Up-regulation of Mitochondrial Uncoupling Protein-2 by the AMP-activated Protein Kinase in Endothelial Cells Attenuates Oxidative Stress in Diabetes

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ABSTRACT

Objective. Recent evidence suggests that the AMP-activated protein kinase (AMPK) is an important therapeutic target for diabetes. The present study was conducted to determine how AMPK activation suppressed tyrosine nitration of prostacyclin synthase (PGIS) in diabetes.

Research Design and Methods. Confluent human umbilical vein endothelial cells or mice were treated with 5-amino-4-imidazole carboxamide riboside (AICAR) for the detection of AMPK phosphorylation and the expression of mitochondrial uncoupling protein (UCP)-2.

Results. Exposure of HUVECs to high glucose (30 mM, HG) increased superoxide anions ($O_2^-$) and PGIS nitration. In addition, overexpression of constitutively active AMPK (Ad-CA-AMPK) or the addition of AICAR, reduced both $O_2^-$ and PGIS nitration caused by HG, whereas adenoviral overexpression of dominant negative AMPK mutants (Ad-DN-AMPK) enhanced the latter effects of HG. Exposure of HUVECs to either AICAR or metformin caused AMPK-dependent up-regulation of both UCP-2 mRNA and UCP-2 protein. Furthermore, over-expression of UCP-2 significantly ablated both $O_2^-$ and PGIS nitration triggered by HG. Further, overexpression of Ad-CA-AMPK increased, whereas overexpression of Ad-DN-AMPK inhibited AICAR-induced phosphorylation of p38 kinase at Thr180/tyr 182. Inhibition of p38 kinase with SB239063, which had no effect on AICAR-induced AMPK-Thr172 phosphorylation, dose-dependently suppressed AICAR-induced up-regulation of UCP-2, suggesting that AMPK lies upstream of p38 kinase. Finally, AICAR markedly increased UCP-2 expression and reduced both $O_2^-$ and PGIS nitration in diabetic wild type mice but not in their AMPK $\alpha_2$ deficient counterparts in vivo.

Conclusion. We conclude that AMPK activation increases UCP-2 resulting in the inhibition of both $O_2^-$ and PGIS nitration in diabetes.

Key words: AMP-activated protein kinase, nitric oxide, 3-nitrotyrosine, prostacyclin synthase, superoxide anions, uncoupling protein-2
AMP-activated protein kinase (AMPK) is a heterotrimer made up of α, β, and γ subunits, each of which has at least two isoforms. Increases in the AMP/ATP ratio activate AMPK by a number of mechanisms, including direct allosteric activation and α subunit phosphorylation (at Thr-172) by at least two AMPK kinases (i.e., LKB1 and CaMKK). AMPK is ubiquitous and is activated in a variety of cell types by inhibition of ATP production (i.e., anoxia, ischemia) or acceleration of ATP consumption (i.e., muscle contraction, fasting). As first noted by Hardie and Carling, AMPK activation appears to be a fundamental component of cellular responses to stresses that threaten cell viability. AMPK is phosphorylated and activated in various tissues by hormones acting through Gq receptors, adiponectin, leptin, α- and β-adrenoreceptor agonists, metformin, thiazolidinediones, and oxidants such as peroxynitrite (ONOO⁻) and H₂O₂. Activation of AMPK leads to the phosphorylation of a number of target molecules resulting in, among other things, increased fatty acid oxidation and muscle glucose transport (to generate more ATP), as well as inhibition of various biosynthetic processes (to conserve ATP). Increasing evidence suggests the functions of AMPK are beyond energy metabolism. For example, both endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) are targets of AMPK in the endothelium and muscle. Winder et al have shown that treatment of rats with AICAR increases the expression of a wide variety of proteins in muscle including the GLUT-4 glucose transporter and several mitochondrial oxidative enzymes. AMPK activation has also been shown to increase the expression of mitochondrial uncoupling protein (UCP)-2 in liver after infection with constitutively active AMPK (Ad-CA-AMPK). Similar effects of AMPK on UCP2 and UCP3 have been reported in skeletal muscle.

