Activation of AMP-Activated Protein Kinase Inhibits the Proliferation of Human Endothelial Cells

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ABSTRACT

AMP-activated protein kinase (AMPK) is an evolutionary conserved energy-sensing enzyme that regulates cell metabolism. Emerging evidence indicates that AMPK also plays an important role in modulating endothelial cell function. In the present study, we investigated whether AMPK modulates endothelial cell growth. Treatment of cultured human umbilical vein endothelial cells with the AMPK activators 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR), 6,7-dihydro-4-hydroxy-3-(2'-hydroxy[1,1'-biphenyl]-4-yl)-6-oxo-thieno[2,3-*b*]pyridine-5-carbonitrile (A-769662), or metformin inhibited cell proliferation and DNA synthesis. The antiproliferative action of AICAR was largely prevented by the adenosine kinase inhibitor 5'-iodotubercidin and mimicked by infecting endothelial cells with an adenovirus expressing constitutively active AMPK. In contrast, pharmacological blockade of endothelial nitric oxide synthase or heme oxygenase-1 activity failed to reverse the inhibition of endothelial cell growth by AICAR. Flow cytometry experiments

revealed that pharmacological activation of AMPK arrested endothelial cells in the G₀/G₁ phase of the cell cycle, and this was associated with increases in p53 phosphorylation and p53, p21, and p27 protein expression and decreases in cyclin A protein expression and retinoblastoma protein phosphorylation. In addition, silencing p21 and p27 expression partially restored the mitogenic response of AMPK-activated cells. Finally, activation of AMPK by AICAR blocked the migration of endothelial cells after scrape injury and stimulated tube formation by endothelial cells plated onto Matrigel-coated plates. In conclusion, these studies demonstrate that AMPK activation inhibits endothelial cell proliferation by elevating p21 and p27 expression. In addition, they show that AMPK regulates endothelial cell migration and differentiation and identify AMPK as an attractive therapeutic target in treating diseases associated with aberrant endothelial cell growth.

Introduction

AMP-activated protein kinase (AMPK) is an evolutionary conserved serine/threonine kinase that functions as an important energy sensor (Hardie et al., 2006). AMPK exists as a heterotrimer comprised of a single catalytic (α) subunit and two regulatory (β and γ) subunits. Multiple mechanisms mediate the activation of AMPK, including changes in the AMP- or ADP-to-ATP ratio, the generation of an AMP mimetic, or alterations in intracellular calcium concentrations.

AMP activates AMPK by competing with ATP for binding to the γ -subunit (Zou and Wu, 2008; Hawley et al., 2010). The AMP-bound γ -subunit allosterically activates the kinase, and the enzyme becomes fully activated after the phosphorylation of the α -subunit at Thr172 by several upstream kinases (Zou and Wu, 2008). More recently, ADP has also been shown to bind the γ -subunit and stimulate Thr172 phosphorylation of the α -subunit but, unlike AMP, it does not directly activate AMPK (Xiao et al., 2011). During periods of metabolic stress that occurs with nutrient deprivation, prolonged exercise, ischemia, or hypoxia, increases in the AMP- or ADP-to-ATP ratio triggers the activation of AMPK, initiating a cellular program to conserve energy by turning on ATP-generating catabolic pathways and switching off ATP-consuming anabolic pathways. These metabolic effects of AMPK are evoked

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ABBREVIATIONS: AMPK, AMP-activated protein kinase; AdAMPK-CA, adenovirus expressing a constitutively active AMPK mutant; AICAR, 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside; ACC, acetyl-CoA carboxylate; eNOS, endothelial nitric oxide synthase; HO, heme oxygenase; HUVEC, human umbilical vein endothelial cell; HAEC, human aortic endothelial cell; AdGFP, adenovirus expressing green fluorescent protein; Iodo, 5-iodotubercidin; PBS, phosphate-buffered saline; siRNA, small interfering RNA; L-NMA, N^G-monomethyl-L-arginine; SnPP, tin protoporphyrin-IX; pRb, phospho-retinoblastoma protein; NT, nontargeting; A-769662, 6,7-dihydro-4-hydroxy-3-(2'-hydroxy[1,1'-biphenyl]-4-yl)-6-oxo-thieno[2,3-*b*]pyridine-5-carbonitrile.

via direct phosphorylation of target proteins and modulation of gene expression (Hardie et al., 2006; Zou and Wu, 2008).

