Suppression of the JNK Pathway by Induction of a Metabolic Stress Response Prevents Vascular Injury and Dysfunction

Eberhard Schulz, MD*; Jörn Dopheide, MD*; Swenja Schuhmacher, BS; Shane R. Thomas, PhD; Kai Chen, MD, PhD; Andreas Daiber, PhD; Philip Wenzel, MD; Thomas Münzel, MD; John F. Keaney, Jr, MD

Background—Oxidative injury and dysfunction of the vascular endothelium are early and causal features of many vascular diseases. Single antioxidant strategies to prevent vascular injury have met with mixed results.

Methods and Results—Here, we report that induction of a metabolic stress response with adenosine monophosphate kinase (AMPK) prevents oxidative endothelial cell injury. This response is characterized by stabilization of the mitochondrion and increased mitochondrial biogenesis, resulting in attenuation of oxidative c-Jun N-terminal kinase (JNK) activation. We report that peroxisome proliferator coactivator 1α is a key downstream target of AMPK that is both necessary and sufficient for the metabolic stress response and JNK attenuation. Moreover, induction of the metabolic stress response in vivo attenuates reactive oxygen species–mediated JNK activation and endothelial dysfunction in response to angiotensin II in wild-type mice but not in animals lacking either the endothelial isoform of AMPK or peroxisome proliferator coactivator 1α.

Conclusion—These data highlight AMPK and peroxisome proliferator coactivator 1α as potential therapeutic targets for the amelioration of endothelial dysfunction and, as a consequence, vascular disease. (Circulation. 2008;118:1347-1357.)

Key Words: angiotensin ■ endothelium ■ hypertension ■ metabolism

The vascular endothelium mediates local tissue homeostasis through the regulation of blood flow, coagulation, and trafficking of both macromolecules and inflammatory cells.1 Dysfunction of the endothelium is an early feature of chronic diseases such as atherosclerosis and diabetes, and the presence of endothelial dysfunction predicts future vascular consequences.2,3 These chronic vascular diseases exhibit excess ambient levels of reactive oxygen species (ROS) in the vascular wall that contribute to endothelial injury and dysfunction.4 Attempts to limit endothelial injury and vascular disease via exogenous ROS scavengers have not been proven effective in clinical settings,5 indicating that the mechanisms of oxidative endothelial injury are not well defined.

Clinical Perspective p 1357

One means of cellular protection against injury is caloric restriction; ample data indicate that this intervention not only extends the lifespan of model organisms but also reduces the risk of chronic degenerative diseases.6 However, the exact mechanisms responsible for these effects have not been fully elucidated. Recent efforts in model systems have uncovered a number of caloric restriction “mimetics” that have proved useful in studying metabolic stress. Among these compounds are 2-deoxy glucose and metformin.7 The former is a non-metabolizable form of glucose that inhibits the phosphohexoisomerase enzyme and extends lifespan in Caenorhabditis elegans.6 Metformin is a drug for improving insulin resistance that induces changes in metabolism and changes in gene expression that closely parallel caloric restriction.8 The specific mechanism(s) whereby caloric restriction and its mimetics provide cellular protection and longevity are not yet clear.

Among the pathways sensitive to nutrient deprivation is the adenosine monophosphate (AMP)–activated protein kinase (AMPK). This ubiquitous kinase is a heterotrimeric enzyme consisting of α, β, and γ subunits that is sensitive to the cellular AMP:ATP ratio and consequently plays a pivotal role in cellular adaptation to energy stress.8 Activation of

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From the Department of Cardiology, 2nd Medical Clinic of the University Hospital Mainz, Johannes Gutenberg University, Mainz, Germany (E.S., J.D., S.S., A.D., P.W., T.M.), and Division of Cardiovascular Medicine, Department of Medicine, University of Massachusetts Medical School, Worcester, Mass (S.R.T., K.C., J.F.K.).

*The first 2 authors contributed equally to this work.