Strong accumulating evidence suggests that oxidative stress, defined as increased formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and/or decreased antioxidant potentials, plays an important role in the development of diabetic complications. This hypothesis is supported by the finding that many biochemical pathways strictly associated with hyperglycemia (glucose auto-oxidation, polyol pathway, prostanoid synthesis, protein glycation) increase the production of free radicals and oxidants. The functions of many proteins are likely affected by increased oxidant levels. We have found that prostacyclin synthase (PGIS), an enzyme releasing vasoprotective prostacyclin (PGI₂), is particularly susceptible to tyrosine nitration by RNS including ONOO⁻. In cultured endothelial cells, hyperglycemic media increases the levels of nitrated PGIS and decreases PGIS activity. Tyrosine nitration of PGIS and consequent thromboxane receptor activation are thought to be important mechanisms contributing to the initiation and progression of vascular complications in diabetes mellitus (reviewed in reference 23). This is due to the down-regulation of the protective actions of nitric oxide (NO) and prostacyclin (PGI₂), as well as accumulation of non-metabolized prostaglandin H₂, which promotes platelet aggregation, atheroma accumulation, and thrombus formation.
Emerging data support a role for ROS and RNS in cell signaling. Griending and co-workers\textsuperscript{27} found that angiotensin II augments $O_2^-$ production in smooth muscle cells via NADH/NADPH oxidase-like enzymatic activity. This enzymatic system now appears to be involved in a number of “maladaptive” characteristics of atherosclerosis, such as PDGF-induced cell proliferation,\textsuperscript{28} smooth muscle cell hypertrophy,\textsuperscript{29} diabetic retinopathy,\textsuperscript{30} and impaired NO bioactivity.\textsuperscript{31} Our earlier results had also demonstrated that pathologically relevant concentrations of ONOO$^-$ are capable of activating AMPK independently of changes in AMP/ATP and that ONOO$^-$-dependent AMPK activation occurs during hypoxia-reoxygenation\textsuperscript{13} and in metformin-treated endothelial cells.\textsuperscript{32} However, the consequences of AMPK activation on cellular oxidative stress remain to be determined. In the present study, we provide evidence that AMPK prevents oxidative stress associated with diabetes, in part, by up-regulating mitochondrial uncoupling protein (UCP)-2.

**MATERIALS AND METHODS**

A full description of Materials and Methods including adenoviral infection, RT-PCR of UCP-2 mRNA, PGIS activity assays, NO bioactivity, immunocytochemistry, and HPLC-detection of 3-nitrotyrosine can be found in the online supplements.

**Animal.** The animal protocol was reviewed and approved by the institutional animal care and use committee. Male AMPK$\alpha_2$ knockout (AMPK$^{-/-}$) mice, which had been crossbred with C57BL6 mice, were bred at the animal house of the University of Oklahoma Health Sciences Center. Their littermates, C57BL6 mice, were obtained from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in temperature-controlled cages with a 12-h light/dark cycle and given free access to water and normal chow. Mice aged 10 weeks were randomly divided into control and treated groups.

AMPK$\alpha_2$ KO mice and age-matched C57BL/6 mice were used to study whether AICAR attenuates diabetes-enhanced UCP-2 expression and PGIS nitration. The mice were made diabetic following five consecutive injection of steptozotocin (50 mg/kg in citrate buffer pH 4.5). Nondiabetic mice were injected with a comparable volume of citrate buffer. Glucose levels were measured in tail blood by a Free Style blood glucose monitoring system (TheraSense, Alameda, CA). Hyperglycemia was confirmed by nonfasting blood glucose >200 mg/dl (11 mmol/l) 1 week after the initial STZ injection. The nondiabetic and diabetic mice were randomly divided into three groups: control, STZ/untreated and STZ but treated with AICAR (250 mg/kg/day, subcutaneously). Six weeks later the animals were euthanized and the aorta was collected for analysis.

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) (American Type Culture Collection, Rockville, MD) were cultured in Ham’s F12 medium with 10% fetal bovine serum, 100 µg/ml heparin, and 30 µg/ml endothelial cell growth supplement. Cells were seeded in 60 x 15-mm culture dishes precoated with 0.1% pig gelatin. When they reached confluence, the cells were maintained in 1% fetal calf serum and exposed to normal glucose (5.5 mmol/l) or high glucose (30 mmol/l) for 3 to 7 days, during which the medium was changed every 2 days. Control groups (to account for media hyperosmolarity) were exposed to mannitol (24.5 mmol/l) in normal medium containing glucose (5.5 mmol/l). After
incubation, the media were collected and assays were conducted as described below. For measurements of $O_2^{•−}$ and cyclic GMP, the growth media was replaced by phosphate-buffered saline (PBS) containing no glucose. Unless otherwise noted, these measurements were performed on cells or the PBS bathing cells, which were stimulated by the calcium ionophore A23187 (10$^{-5}$ mol/l, 2 h).

