent discoveries have now provided novel evidence that it may function as a protector of endothelial function. AMPK has pleiotropic effects that may be

beneficial to endothelial function and anti-atherogenesis, including: (i) induction of the eNOS/NO pathway resulting in increased NO bioavailability; (ii) suppression of endothelial ROS production induced by deleterious stimuli, such as hyperglycaemia or high FFAs; (iii) improving endothelial FFA oxidation, leading to suppression of lipid accumulation; and (iv) anti-apoptotic and anti-inflammatory effects, as well as adjustments of vascular tone (Fig. 1). Together, additional insights into AMPK physiology in the endothelium and the consequences of its modification may suggest novel therapeutic targets for cardiovascular disease. Indeed, two major classes of insulin-sensitizing drugs, namely metformin and thiazolidinediones, may have great potential for promoting vascular health and lessening diabetes risk in insulin-resistance syndromes mainly by activating the AMPK system. However, both these drug classes appear to activate AMPK indirectly, either by inhibiting the respiratory chain or by stimulating the release of adiponectin. Moreover, gastrointestinal side-effects, weight gain, renal and liver toxicity and a slight risk of lactic acidosis would make these drugs logistically difficult to use widely for the many millions of individuals who are susceptible to insulin-resistant and cardiovascular diseases. Therefore, a better-tolerated, safer compound that directly and effectively activates AMPK in endothelial cells, yielding the same beneficial therapeutic effects of existing drugs while avoiding unwanted side-effects, may prove to have substantial practical value for promoting vascular health.

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List of abbreviations

ACC	Acetyl CoA carboxylase
AICAR	5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside
AMPK	AMP-activated protein kinase
AMPKK	AMPK kinase
BH ₄	Tetrahydrobiopterin
CaMKK	Calcium calmodulin-dependent kinase kinase
eNOS	Endothelial nitric oxide synthase
FFAs	Free fatty acids
NF- κ B	Nuclear factor- κ B
NO	Nitric oxide
PKC	Protein kinase C
PP2A	Protein phosphatase 2A
PP2C	Protein phosphatase 2C
ROS	Reactive oxygen species
SOD	Superoxide dismutase

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Fig. 1.

Pathways that regulate P-activated protein kinase (AMPK) activation and AMPK-regulated pathways in endothelial cells. When the γ -subunit binds AMP, AMPK is allosterically activated and becomes a better substrate for AMPK kinase LKB1. AMPK is also regulated by upstream kinase calcium calmodulin-dependent kinase kinase β (CaMKK β) and protein phosphatase 2A and 2C (PP2C and PP2A, respectively). Available studies suggest that AMPK activation improves endothelial function through multiple pathways, as indicated. See text for details. PKC ζ , protein kinase C ζ ; FFAs, free fatty acids; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; NF- κ B, nuclear factor- κ B; ROS, reactive oxygen species.