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Correspondence to Eberhard Schulz, MD, Department of Cardiology, Johannes Gutenberg University, 55101 Mainz, Germany. E-mail dreberhard.schulz@nexgo.de

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1347
AMPK attenuates anabolic processes such as the synthesis of proteins, fatty acids, and cholesterol, and it stimulates ATP-generating catabolic pathways.9 Accordingly, downstream targets of AMPK include key enzymes of glucose and lipid metabolism,10 mitochondrial enzymes,11 and transcriptional coactivators controlling mitochondrial biogenesis.12 The precise role of AMPK and its molecular targets in more generalized stress responses, however, is not well defined.8 Therefore, the purpose of this study was to examine the implications of AMPK in mediating the response to oxidative stress, a key feature of many chronic diseases.

Methods

Materials

Cell culture reagents were obtained from Invitrogen (Carlsbad, Calif) and Cambrex (East Rutherford, NJ). 5-Aminooimidazole-4-carboxamide riboside (AICAR) was obtained from Toronto Research Chemicals (Toronto, Canada). Tumor necrosis factor alpha-α (TNF-α) was purchased from R&D systems (Minneapolis, Minn). Compound C was a kind gift from Merck. Polyclonal antibodies against phosho-AMPK (Thr-172), α-AMPK, and phospho-acyetyl CoA carboxylase (ACC; Ser-79) were from Cell Signaling Technology (Beverly, Mass). We obtained antibodies for catalase and mitochondrial transcription factor A from Abcam (Cambridge, Mass) and the heme oxygenase-1 antibody from Stressgen (Victoria, BC, Canada). Antibodies against ACC, α-AMPK, α2-AMPK, superoxide dismutase (SOD) 1, and SOD2 were from Upstate Biotechnology (Lake Placid, NY), as well as the small interfering RNA (siRNA) constructs SMARTPool with controls. [32P]-ATP (250 μCi, 10 mCi/mL) was obtained from Perkin Elmer Life Sciences (Boston, Mass). Dihydrorhodamine, JC-1, nonyl acridine orange (NAO), and tetramethylrhodamine carboxamide riboside (AICAR) was obtained from Toronto Research Science, Indianapolis, Ind) normalized to 1 well treated with 1% Triton X-100 for maximum LDH release. Cell viability was also assessed by the LDH release assay, we used the cytotoxicity detection kit (Roche Applied Science, Indianapolis, Ind) normalized to 1 well treated with 1% Triton X-100 for maximum LDH release. Cell viability was also assessed by the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, San Luis Obispo, Calif) with an internal Renilla luciferase control plasmid to normalize for transfection efficiencies.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 medium (Clonetics) with all supplements and used between passages 3 and 6. Four hours before AMPK activation, HUVECs were cultivated in a reduced-serum medium containing 0.4% FBS with all EGM-2 supplements in a 1:5 dilution. COS-7 cells were cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. For experiments, confluent cells were used on either 6-well or 12-well plates. Porcine aortic endothelial cells (PAECs) were cultured as described.14 Overnight, AICAR (1 mmol/L) or metformin (5 mmol/L) treatment was directly added to the cell medium, and before H2O2 exposure, cells were washed in HEPES-buffered physiological salt solution (PSS) as described.14

Cellular ATP Content and AMPK Activity Assay

Measurement of ATP was performed with the bioluminescent somatic cell assay kit (Sigma) according to instructions. Determination of AMPK catalytic activity was performed by incorporation of [32P] into the specific AMPK target sequence HMRSAMSGLHLVKKR (SAMS peptide) as described previously.15

Mitochondrial ROS Production

Mitochondrial ROS production was assessed by measuring dihydronicotinamide 123 fluorescence. After treatments, PAECs were washed and incubated for 30 minutes with 10 μmol/L dihydronicotinamide in PSS, washed in PSS, and treated with 200 μmol/L H2O2 for 30 minutes. Cells were then washed, scraped in ice-cold PBS, and dispersed by repeated pipetting, and aliquots were added to either coverslips or 96-well plates. Dihydrorhodamine fluorescence was assessed with a fluorescent plate reader (Molecular Devices, Sunnyvale, Calif) with excitation at 480 nm and emission at 535 nm.