**Adenovirus infection.** Human umbilical vein endothelial cells (HUVECs) were infected with adenovirus encoding either Ad-DN-AMPK or Ad-UCP-2, as described previously.13-14,32 Adenoviruses encoding green fluorescent protein (GFP) served as a control.

**Measurement of intracellular ROS.** Intracellular $O_2^{•−}$ was measured using the dihydroethidium (DHE) fluorescence/HPLC assay with minor modification. Briefly, HUVECs were incubated with DHE (0.5 µM) for 30 min, harvested, and then methanol extracted. Oxyethidium (a product of DHE and $O_2^{•−}$) and ethidium (a product of DHE auto-oxidation) were separated and quantified using a C-18 HPLC column (mobile phase: gradient of acetonitrile and 0.1% trifluoroacetic acid). $O_2^{•−}$ production was determined by the conversion of DHE into oxyethidium. ROS production in aorta was assayed using lucigenin (5µM) chemiluminescence as described previously.24-26

**Statistical analysis.** Statistical comparison was performed using a one- or two-way ANOVA, and intergroup differences were determined using the Bonferroni inequality. Values are expressed as mean ± SEM. $P < 0.05$ was considered significant.

The authors have full access to the data and take full responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

**RESULTS**

**AICAR reduces high glucose-induced oxidative stress in HUVECs.** Our earlier studies demonstrated that hyperglycemia not only increases $O_2^{•−}$ production and tyrosine nitration of PGIS, but also reduces NO bioactivity, as determined by cyclic GMP levels. A subsequent study revealed that the AMPK activator, metformin, dramatically attenuates the latter effect. To understand whether the beneficial effects of AMPK activation may be attributable to its ability to reduce oxidative stress, we tested the effect of AICAR on markers of oxidative stress in HUVECs. Confluent HUVECs were exposed to 5 mM D-glucose (NG), 30 mM D-glucose (HG), or 5 mM D-glucose plus 25 mM mannitol (OG) for 72h with or without 0.5 mM AICAR. HG but not the osmotic control (i.e., 5 mM D-glucose plus 25 mM mannitol), caused a 3-fold increase of $O_2^{•−}$ in HUVECs (Figure 1A). Administration of 0.5 mM AICAR had no effect on basal $O_2^{•−}$ production, but attenuated HG-enhanced $O_2^{•−}$ release. We also examined the effect of AICAR on NO bioactivity, which depends on the overall production and/or depletion of NO by $O_2^{•−}$. In line with elevated $O_2^{•−}$ release, HG significantly reduced the levels of cyclic GMP, and AICAR prevented this effect (Figure 1B). Further, AICAR increased the phosphorylation of eNOS-Ser1177 whereas it had no effects on of the total amount of eNOS protein in HG-exposed HUVECs (data not shown). These results suggest that AICAR might maintain NO bioactivity under HG conditions by increasing NO release and/or counteracting oxidative stress.

Further analysis of the antioxidant effects of AICAR was performed by
measuring 8-iso-prostaglandin F$_{2\alpha}$, a marker of lipid peroxidation. As shown in Figure 1C, HG markedly increased 8-iso-prostaglandin F$_{2\alpha}$ levels. Although AICAR had no effect on basal levels of 8-iso-prostaglandin F$_{2\alpha}$, it partially but significantly reduced HG-induced increases in 8-iso-prostaglandin F$_{2\alpha}$. AICAR didn’t completely abolish HG-enhanced 8-iso-prostaglandin F$_{2\alpha}$ in HUVEC (p<0.05, NG vs. HG plus AICAR). To understand whether AICAR acts as an oxidant scavenger, we exposed HUVECs to chemically synthesized ONOO$^-$. ONOO$^-$ significantly increased 8-iso-prostaglandin F$_{2\alpha}$ levels; however, co-treatment with AICAR did not alter this effect (Figure 1C). Additional experiments revealed that AICAR had no effect on the reduction of cytochrome C caused by xanthine/xanthine oxidase (data not shown). Thus, the reduction of oxidative stress by AICAR cannot be attributed to its ability to directly scavenge O$_2^-$ or ONOO$^-$. **AICAR inhibits high glucose-induced nitration and inactivation of PGIS.** Our earlier studies$^{24-25}$ had demonstrated that HG increases tyrosine nitration of PGIS, which inhibits PGIS activity. Analysis of 6-keto-PGF$_{1\alpha}$ concentrations revealed that HG, but not mannitol, significantly suppressed PGIS activity in HUVECs (Figure 1D). Interestingly, AICAR (1 mM) significantly attenuated HG-induced reduction of 6-keto-PGF$_{1\alpha}$ but had no effect on the levels of 6-keto-PGF$_{1\alpha}$ in cells exposed to mannitol (Figure 1D).