Numerous pharmacological compounds have been developed to activate AMPK. 5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) is an established, cell-permeable activator of AMPK that is rapidly taken up by cells and phosphorylated by adenosine kinase to 5-aminoimidazole-4-carboxamide, an AMP analog that mimics the effect of AMP on AMPK activation (Corton et al., 1995). The thienopyridone compound 6,7-dihydro-4-hydroxy-3-(2'-hydroxy[1,1'-biphenyl]-4-yl)-6-oxo-thieno[2,3-*b*]pyridine-5-carbonitrile (A-769662) is a selective small-molecule activator of AMPK that directly binds and activates the kinase in a fashion similar to AMP (Göransson et al., 2007). Metformin, the most widely prescribed oral hypoglycemic agent, also activates AMPK in endothelial cells. The mechanism of activation by metformin is complex, but probably involves the inhibition of complex I of the mitochondrial respiration, which eventually precipitates an elevation of the AMP-to-ATP ratio (El-Mir et al., 2000; Zhou et al., 2001).

Aside from its role in energy homeostasis, emerging evidence indicates that AMPK regulates endothelial cell function. AMPK phosphorylates and activates endothelial nitric oxide synthase (eNOS), resulting in the liberation of nitric oxide, a critical modulator of vascular tone (Morrow et al., 2003; Davis et al., 2006). In addition, AMPK exerts important anti-inflammatory effects by blocking the activation of nuclear factor- κ B and the expression of adhesion molecules and chemokines by endothelial cells (Hattori et al., 2006; Ewart et al., 2008). Moreover, we reported previously that AMPK activation attenuates postischemic adhesion of leukocytes to murine postcapillary venular endothelium in a heme oxygenase (HO)-dependent manner (Gaskin et al., 2009). Finally, multiple studies demonstrate that AMPK preserves endothelial cell viability in response to metabolic, oxidative, and inflammatory stress (Ido et al., 2002; Colombo and Moncada, 2009; Li et al., 2009b; Liu et al., 2011). Significantly, many of the beneficial actions of AMPK on endothelial cell function are related to the activation of eNOS or the induction of the vasoprotective protein HO-1 (Gaskin et al., 2007, 2009; Liu et al., 2011).

Although the ability of AMPK to preserve endothelial cell viability is well appreciated, the role of AMPK in regulating endothelial cell proliferation is limited and not well understood (Reihill et al., 2011). In the present study, we examined whether pharmacological or molecular activation of AMPK regulates the proliferation of human endothelial cells derived from both the venous and arterial circulation. In addition, we determined the molecular mechanism underlying the effect of AMPK on endothelial cell growth. Finally, we also investigated the influence of AMPK on endothelial cell migration and tube formation.

Materials and Methods

Materials. Penicillin, streptomycin, gelatin, heparin, 5-iodobercidin, dithiothreitol, SDS, NaCl, EDTA, trichloroacetic acid, trypan blue, propidium iodide, RNase A, AICAR, methyl-L-arginine, and metformin were from Sigma-Aldrich (St. Louis, MO). M199 medium, L-glutamine, and bovine calf serum were from Invitrogen (Carlsbad, CA). A-769662 was from Tocris Bioscience (Ellisville, MO). Endothelial cell growth factor was from BD Biosciences (San Jose, CA), and tin protoporphyrin-IX was from Frontier Scientific (Logan, UT). Antibodies against cyclin D1, cyclin E, cyclin A, p21, p27, p53, and β -actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A pan-specific antibody against AMPK α and phosphorylation-specific antibodies against AMPK α , p53, retinoblastoma protein, and acetyl-CoA carboxylate (ACC) were from Cell Signaling Technology (Danvers, MA). [3 H]thymidine (20 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Waltham, MA).

Cell Culture. Human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) were purchased from Lonza Incorporated (Allendale, NJ) and subcultured on gelatin-coated dishes as described previously (Wei et al., 2009). Cells were grown in M199 medium supplemented with 20% bovine calf serum, 2 mM L-glutamine, 50 μ g/ml endothelial cell growth factor, 90 μ g/ml heparin, and 100 U/ml of penicillin and streptomycin at 37°C in a humidified 95% air-5% CO₂ atmosphere.