Mitochondrial Membrane Potential

Mitochondrial membrane potential was estimated by fluorescence of JC-1 aggregates that are formed as a function of inner mitochondrial membrane potential.16 PAECs were washed twice with PSS, equilibrated for 30 minutes, and then treated with H2O2 or vehicle for 1 hour. After treatment, cells were carefully washed 2 times and incubated with 2.5 μg/mL JC-1 for 15 minutes in PSS, washed 3 times in PSS, and subjected to fluorescence (for red fluorescence: excitation, 550 nm; emission, 600 nm; for green fluorescence: excitation, 485 nm; emission, 535 nm) ratio detection.

Transcriptional Activation Assays and Immunoblotting

All experiments were performed with the Dual Luciferase Reporter Assay (Promega, San Luis Obispo, Calif) with an internal Renilla luciferase control plasmid to normalize for transfection efficiencies. COS-7 cells were transiently transfected for 40 hours with the human mitochondrial transcription factor A luciferase promoter as described.13 Immunoblotting was performed as previously described.14

Cell Death Assays

PAECs in 12-well plates were treated with vehicle, AICAR, or metformin overnight in regular medium. Cells were then kept in HEPES-buffered PSS (30 minutes) and exposed to increasing concentrations of H2O2 for 2 hours. For the lactate dehydrogenase (LDH) release assay, we used the cytotoxicity detection kit (Roche Applied Science, Indianapolis, Ind) normalized to 1 well treated with 1% Triton X-100 for maximum LDH release. Cell viability was also assessed by the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) following the manufacturer’s instructions. Briefly, the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) is bioreduced by metabolically active cells into a formazan product and can be measured by the amount of 490 nm absorbance in the tissue media.

Mitochondrial Mass Determination

Mitochondrial mass was estimated by fluorescence of NAO or MitoTracker Green FM, both mitochondrial specific dyes, independently of the mitochondrial membrane potential.16,17 After various treatments, HUVECs were washed twice with PBS and then incubated in full growth medium including 100 nmol/L NAO or MitoTracker Green FM for 30 minutes at 37°C and 5% CO2. After 3 washes, cells were subjected to fluorescence detection (for MitoTracker Green FM: excitation, 490 nm; emission, 516 nm; for NAO: excitation, 495 nm; emission, 519 nm) as an indicator of mitochondrial mass.

Gene Silencing by siRNA

Double-stranded RNAi was transfected into cells with RNAiFect (Qiagen, Valencia, Calif). After 72 hours of transfection, cells were incubated with 1 mmol/L AICAR overnight for chronic AMPK activation. Then, 100 μmol/L H2O2 for 2 hours was applied to the cells for cell death and cell survival assays. Scrambled RNAi (Upstate Biotechnology Inc, Waltham, Mass) was used as control.

Experimental Animals

Animal experiments were in accordance with the Declaration of Helsinki and National Institutes of Health guidelines and were
performed with approval of the Ethics Committee of the University Hospital Mainz. To study in vivo AMPK activation by AICAR, we used male C57Bl6 mice. The mice were anesthetized by isoflurane inhalation and treated with a subcutaneous osmotic minipump (Alzet model 1007) containing either angiotensin II or solvent (NaCl 0.9%) for 7 days. Angiotensin infusion rate averaged 1 mg · kg\(^{-1}\) · d\(^{-1}\). Animals from both groups were randomized to receive either AICAR (200 mg · kg\(^{-1}\) · d\(^{-1}\)) or vehicle (NaCl 0.9%) via subcutaneous injection once daily, starting at the time of the angiotensin II–containing minipump implantation. To probe the role of AMPK, we used 100\(^{-1}\)-AMPK knockout mice\(^{20}\) and corresponding littermate wild-type mice (C57Bl6/129Sv/FVB-N background) as controls. To probe the role of PGC-1\(^{\alpha}\), we used animals with severely diminished PGC-1\(^{\alpha}\) levels as described previously.\(^{21}\) In a second approach, endothelial dysfunction was induced in wild-type mice by a single intraperitoneal injection of lipopolysaccharide (15 mg/kg), and the mice were killed 24 hours later. Mice were randomized to receive either AICAR (200 mg · kg\(^{-1}\) · d\(^{-1}\)) or vehicle (NaCl 0.9%) via subcutaneous injection once daily, starting 2 days before lipopolysaccharide treatment. To dissect the role of AMPK, lipopolysaccharide/AICAR injections also were performed in \(100^{-1}\)-AMPK knockout mice. After all treatment protocols, animals were killed, and tissues were removed and subjected to further analysis.