We next determined if increased PGIS activity was due to increased PGIS expression. Under normal glucose conditions, AICAR (1 mM, 1 to 72 h exposure) altered neither PGIS protein levels (Figure 2A) nor PGIS activity (Figure 2B). These results suggest AICAR had no effect on PGIS activity or PGIS expression in HUVECs.

As HG exposure increased PGIS nitration and inactivation, we next determined if the protective effects of AICAR on PGIS activity was due to the reduction of PGIS nitration caused by HG. Consistent with the idea that AMPK reduces oxidative stress, AICAR (0.5 mM) prevented PGIS nitration in HUVECs exposed to HG (Figure 2C and D). **AMPK activation is required for AICAR-induced reduction of oxidative stress.** Incubation of HUVECs with AICAR resulted in time-dependent AMPK activation, as determined by Thr172 phosphorylation of AMPK (Figure 3A). Similarly, a 24-h incubation with metformin (1 mM) markedly increased Thr172-AMPK phosphorylation (Figure 3A). Since either AICAR (Figure 1A) or metformin (data not shown) significantly reduced ROS production under HG conditions, we tested whether AMPK activation was required for this antioxidant effect. Under normal glucose conditions, inhibition of AMPK by adenoviral overexpression of dominant negative AMPK (Ad-DN-AMPK) significantly increased ROS in HUVECs, while GFP overexpression had no effect (Figure 3B). Moreover, Ad-DN-AMPK overexpression significantly accentuated HG-induced ROS production (Figure 3B). These results suggest that AMPK functions as an endogenous protector against ROS in endothelial cells. **AMPK attenuates high glucose-induced oxidative stress and PGIS nitration through up-regulation of UCP-2.** To further investigate the mechanism by which AMPK activation reduces oxidative stress, we analyzed UCP-2 expression in HUVECs. Under normal conditions, UCP-2 mRNA was abundant but very low levels of UCP-2 protein were detected (Figure 3C). However, exposure of HUVECs to AICAR significantly increased both UCP-2 mRNA
(Figure 3C) and UCP-2 protein expression (Figure 3D). Importantly, inhibition of AMPK with Ad-DN-AMPK, but not control Ad-GFP, significantly attenuated AICAR-induced UCP-2 expression, implying that AMPK up-regulates UCP-2 expression in HUVECs (Figure 3D).

Next, we tested whether adenoviral overexpression of UCP-2 in HUVECs altered HG-induced ROS production. Overexpression of Ad-UCP-2 markedly increased UCP-2 protein in HUVECs (Figure 4A). In addition, adenoviral overexpression of UCP-2 significantly reduced HG-induced increases in ROS (Figure 4B) and 3-nitrotyrosine (Figure 4C), with GFP overexpression having no effect on either parameter. Consistent with this result, UCP-2 overexpression markedly inhibited HG-induced PGIS nitration (Figure 4D) and PGIS inactivation (Figure 4E).

**Activation of p38 kinase is required for AMPK-dependent UCP-2 expression.** The fact that both AMPK and p38 kinase are activated by extracellular stresses (e.g., hypoxia/reoxygenation and osmotic stress),34-35 prompted us to investigate whether p38 kinase is required for AMPK-dependent UCP-2 expression. As shown in Figure 5A, the phosphorylation of p38 kinase at Thr180/Tyr 182 was markedly increased for up to 4h after AICAR treatment. In parallel, AICAR increased the phosphorylation of c-Jun, a downstream enzyme of p38 kinase. Adenoviral overexpression of constitutively active AMPK for 24h prior to AICAR treatment (2 mM for 2 h) resulted in increase in c-Jun and p38 kinase phosphorylation that was even greater than that elicited by AICAR alone (Figure 5B). Conversely, overexpression of Ad-DN-AMPK inhibited AICAR-induced phosphorylation of p38 kinase and c-Jun (Figure 5B).