Small Interfering RNA and Adenovirus Infection Protocol. Gene expression was silenced by using siRNAs targeting p21 and p27. The experimental and control nontargeting siRNAs were obtained from Santa Cruz Biotechnology Inc. and delivered to endothelial cells (100 nM) by using a commercial transfection reagent (Invitrogen). A replication-defective adenovirus expressing a constitutively active AMPK α mutant (AdAMPK-CA) was generously provided by Dr. Ming-Hui Zou (University of Oklahoma Health Sciences Center, Oklahoma City, OK). Endothelial cells were infected with AdAMPK-CA or an adenovirus expressing green fluorescent protein (AdGFP) at a multiplicity of infection of 50. Cells were transfected or infected with appropriate constructs 48 h before treatment.

Cell Proliferation and DNA Synthesis. Endothelial cells were seeded (2×10^4 cells/well) onto six-well plates in serum-containing media. After 24 h, cells were washed and treated with serum-containing media in the presence and absence of various test compounds. Cell number determinations were performed at various times by dissociating cells with trypsin and counting cells in a Beckman Z1 Coulter Counter (Beckman Coulter, Fullerton, CA). Endothelial cell proliferation was also monitored by measuring DNA synthesis as described previously (Peyton et al., 2002). Cells were incubated with [3 H]thymidine for 4 h, washed three times with ice-cold PBS, and fixed with 10% trichloroacetic acid for 30 min at 4°C. DNA was then extracted with 0.2% SDS/0.2 N NaOH, and radioactivity was determined by scintillation spectroscopy.

Cell Viability. Cell viability was monitored by measuring the uptake of the membrane-impermeable stain trypan blue. Cells were treated with trypsin (0.25%), collected, diluted (1:4) with trypan blue, and examined by microscopy. Viability was determined by the percentage of cells that excluded the dye (Wei et al., 2009).

Cell Cycle Analysis. Cell cycle progression was assessed in cells grown to 70 to 80% confluence by flow-activated cell sorting as reported previously (Peyton et al., 2009). Endothelial cells were collected, suspended in PBS, and pelleted by centrifugation at 1000g for 5 min. Pellets were washed twice with PBS, suspended in 70% ethanol, and fixed overnight at 4°C. Fixed cells were then incubated with propidium iodide (50 μ g/ml) and RNase A (100 μ g/ml) for 1 h at room temperature, and DNA fluorescence was measured in a Beckman Coulter CyAN ADP Cytometer.

Western Blotting. Endothelial cells were collected in electrophoresis buffer (125 mM Tris, pH 6.8, 12.5% glycerol, 2% SDS, and trace bromophenol blue), and proteins were separated by SDS-polyacrylamide gel electrophoresis. After electrophoretic transfer to nitrocellulose membranes, membranes were blocked with PBS and nonfat milk (5%) and then incubated with antibodies against cyclin D1 (1:200), cyclin E (1:100), cyclin A (1:500), p27 (1:250), p21 (1:250), p53 (1:200), phospho-p53 (1:100), phospho-retinoblastoma protein (pRb; 1:100), AMPK α (1:500), ACC (1:500), phospho-AMPK α (1:100), phospho-ACC (1:100), or β -actin (1:200). Membranes were washed in PBS, incubated with horseradish peroxidase-conjugated goat anti-

Fig. 1. AICAR inhibits the proliferation of HUVECs in an AMPK-dependent manner. **A**, AICAR (500 μM) induced a sustained increase in AMPK activity, as reflected by the phosphorylation of AMPK (AMPK-P). **B**, time-dependent increase in HUVEC number was blocked by AICAR. Cells were incubated in the absence and presence of AICAR (500 μM) for 3 days. **C**, AICAR inhibited the proliferation of HUVECs in a concentration-dependent manner. HUVECs were incubated in the absence or presence of different concentrations of AICAR (5–500 μM) for 3 days. **D**, inhibition of AICAR (500 μM for 24 h)-mediated AMPK activation by Iodo (0.3 μM), as reflected by inhibition of the phosphorylation of the AMPK substrate ACC (ACC-P). **E**, effect of AMPK inhibition on AICAR-mediated inhibition of HUVEC proliferation. Cells were treated with AICAR (500 μM) in the absence or presence of Iodo (0.3 μM) for 3 days. Results are means $\pm$ S.E.M. ($n = 4-6$). *, statistically significant effect of AICAR. †, statistically significant effect of Iodo.

rabbit or rabbit anti-goat antibodies, and developed with commercial chemoluminescence reagents (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Protein expression was quantified by densitometry and normalized with respect to β -actin.