**Assessment of Endothelial Function and Superoxide**

Endothelial function was assayed as endothelium-dependent arterial relaxation in segments of thoracic aorta as described previously.\(^{22}\)
Vascular superoxide was estimated with dihydroethidium staining, and NADPH-oxidase activity was estimated in heart membrane fractions as described.

Statistical Analysis
All immunoblots are representative of 3 to 4 independent experiments. Numerical data are presented as mean ± SEM. Comparisons among treatment groups were performed with 1-way ANOVA and an appropriate posthoc Dunnett or Tukey comparison. Statistical significance was accepted if the null hypothesis was rejected with values of P < 0.05.

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

Results

Peroxide Induces AMPK Activation in the Endothelium
Because AMPK isoform composition is known to affect its function in various cell types, we used reverse-transcriptase polymerase chain reaction and immunoblotting to determine that endothelial cells almost exclusively harbor the α catalytic isoform (Figures IA and IB of the online Data Supplement). There is also a predominance of the β and γ isoforms (Figure IA). We then treated endothelial cells with H₂O₂ and observed concentration-dependent AMPK activation as assessed by Western blot and AMPK activity assay (Figure 1A and 1B). Inhibitors targeting phosphoinositide 3-kinase, protein kinase C, src-family kinases, general tyrosine kinases, mitogen-activated protein kinases, or intracellular calcium transients did not block H₂O₂-induced AMPK activation (data not shown). We next examined cellular energy status and found that endothelial ATP levels decline rapidly after H₂O₂ exposure (Figure 1C), suggesting that AMPK activation is, at least in part, mediated by the resulting rise in cellular AMP that can allosterically activate AMPK.

To determine whether AMPK activation promotes survival, we inhibited H₂O₂-mediated AMPK activation with compound C but observed no impact on cell death (Figure 1D). Similarly, overexpression of a dominant-negative AMPK mutant had no impact on H₂O₂-mediated cell death (Figure 1E) despite significant inhibition of AMPK activation (Figure 1F). These data indicate that acute AMPK activation has limited implications for endothelial cell death in response to H₂O₂.

Chronic AMPK Activation Induces Stress Adaptation in Endothelium
We were able to elicit sustained endothelial cell AMPK activation with AICAR treatment over 20 hours on the basis of AMPK activity and phosphorylation of its downstream target, ACC (Figure 2A and 2B). This chronic AMPK activation before H₂O₂ challenge produced a significant attenuation in the response to H₂O₂ that was reversed by either pharmacological (Figure 2C) or molecular (Figure 2D) inhibition of AMPK. Shorter AICAR treatment for 30 minutes or 4 hours did not mimic the effects observed with a 20-hour treatment period (supplementary Figure II). To
determine the general nature of these findings, we chronically activated AMPK with the drug metformin (Figure 2E) and observed a significant inhibition of H₂O₂-induced cell death that was quantitatively similar to that observed with AICAR (Figure 2F). Thus, chronic AMPK activation attenuates H₂O₂-induced endothelial cell death.

AMPK-Mediated Stress Adaptation Involves the Mitochondrion

Cell viability after H₂O₂ exposure is, in part, dictated by the relative activity of death versus survival pathways. We probed Akt and mitogen-activated protein (MAP) kinase activation and found that AICAR and metformin had no impact on H₂O₂-induced Akt or p38 MAP kinase activation (Figure 3A), whereas c-Jun N-terminal kinase (JNK) activation determined as c-Jun phosphorylation was abrogated by AMPK (Figure 3A). We did not find any change in cytosolic antioxidant enzymes with AICAR or metformin; however, we did detect a 30% to 40% increase in mitochondrial SOD (Figure 3B and supplementary Figure III).

Because JNK is a downstream component of mitochondrial death signals and AMPK activation increased mitochondrial SOD, we explored the implications of chronic AMPK activation for mitochondrial response(s) to toxic stimuli. Endothelial cell H₂O₂ treatment reduced mitochondrial membrane potential, and chronic AMPK activation with AICAR (Figure 3C and 3D) or metformin (not shown) prevented this effect. Similarly, the H₂O₂-induced mitochondrial ROS signal determined by dihydrorhodamine fluorescence was attenuated by chronic AMPK activation with either metformin or AICAR (Figure 3E). We also could mimic the effect of AICAR to suppress the mitochondrial ROS signal and JNK activation in response to H₂O₂ using the mitochondria-targeted antioxidant MitoQ (Figure 3F and 3G). Collectively, these data indicate that chronic AMPK activation modifies mitochondrial responses to H₂O₂.