To determine whether p38 kinase is required for AMPK-dependent UCP-2 expression, we treated HUVECs with SB239063, a potent p38 kinase inhibitor. Incubation of HUVECS with SB239063 abolished AICAR-induced phosphorylation of both p38 kinase (Figure 5C) and c-Jun (data not shown). Importantly, SB239063 also suppressed AICAR-induced up-regulation of UCP-2 in a dose-dependent manner (Figure 5D). When used at concentrations up to 20 µM, SB239063 did not alter AICAR-induced AMPK-Thr172 phosphorylation (Figure 5E), suggesting that AMPK lies upstream of p38 kinase.

**Chronic stimulation of AMPK with AICAR attenuates diabetes-induced oxidative stress and PGIS nitration.** To extend our *in vitro* findings, we investigated the effect of AICAR on PGIS nitration and UCP-2 expression associated with streptozotocin (STZ)-induced diabetes. Ten-week-old C57BL6 mice or AMPK α2 KO mice were subjected to five consecutive injections of STZ (50 mg/kg in citrate buffer, pH 4.5) or an equivalent volume of citrate buffer. Two weeks after STZ injection, animals received daily subcutaneous injections of 250 mg/kg AICAR (n = 7) or a corresponding volume of 0.9% NaCl for 6 weeks. One week after injection, STZ-treated mice had significantly higher serum glucose levels than their control counterparts (WT: 488 ± 25 vs.119 ± 7 mg/dL, P < 0.001, n = 11; AMPKα2 KO: 493 ± 27 vs.129 ±11 mg/dL, P < 0.001, n =9). In addition, diabetic animals had significantly lower body weights (WT: 16.0 ± 0.8 vs. 18.8 ± 1 grams, -14.9%, P < 0.05, n = 11; AMPKα2 KO: 15.8 ± 0.9 vs.17.9 ±0.7 grams, P < 0.05, n =10) and plasma insulin concentrations (WT: 0.3 ±
AMPKα2 KO: 0.4 ± 0.2 vs. 1.0 ± 0.2 ng/ml, P < 0.01, n = 11; AMPKα2 KO: 0.4 ± 0.2 vs. 1.0 ± 0.2 ng/ml) than controls. Diabetic and non-diabetic animals had a similar heart weight (WT: 70 ± 3.5 vs. 75 ± 2.6 mg). Comparison of AICAR and non AICAR-treated diabetic mice revealed that both groups had similar blood glucose levels (WT: 488 ± 25 vs. 461 ± 28 mg/dL; AMPKα2 KO: 493 ± 27 vs. 509 ± 27 mg/dL, P < 0.001, n = 9), water consumption (16.1 ± 0.7 vs. 15.1 ± 0.5 ml/d), and body weight (17.5 ± 0.8 vs. 19.1 ± 0.1 g).

Next, isolated aortas were analyzed for O$_2$- levels, PGIS nitration, and UCP-2 expression. A comparison of aortas from non-diabetic C57BL6 mice and AMPK α2 KO mice revealed that AMPK α2 KO aortas exhibited higher O$_2$- levels and PGIS nitration, but had lower levels of PGIS activity (Figures 6A, 6B and 6C). Compared to aortas from non-diabetic mice, those from diabetic mice had markedly increased O$_2$- levels (Figure 6A), increased PGIS nitration (Figure 6C), and decreased PGIS activity (Figure 6C) (P < 0.01, n = 7). STZ injection exacerbated aortic O$_2$- levels and PGIS nitration in AMPK α2 KO mice. Activation of AMPK with AICAR significantly inhibited O$_2$- release, PGIS nitration, and PGIS inactivation in diabetic WT mice (p<0.01, n=7) but AICAR administration had no effect in AMPKα2 KO mice. Taken together, these results suggest that AMPK activation is required for the suppression of aortic O$_2$- formation and PGIS nitration by AICAR in vivo.