AMPK Activation. AMPK activity was determined by Western blotting using phospho-specific antibodies directed against AMPK α or ACC (Liu et al., 2011).

Cell Migration. Cell migration was determined by using a previously described scratch wound assay (Peyton et al., 2011). Confluent endothelial cell monolayers were scraped with a pipette tip to generate a wound. Cell debris was removed by several washes with PBS, and injured monolayers were incubated in serum-containing media in the presence and absence of various test compounds. Cell monolayers were photographed immediately and 24 h after scratch injury with a digital camera (Q-Imaging, QICAM; Hirschfeld Instruments, Incorporated, St. Louis, MO), and the degree of wound closure was determined by planimetry.

Endothelial Cell Tube Formation. The endothelial cell tube formation assay was performed by using growth factor-reduced Matrigel (BD Biosciences, San Jose, CA). Cells (2×10^4 cells/well) were plated in 96-well plates that had been precoated with Matrigel (50 μl/well). After incubation for 6 h in serum-containing media, images of tube morphology were taken by an inverted Olympus CKX41 microscope (Olympus America, Inc., Center Valley, PA), and the extent of tube formation was quantified by counting the number of tubes.

Statistics. Results are expressed as mean $\pm$ S.E.M. Statistical analyses were performed with the use of a Student's two-tailed t test and an analysis of variance with the Tukey post hoc test when more than two treatment regimens were compared. P values <0.05 were considered statistically significant.

Results

Treatment of HUVECs with AICAR (500 μM) resulted in a persistent, time-dependent increase in AMPK activity, as reflected by the phosphorylation of AMPK (Fig. 1A). Induction of AMPK activity was detected 1 h after AICAR administration, and AMPK activity remained elevated during 24 h of AICAR exposure. Incubation of HUVECs with serum-containing media stimulated a time-dependent increase in cell number that was blocked by AICAR (Fig. 1B). The inhibition of HUVEC growth by AICAR was concentration-dependent (Fig. 1C). A significant inhibition of cell growth by AICAR was noted at a concentration of 50 μM, and near-total ablation of proliferation was noted with 500 μM. The antiproliferative effect of AICAR was not related to any change in cell viability, as assessed by trypan blue exclusion [control: $95.4 \pm 3.7\%$ viable versus AICAR (0.5 mM): $94.6 \pm 3.4\%$ viable]. However, the adenosine kinase inhibitor 5'-iodotubercidin (Henderson et al., 1972), which blocks the intracellular conversion of AICAR to 5-aminoimidazole-4-carboxamide that is required for AMPK activation, abolished the activation of AMPK by AICAR, as reflected by the phosphor-

Fig. 2. Activation of AMPK inhibits the proliferation of HUVECs. **A**, treatment of HUVECs with AICAR (500 μM), A769662 (300 μM), or metformin (2 mM) for 24 h stimulated AMPK activity, as reflected by the phosphorylation of AMPK (AMPK-P). **B**, AMPK activators inhibited the proliferation of HUVECs. Cells were incubated in the absence or presence of AICAR (500 μM), A769662 (300 μM), or metformin (2 mM) for 3 days. **C**, AMPK activators inhibited HUVEC DNA synthesis. Cells were incubated in the absence or presence of AICAR (500 μM), A769662 (300 μM), or metformin (2 mM) for 24 h. **D**, AICAR-mediated inhibition of DNA synthesis depends on AMPK activity but is independent of heme oxygenase or endothelial nitric-oxide synthase activity. Cells were treated with AICAR (500 μM) in the absence or presence of Iodo (0.3 μM), N^G -monomethyl-L-arginine (L-NMA; 1 mM), or tin protoporphyrin-IX (SnPP; 5 μM). **E**, infection of HUVECs with AdAMPK-CA but not AdGFP inhibited DNA synthesis. Results are means $\pm$ S.E.M. ($n = 4-6$). *, statistically significant effect of AMPK activators or AdAMPK-CA. †, statistically significant effect of Iodo.