Figure 3. AMPK-mediated adaptation involves the mitochondrion. A, PAECs in 6-well plates were exposed to AICAR, metformin (MET), or buffer alone (CTL) as in Figure 2, followed by assessment of JNK, Akt, and p38 MAP kinase activation (p-) as described. B, PAECs treated as in A were washed and exposed to 100 μmol/L H₂O₂ (60 minutes) before loading with 2.5 μg/mL JC-1 (final concentration) and then examined either qualitatively by microscopy (C) or quantitatively in a plate reader (D) for red (excitation, 550 nm; emission, 600 nm) and green (excitation, 485 nm; emission, 535 nm) fluorescence. Images are representative of 3 independent experiments, and quantitative analysis represents mean ± SEM of 3 independent experiments. *P < 0.05 vs without H₂O₂ by 2-way ANOVA and a Tukey posthoc test. C, PAECs treated with AICAR as in A were washed and exposed to 100 μmol/L H₂O₂ (60 minutes) before loading with 2.5 μg/mL JC-1 (final concentration) and then examined either qualitatively by microscopy (C) or quantitatively in a plate reader (D) for red (excitation, 550 nm; emission, 600 nm) and green (excitation, 485 nm; emission, 535 nm) fluorescence. Images are representative of 3 independent experiments, and quantitative analysis represents mean ± SEM of 3 independent experiments. *P < 0.05 vs without H₂O₂ by 2-way ANOVA and a Tukey posthoc test.
Chronic AMPK Activation Induces PGC-1α-Dependent Mitochondrial Biogenesis

One recognized link between AMPK and the mitochondrion is mitochondrial biogenesis that appears dependent on PGC-1α in many tissues. In agreement with these data, AICAR treatment increased the abundance of markers associated with mitochondrial biogenesis such as PGC-1α, mitochondrial transcription factor A, and cytochrome c (Figure 4A and supplementary Figure IV). Consistent with this observation, AICAR treatment produced PGC-1α-driven gene transcription assessed by the mitochondrial transcription factor A promoter linked to luciferase (Figure 4B). Similarly, chronic AMPK activation produced an increase in endothelial cell mitochondrial mass (Figure 4C), and this effect was recapitulated with adenoviral overexpression of either α1-AMPK or PGC-1α (Figure 4D and 4E). Finally, AICAR stimulation increased mitochondrial mass in an α1-AMPK– and PGC-1α-dependent manner (Figure 4F). Collectively, these data indicate that chronic AMPK activation increases endothelial cell mitochondrial biogenesis and mass.

Stimulation of Mitochondrial Biogenesis Enhances Endothelial Cell Resistance to H2O2

The endothelial cell resistance to H2O2-induced death and dysfunction afforded by AMPK extended beyond H2O2 because TNF-α–induced cell death (measured as JNK activation; Figure 5A) also was inhibited by AMPK. Because we believed that the action of AICAR and metformin involved the mitochondrion (Figure 3) and that AMPK facilitates mitochondrial biogenesis (Figure 4), we examined the link between mitochondrial biogenesis and endothelial cell resistance to stress. We found that AMPK activation reduced H2O2-induced cell death, and this response was recapitulated with PGC-1α overexpression via adenoviral transfection (Figure 5B and 5C). The effect of AICAR against H2O2-mediated cell death was lost by siRNA-mediated suppression of either α1-AMPK or PGC-1α (Figure 5D and 5E). We also found that overexpression of PGC-1α selectively attenuates H2O2-induced JNK activation with preserved p38 MAP kinase activation (Figure 5F). In total, these data link stimulation of mitochondrial biogenesis to endothelial cell protection from cell death and indicate that PGC-1α overexpression is both necessary and sufficient for this response.