As several cellular types exhibited reduced AMPK activity in the presence of high glucose and high glucose increases ROS production, we next determined if the addition of catalase altered UCP-2 expression in HUVEC. As shown in Figure 7, short exposure (2 hours) of HUVEC to HG significantly increased the phosphorylation of AMPK Thr172 along with increased detection of UCP-2 in HUVEC. Administration of catalase altered neither AMPK phosphorylation nor UCP-2 expression in HUVEC exposed to high glucose.

**DISCUSSION**

In the present study, we have found that AICAR, an activator of AMPK, reduces oxidative stress (O$_2^-$ and 3-nitrotyrosine) and increases UCP-2 expression in cultured endothelial cells as well as in aorta from diabetic mice. In HG-exposed HUVECs, AMPK inhibition of O$_2^-$ formation and PGIS nitration was accompanied by increased NO bioactivity. These protective effects of AMPK were confirmed by the finding that AMPK gene deletion not only exacerbated STZ-induced O$_2^-$ production and PGIS
nitrination, but also rendered AICAR incapable of protecting against increased $O_2^-$. The ability of AICAR to up-regulate UCP-2 expression in diabetic C57BL6 mice, but not in AMPK α2 KO mice, suggests that AMPK-dependent UCP-2 expression is essential for reduction of oxidative stress by AMPK.

Uncoupling proteins are mitochondrial transporters that are present in the inner mitochondrial membrane and belong to a family of mitochondrial anion carriers, which includes adenine nucleotide transporters. Mild uncoupling of respiration is known to diminish mitochondrial ROS formation by complex I and II. Recent evidence implies that the basic role of all UCPs is to prevent oxidative tissue injury by reducing oxidative stress. A role for UCP-2 in the down-regulation of mitochondrial ROS production is plausible, since available evidence suggests that this protein is expressed in numerous mammalian tissues. Macrophages of leptin-deficient ob/ob mice have low UCP-2 levels compared to those of normal mice, and these low UCP-2 levels are associated with increased mitochondrial ROS production. In addition, ROS levels in macrophages of UCP-2<sup>−/−</sup> mice and muscle tissue of UCP-3<sup>−/−</sup> mice exceed wild type levels. UCP-2 has the ability to reduce ROS not only in mitochondria, but also within the remainder of the cell and even in the extracellular space. Duval et al. have recently shown that UCP-2-mediated uncoupling in endothelial cells decreases extracellular ROS. Lee et al. have demonstrated that adenoviral transfer of the UCP-2 gene into human airway epithelial cells profoundly suppresses ROS generation, decreases NF-κB activity, enhances eNOS transcription, and improves endothelium-dependent vascular relaxation. Nevertheless, the molecular mechanisms underlying UCP-2 expression remain poorly defined. We have provided the first evidence that AMPK is essential for UCP-2 expression in endothelial cells in vivo. In line with this hypothesis, we have found that activation of AMPK with AICAR prevents $O_2^-$. formation, NO inactivation, and PGIS nitration that accompanies prolonged exposure of HUVECs to HG. Also in line with this hypothesis, pharmacological or genetic inhibition of AMPK abolished the ability of AICAR to not only reduce oxidative stress, but also up-regulate UCP-2 expression. The finding that STZ amplified $O_2^-$. production and PGIS nitration in AMPK α2 KO mice strongly suggests that AMPK suppresses oxidative stress. However, the most conclusive evidence for this idea is provided by the finding that AICAR failed to alter markers of oxidative stress or UCP-2 expression in the AMPK-α2 KO mice. Consistent with our results, AMPK activation has also been shown to increase the expression of UCP-2 in liver and in skeletal muscle. In pre-diabetic (impaired glucose tolerance) subjects, a one-year lifestyle diabetes prevention program involving increased physiological exercise improves metabolic control and increases UCP-3 levels by two-fold. Physiological exercise is known to lead to AMPK activation. Activation of AMPK leads to a reduction of oxidative stress and vascular function.