Fig. 3. Activation of AMPK inhibits the proliferation of HAECs. A, AMPK activators inhibited the proliferation of HAECs. Cells were incubated in the absence or presence of AICAR (500 μ M), A769662 (300 μ M), or metformin (2 mM) for 3 days. B, infection of HAECs with AdAMPK-CA but not AdGFP inhibited DNA synthesis. Results are means $\pm$ S.E.M. ($n = 4$). *, statistically significant effect of AMPK activators or AdAMPK-CA.

ylation of the AMPK substrate ACC (Fig. 1D), and largely reversed the antiproliferative action of AICAR (Fig. 1E).

The ability of AMPK to inhibit HUVEC growth was also corroborated by using two other distinct activators of AMPK. As observed with AICAR, A-769662 (300 μ M) and metformin (2 mM) activated AMPK in HUVECs (Fig. 2A) and led to a significant decline in cell growth (Fig. 2B). Furthermore, all three AMPK activators markedly suppressed HUVEC DNA

synthesis (Fig. 2C). The ability of AICAR to block DNA synthesis depended on AMPK activation because it was largely reversed by 5'-iodotubercidin (Fig. 2D). Because we reported previously that eNOS and HO-1 contribute to the biological actions of AMPK on endothelial cells, their roles in mediating the antiproliferative action of AICAR were examined (Gaskin et al., 2009; Liu et al., 2011). The contribution of eNOS to the growth-suppressing activity of AICAR was determined by incubating endothelial cells with the eNOS inhibitor methyl-L-arginine (Palmer et al., 1988), whereas the involvement of HO-1 was assessed by treating endothelial cells with the HO inhibitor tin protoporphyrin-IX (Drummond and Kappas, 1981). However, both pharmacological inhibitors failed to reverse the inhibition of DNA synthesis by AICAR (Fig. 2D). The role of AMPK in regulating HUVEC proliferation was also evaluated by using AdAMPK-CA. Infection of HUVECs with AdAMPK-CA resulted in a significant decline in DNA synthesis, whereas infection with a control adenovirus had no effect (Fig. 2E). Pharmacological activators of AMPK or infection of cells with AdAMPK-CA also blocked DNA synthesis in HAECs (Fig. 3).

Subsequently, we determined the effect of AMPK activation on endothelial cell cycle progression. Administration of AICAR, A-769662, or metformin arrested cells in the G_0/G_1 phase of the cell cycle, as demonstrated by an increase in the percentage of cells in G_0/G_1 with a corresponding decline in the fraction of cells in S and G_2/M phases (Fig. 4). In addition, no apparent toxicity was noted by any of the AMPK activators as reflected by the lack of a sub- G_0/G_1 fraction (Fig. 4A).

To determine the mechanism by which AMPK disrupts cell cycle progression in HUVECs, we examined the effect of AICAR on the expression of cell cycle regulatory proteins. AICAR dramatically reduced the expression of cyclin A and the phosphorylation of retinoblastoma protein (Fig. 5). In addition, AICAR strongly induced the expression of the cyclin-dependent kinase inhibitors p21 and p27 and elevated the expression and phosphorylation of p53. In contrast,

Fig. 4. Activation of AMPK inhibits cell cycle progression by HUVECs. A, representative histograms of HUVECs incubated in the absence or presence of AICAR (500 μ M), A-769662 (300 μ M), or metformin (2 mM) for 24 h. B, effect of AICAR (500 μ M), A-769662 (300 μ M), or metformin (2 mM) exposure for 24 h on the distribution of HUVECs in the cell cycle. Results are means $\pm$ S.E.M. ($n = 3$). *, statistically significant effect of AMPK activators.

Fig. 5. Effect of AICAR on the expression and phosphorylation (P) of cell cycle regulatory proteins in HUVECs. A, cells were incubated in the absence or presence of AICAR (500 μ M) for 24 h, and protein expression and phosphorylation were determined by Western blotting. B, quantification of protein expression or phosphorylation relative to control, untreated cells. Results are means $\pm$ S.E.M. ($n = 4$). *, statistically significant effect of AICAR.

AICAR had no significant effect on the expression of cyclin D1 and cyclin E.