In Vivo AMPK Activation Prevents Endothelial Dysfunction in a PGC-1α-Dependent Manner

To determine whether our cell culture data are operable in vivo, we used a model of angiotensin infusion that produces vascular dysfunction, in part, through increased vascular oxidative stress via ROS production. Accordingly, we induced endothelial dysfunction in mice with a 7-day infusion of angiotensin II. Aortas were harvested and examined for endothelial function as acetylcholine-induced endothelium-dependent arterial relaxation. We found that angiotensin II–induced endothelial dysfunction (Figure 6A) was characterized by an increased superoxide signal in media of the
thoracic aorta (Figure 6B), increased myocardial NADPH oxidase activity (Figure 6C), and JNK activation (Figure 6D). In this model, chronic AMPK activation with AICAR attenuated endothelial dysfunction (Figure 6A) and NADPH oxidase activation (Figure 6B and 6C) without material alteration in the blood pressure response (supplementary Figure V). Moreover, AICAR was ineffective in preserving endothelial function in mice lacking either the α1-AMPK isoform (Figure 6E) or PGC-1α (Figure 6F), validating our paradigm in vivo.

To determine whether chronic AMPK activation was generally protective, we used a lipopolysaccharide-induced model of endothelial dysfunction. Treatment of mice with lipopolysaccharide induced both endothelial dysfunction (Figure 7A) and an increased vascular superoxide signal (Figure 7B) that were prevented by chronic AMPK activation in wild-type but not α1-AMPK–null mice. Thus, taken together, these data indicate that chronic AMPK activation in vivo also protects the endothelium against the injurious actions of angiotensin II and lipopolysaccharide.
Discussion

In this study, we found that induction of metabolic stress in the form of chronic AMPK activation was highly effective in protecting endothelial cells against both H2O2 and TNF-α. This stress resistance was related to AMPK-mediated modulation of the mitochondrial redox state that was dependent on the transcriptional coactivator PGC-1α. Indeed, PGC-1α was sufficient for this response as determined by overexpression of PGC-1α, and the redox modulation was mimicked by a mitochondria-targeted antioxidant. We also found that the AMPK–PGC-1α pathway selectively attenuated stress-related JNK activation, an effect one might expect to suppress stress-related cellular injury and death. Our findings were physiologically relevant because metabolic stress induction via AICAR in vivo prevented angiotensin II–mediated JNK activation and endothelial injury, a process dependent on oxidative stress and vascular ROS production. Moreover, lipopolysaccharide-induced endothelial dysfunction also was prevented by chronic AMPK activation. This protective effect of AICAR was lost in mice lacking α1-AMPK (the isofrom that predominates in the endothelium) and PGC-1α. Thus, these data indicate that AMPK can direct adaptive changes in the mitochondrion via PGC-1α, which enhances mitochondrial biogenesis and cellular resistance to stress.

Previous studies addressing the role of AMPK in cell death have yielded equivocal results. Some of these previous
negative studies relied solely on AICAR as the means to increase AMPK activity. However, AICAR may affect other AMP-dependent enzymes, and long-term AICAR treatment might increase cellular nucleotide levels. In this study, we used metformin as a complementary means of AMPK activation and found comparable effects on endothelial cell survival. Because the mechanism of metformin-mediated AMPK activation differs from that of AICAR, there is confidence that the results with AICAR are indeed due to AMPK activation. This contention is supported by our observations that both genetic and pharmacological approaches to block AICAR-mediated AMPK activation reversed the protective effects of AICAR in vitro and in vivo.

It is now clear that different AMPK isoform compositions may dictate distinct sequelae of AMPK activation. For example, metabolic consequences of AMPK activation such as insulin sensitivity have largely been attributed to the α2 isoform. The AMP dependency of AMPK is also greater in α2-bearing enzyme complexes. Moreover, differences in substrate specificity have been observed for the 2-α-subunits in vitro. Thus, the fact that endothelial cells exclusively harbor the α1-AMPK isoform may indicate a functional role distinct from the well-characterized metabolic features of AMPK. For example, one could speculate that the α1-AMPK enzyme is protective against cell death, whereas α2-containing enzymes might promote it. In this regard, studies in neuronal or pancreatic cells (with a preponderance of the α2 isoform) exhibit increased cell death on AMPK activation, whereas data presented here and another study in endothelial cells (with the α1 isoform) show that AMPK activation is cytoprotective.