AMPK is activated by multiple stimuli, including oxidants such as ONOO<sup>−</sup> and H$_2$O$_2$. Thus, the production of oxidants might be required for AMPK-dependent UCP-2 expression. This idea is supported by the fact that UCP-2 was weakly expressed in endothelial cells from both wild type and AMPK α2 KO mice (Figure 6D). The ability of STZ to induce aortic $O_2^-$. formation, PGIS nitration, and UCP-2
expression in wild type mice, taken with its inability to induce UCP-2 expression in AMPK α2 KO mice (Figure 6D), strongly suggests that intracellular ROS activate AMPK, which then stimulates transcription of the UCP-2 gene. Consistent with this hypothesis, Brand’s group\textsuperscript{48,49} suggested that \( \text{O}_2^- \) itself activates UCP-2 within the matrix by an unspecified mechanism. We propose that ROS-activated AMPK, in return, limits ROS production by increasing mitochondrial UCP-2 expression. That is, AMPK-dependent UCP-2 up-regulation is a compensatory mechanism aimed at counteracting intracellular oxidative stress. Collectively, our findings suggest that AMPK is a physiologic regulator of ROS that protects endothelial cells against the adverse effects of hyperglycemia by inhibiting the processes that generate oxidants.

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**Disclosures**

None.
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FIGURE 1. AMPK ACTIVATION WITH AICAR (0.5 MM) REDUCES HIGH GLUCOSE-INDUCED OXIDATIVE STRESS AND PGIS NITRATION IN HUVECS. CONFLUENT HUVECS WERE EXPOSED TO 5 MM D-GLUCOSE (NG), 30 MM D-GLUCOSE (HG), OR 5 MM D-GLUCOSE PLUS 25 MM MANNITOL (OG) FOR 72H WITH OR WITHOUT 0.5 MM AICAR. AFTER THE INCUBATION, O$_2^-$ (1A), 8-ISO-PGF$_2\alpha$ (1B), CYCLIC GMP (NO BIOACTIVITY, 1C), AND PGIS ACTIVITY (AS REFLECTED BY THE CONVERSION OF PGH$_2$ TO 6-KETO-PGF$_{1\alpha}$, 1D) WERE ASSAYED (N = 12, *P < 0.01 HG VS. NG, ♣P < 0.01 HG VS. HG PLUS AICAR; †P<0.05 NG VS. HG PLUS AICAR).
Figure 2. Chronic administration of AICAR attenuates high glucose-enhanced PGIS nitration in HUVECs. Confluent HUVECs were exposed to 0.5 mM AICAR for the indicated time. PGIS, nitrated PGIS, and PGIS activity were assayed as described in Materials and Methods. 2A. Effect of AICAR on PGIS protein expression in HUVECs. N=7; 2B. Effect of AICAR (0.5 mM) on PGIS activity in HUVECs. N=7; 2C & 2D. AICAR administration attenuates HG-enhanced PGIS nitration in HUVECs. N = 5, #P < 0.01 HG vs. NG, *P < 0.01 HG vs. HG plus AICAR).
Figure 3. AICAR-induced UCP-2 expression in HUVECs is AMPK-dependent. 3A. AMPK activation by AICAR or metformin. The blot is a representative blot of four blots from four individual experiments. 3B. Effect of Ad-DN-AMPK on $O_2^-$ production induced by HG (n = 3, $^\#P < 0.05$ NG vs. HG or HG/Ad-GFP, $^*P < 0.05$ Ad-GFP/HG/AICAR vs. GFP/HG or HG, $^{+}P < 0.05$ Ad-DN-AMPK vs. NG, $^{\dagger}P < 0.05$, AD-DN-AMPK/AICAR/HG vs. Ad-GFP/HG/AICAR). 3C. Increase of UCP-2 mRNA by AICAR in HUVECs (n=3, $^*P < 0.05$ control vs. metformin or AICAR). 3D. AICAR-enhanced UCP-2 expression is AMPK-dependent. n = 3, $^*P < 0.05$ vs. GFP vs. GFP plus AICAR, $^{+}P < 0.05$ AICAR plus GFP vs. AICAR plus AD-DN-AMPK;
Figure 4. UCP-2 overexpression suppresses high glucose-induced ROS generation. 4A. Overexpression of UCP-2 increases UCP-2 protein in HUVECs. The blot is a representative of three blots from three individual experiments. 4B. Overexpression of UCP-2 suppresses HG-induced \( \text{O}_2^- \) production. \( n = 3 \), \( *P < 0.05 \) GFP plus HG vs. GFP plus NG, \( \text{**P} < 0.05 \) GFP/HG vs. UCP-2/HG. 4C. Overexpression of UCP-2 suppresses HG-enhanced-3-nitrotyrosine. \( n = 5 \), \( \text{*P} < 0.05 \) GFP plus HG vs. GFP plus NG, \( \text{**P} < 0.05 \) GFP plus HG vs. UCP-2 plus HG. 4D. Overexpression of UCP-2 attenuates HG-induced PGIS nitration in HUVECs. \( n = 5 \), \( \text{#P} < 0.05 \) HG vs. NG; GFP/HG vs. GFP/NG; \( \text{*P} < 0.05 \) GFP/HG vs. HG or OG; \( \text{†P} < 0.05 \) HG/GFP vs. HG/UCP-2; 4E. Effect of UCP-2 overexpression on HG-induced PGIS inactivation \( n = 5 \), \( \text{#P} < 0.05 \) NG vs. HG or HG/GFP, \( \text{*P} < 0.05 \) GFP/HG vs. HG/UCP-2.
Figure 5. p38 kinase is required for AMPK-dependent UCP-2 expression. 5A. Effects of AICAR on the phosphorylation of c-Jun and p38 kinase in HUVECs. Confluent HUVECs were treated with 0.5 mM AICAR at times indicated. The blot is a representative of three blots from three independent experiments. 5B. AICAR-increased phosphorylation of C-Jun and p38 is AMPK-dependent. HUVEC were infected with either Ad-AMPK-CA or AD-AMPK-DN. The blot is a representative of three blots from three independent experiments. 5C. Effects of SB239063 on AICAR-enhanced phosphorylation of p38 in HUVECs. HUVECs were treated with 0.5 mM AICAR in the presence or absence of different concentrations of SB239063, a selective p38 inhibitor. n = 4, #P < 0.05 AICAR vs. control, *P < 0.05 AICAR vs. AICAR plus SB239063; 5D. Effects of SB239063 on up-regulation of UCP-2 expression by AICAR. n = 4, #P < 0.05 AICAR vs. control, *P < 0.05 AICAR vs. AICAR plus SB239063; 5E. Effect of SB239063 on AMPK-Thr172 phosphorylation caused by AICAR in HUVECs. n = 4, #P < 0.05 AICAR vs. control, *P < 0.05 AICAR vs. AICAR plus SB239063.
Figure 6. AMPK reduces oxidative stress, PGIS nitration, and UCP-2 expression associated with STZ-induced diabetes. 6A. Effect of AICAR on aortic O$_2^-$ levels in wild type and AMPKα2 KO mice with and without diabetes (n = 6 – 8, *P < 0.05 STZ vs. control; †P < 0.05 STZ plus AICAR vs. STZ, ‡P < 0.05 STZ plus AICAR in AMPKα2 KO vs. wild type STZ plus AICAR; ††P<0.05, AMPK KO control vs. wild type control; 6B. Effect of AICAR on aortic PGIS activity in wild type and AMPKα1 KO mice with and without diabetes. n = 6 – 8, *P < 0.05 STZ vs. control, *P < 0.05 STZ plus AICAR vs. STZ; †P < 0.05 STZ plus AICAR in AMPKα2 KO vs. STZ in AMPK α2 KO; ‡P<0.05, STZ plus AICAR in wild type vs. STZ plus AICAR in AMPKα2 KO; 6C. Aortic PGIS nitration in diabetic mice treated with and without AICAR. n = 6 – 8, *P < 0.05 STZ vs. control, *P < 0.05 STZ plus AICAR vs. STZ in AMPKα2 KO; †P<0.05, Wild type control vs. AMPKα2 KO control; 6D. Top panel: Immunohistochemical staining of UCP-2 levels in AICAR-treated diabetic wild type and AMPK α2 KO mice; Bottom panel: AMPK-dependent UCP-2 expression by AICAR in vivo. n = 6 – 8, *P < 0.05 STZ vs. control, *P < 0.05 STZ plus AICAR vs. STZ in wild type; †P < 0.05 STZ plus AICAR in AMPKα2 KO vs. STZ in AMPKα2 KO.
Figure 7. Effects of catalase on high glucose-enhanced expression of UCP-2 in HUVECs. Confluent HUVECs were exposed to HG or NG in presence or absence of catalase (500 Units/ml) for 2 hours. The levels of AMPK-Thr172 and UCP-2 were examined in western blots by using the specific antibody. n = 3 – 5, *P < 0.05 NG vs. HG or HG plus catalase; †P < 0.05 HG plus HG plus catalase.