In the present study, we found that AMPK activation induced mitochondrial biogenesis in the endothelium, consistent with reports from skeletal muscle. A previous report suggests that modulation of nitric oxide levels in endothelium parallel PGC-1α-dependent mitochondrial biogenesis. Because previous in vivo data implicate endothelial nitric oxide synthase–derived nitric oxide in mitochondrial biogenesis, it is tempting to speculate that our effects with AICAR are due to endothelial nitric oxide synthase activation. Such speculation would be consistent with reports that AMPK is responsible for vascular endothelial growth factor–mediated endothelial nitric oxide synthase activation. However, recent data that AMPK directly phosphorylates PGC-1α would seem to refute the requirement for nitric oxide production. Understanding the precise details of AMPK-mediated PGC-1α activation in the endothelium requires further investigation.

Signaling cascades that lead to cell death often require mitochondrial ROS production and a loss of mitochondrial membrane potential. We have implicated stabilization of the mitochondrial membrane potential and reduced mitochondrial ROS as key targets for metabolic stress–mediated prevention of endothelial cell death. Our findings implicate PGC-1α in this process and provide a mechanism whereby the observed contribution of PGC-1α to cellular oxidant defense leads to attenuation of cell death. Indeed, the fact that a mitochondria-targeted antioxidant also limits stress-induced mitochondrial ROS and cell death supports our contention that the mitochondrion is a key component of AMPK-mediated protection.

Our data indicate that metabolic stress provides cellular protection, at least in part, through the attenuation of JNK activation. It is well known that JNK mediates apoptosis and cell death in response to environmental stress; thus, it is plausible that the salutary effects of metabolic stress stem from JNK inhibition. However, the specific means whereby AMPK and PGC-1α signaling impact JNK is not yet clear. One possibility relates to observations that prolonged JNK activation requires intracellular ROS, which inactivates JNK phosphatases. In our study, we found that AMPK activation attenuated intracellular and mitochondrial ROS signals, perhaps preventing the phosphatase inhibition needed for JNK activation. Published data indicate that manganese SOD prevents TNF-α–induced JNK activation, consistent with our findings that AMPK increases endothelial cell manganese SOD content. Thus, suppression of intracellular ROS appears to be a plausible explanation for our findings that AMPK and PGC-1α attenuate endothelial JNK activation and injury.

The present study has important implications for endothelial cell biology. Oxidative stress is a common feature of many vascular diseases and is known to impair endothelial function. Indeed, endothelial damage and reendothelialization are important factors that determine endothelial function. In this context, the endothelium has emerged as a target for the development of new therapies for vascular disease. However, the implications of increased oxidative stress on endothelial cell viability have garnered surprisingly little attention. We show here that moderate levels of oxidative stress (≈10 μmol/L H2O2) lead to significant endothelial cell necrosis; thus, it seems reasonable that finding new molecular targets to limit endothelial cell death should favorably affect vascular disease. Our study identifies PGC-1α, a key regulator of mitochondrial biogenesis, as a potential molecular target to improve endothelial cell viability and function in vascular disease.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Experimental and clinical studies defined endothelial dysfunction as an early marker of atherosclerotic vascular disease. Therefore, preservation of endothelial function is an important goal to avoid vascular disease and to improve prognosis. In the present study, we establish the activation of adenosine monophosphate–activated protein kinase (AMPK), a key enzyme in the adaptation to metabolic stress, as a way to prevent endothelial injury and finally vascular disease. Interestingly, antidiabetic drugs (metformin, glitazones) and statins have AMPK-activating properties, which may, at least in part, contribute to their desired metabolic effects. Our study also suggests that these drugs will exert beneficial effects on the vasculature through AMPK activation apart from those secondary to improved metabolic control. Because cardiovascular events largely determine the prognosis of patients with metabolic disorders, the choice of a drug with AMPK-activating properties seems reasonable because it will improve both endothelial function and metabolic control.
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Supplementary Methods

RT-PCR - HAEC in 100mm dishes were washed in phosphate buffer saline (PBS) and subjected to RNA isolation. After RNA extraction, reverse transcriptase polymerase chain reaction (RT-PCR) was performed with forward (sense) and reverse (antisense) primers. Nucleotide sequences for human AMPK isoforms were designed as follows: α1 sense 5’→3’: GAG CCT TGA TGT GGT AGG, α1 antisense 5’→3’: GGA AGG GTT CCA CAT AAT, α2 sense 5’→3’: TCT GGA GGT GAA TTA TTT, α2 antisense 5’→3’: CCA TTC ATG CTC TCT TAT, β1 sense 5’→3’: CAA CGG TGT TTC GAT GGA, β1 antisense 5’→3’: AGG TGG TGA CGT ACT TCT, β2 sense 5’→3’: GAT TTG GAG GAC TCC GTA, β2 antisense 5’→3’: AAG GAC CAT CAC ACT GTC, γ1 sense 5’→3’: ATG AAG TCT CAT CGC TGC, γ1 antisense 5’→3’: CCA ATC TGT AGC TCT TCC, γ2 sense 5’→3’: CAT GCG ATT CAT GAG GTC, γ2 antisense 5’→3’: ATT CCA AGC TCA TCC AGG, γ3 sense 5’→3’: TTG AGA TGG ACA AGG CAG, γ3 antisense 5’→3’: CAG AGC AAA GAA GGC CTT.

Measurement of Blood Pressure – Wistar rats (n = 6) Male wistar rats (~400g) were obtained from Charles River (Sulzfeld, Germany). During intraperitoneal anesthesia with
ketamine/xylazine, radiotelemetry transmitters (PA-C40, Data Sciences International, Roseville, MN) were implanted into the abdominal aorta via laparotomy under aseptic conditions. Following surgery, animals were individually housed and allowed to recover for 14 days. After that period, osmotic minipumps (Alzet model 2001) containing angiotensin II (1.0mg/kg/d) were implanted subcutaneously at the back. Half of the animals received a parallel infusion of either AICAR (200mg/kg/d) or solvent (DMSO) via a second osmotic minipump (alzet model 2ML1). Blood pressure data was collected every 15 minutes via individual RPC-1 Receiver (Data Sciences International, Roseville, MN) and recorded for the next 7 days.
Figure S1. **AMPK isoforms in endothelial cells.** (A) Total RNA from HAEC was used for RT-PCR with primers designed for each AMPK isoform (see “Methods”). (B) Immunoblotting was performed to confirm PCR results for the α-AMPK subunit at the protein level. Endothelial cells of different origin (PAEC: porcine aortic endothelial cells, BAEC: bovine aortic endothelial cells, HAEC: human aortic endothelial cells, HUVEC: human umbilical vein endothelial cells) were lysed and immunoblotting was performed with isoform specific (α1, α2) antibodies against the α-AMPK subunit. COS-7 cells were used as a positive control for the α2-AMPK isoform. RT-PCR and Immunoblots are representative of 3 independent experiments.
Figure S2. **Time course of AMPK-dependent resistance to cell death.** PAECs were treated with AICAR as indicated, followed by challenge with H$_2$O$_2$ as indicated. Data represent mean ± SEM of 4 observations. *p<0.05 vs 0 hours by one way ANOVA with Dunnet’s test.
Figure S3. Chronic AMPK activation increases SOD2. PAECs in 6-well plates were exposed to AICAR, metformin (MET), or buffer alone (CTL) as in Fig. 3 followed by assessment of SOD2 levels by immunoblot and densitometry as described. Data represent mean ± SEM of 4 independent observations. *P<0.05 vs CTL by one-way ANOVA with repeated measures and a post hoc Dunnet’s test.
Figure S4. **Chronic AMPK activation increases mitochondrial transcription factor A.** PAECs in 6-well plates were exposed to AICAR for the indicated times followed by assessment of mitochondrial transcription factor A Mt-TFA levels by immunoblot and densitometry as described. Data represent mean ± SEM of 4 independent observations. *P<0.05 vs CTL by one-way ANOVA with repeated measures and a post hoc Dunnet’s test.
Figure S5. Activation of AMPK does not impact angiotensin II-induced hypertension. Wistar rats were implanted with radiotlemetry blood pressure transducers before being exposed to angiotensin II (1.0 mg/kg/d) or vehicle as described in "Methods."