In subsequent experiments, siRNA knockdown of p21 and p27 was used to test the role of these cyclin-dependent kinase inhibitors in mediating the inhibition of HUVEC proliferation by AMPK. We found that siRNA targeting p21 or p27 suppressed the AICAR-induced expression of p21 or p27, respectively, whereas the control nontargeting siRNA had no effect on the expression of either protein (Fig. 6, A and B). The knockdown of p21, p27, or the combination of both proteins failed to alter basal endothelial cell DNA synthesis (data not shown). In contrast, individual knockdown of either p21 or p27 partially restored DNA synthesis, whereas knockdown of both p21 and p27 further returned the proliferative response of AICAR-treated cells. However, 5'-iodotubercidin was more effective in restoring endothelial cell growth in AICAR-exposed cells.

Finally, we investigated the effect of AMPK on endothelial cell migration and differentiation. Treatment of HUVECs with AICAR resulted in a concentration-dependent decrease in cell migration (Fig. 7A). An approximate 60% decline in cell migration by AICAR was observed with 500 μ M, which was largely reversed in the presence of 5'-iodotubercidin (Fig. 7B). It is noteworthy that AICAR stimulated endothelial cell tube formation that was completely abolished by 5-iodotubercidin (Fig. 7C). In the absence of AICAR, 5-iodotubercidin had no effect on endothelial cell migration or tube formation (Fig. 7, B and C).

Discussion

The present study demonstrates that AMPK is a potent inhibitor of human endothelial cell proliferation. The inhibition of endothelial cell proliferation was observed after pharmacological or molecular activation of AMPK and was seen in endothelial cells obtained from both the arterial and venous

circulation. In addition, AMPK arrests endothelial cells in the G_0/G_1 phase of the cell cycle, and this is associated with alterations in the expression and phosphorylation of several cell cycle regulatory proteins, including increases in p21 and p27 expression. Significantly, enforced knockdown of p21 and p27 partially abrogates the antiproliferative effects of AMPK, indicating a critical role for these cyclin-dependent kinase inhibitors in mediating the growth-suppressive action of AMPK on endothelial cells. We also found that AMPK inhibits the migration of endothelial cells but stimulates the differentiation of endothelial cells into tube-like structures. The ability of AMPK to block endothelial cell growth and migration identifies this kinase as a novel therapeutic target in ameliorating pathophysiological processes that depend on the proliferation and movement of endothelial cells.

In the current study, we found that the AMPK activator AICAR is a robust inhibitor of endothelial cell proliferation and DNA synthesis. The antiproliferative effect of AICAR is concentration-dependent and largely reliant on AMPK activ-

Fig. 6. AMPK-mediated inhibition of HUVEC proliferation depends on p21 and p27 protein expression. A, silencing p21 protein expression in HUVECs. B, silencing p27 protein expression in HUVECs. C, effect of silencing p21 and/or p27 protein expression or blocking AMPK activation on the inhibition of DNA synthesis by AICAR. Cells were transfected with siRNA to p21 (p21 siRNA; 100 nM), p27 (p27 siRNA; 100 nM), and/or nontargeting siRNA (NT siRNA; 100 nM) for 48 h and then treated with AICAR (500 μ M) in the absence or presence of Iodo (0.3 μ M) for 24 h, at which point cells were collected for Western blotting or pulsed with [3 H]thymidine for DNA synthesis determination. Results are means $\pm$ S.E.M. ($n = 4$). *, statistically significant effect of AICAR. †, statistically significant effect of siRNA transfection or Iodo.

Fig. 7. Effect of AMPK activation on migration and tube formation by HUVECs. **A**, AICAR inhibited the migration of HUVECs in a concentration-dependent manner. Cells were incubated in the absence or presence of different concentrations of AICAR (5–500 μ M) for 24 h after wound injury. **B**, effect of AMPK inhibition on AICAR-mediated inhibition of HUVEC migration. Cells were treated with AICAR (500 μ M) for 24 h after wound injury in the absence or presence of Iodo (0.3 μ M). **C**, AMPK activation stimulated tube formation by HUVECs. Cells were treated with AICAR (500 μ M) for 6 h after plating onto Matrigel in the absence or presence of Iodo (0.3 μ M). Results are means $\pm$ S.E.M. ($n = 4$). *, statistically significant effect of AICAR. †, statistically significant effect of Iodo.

ity, because pharmacological inhibition of AMPK activation drastically reduces the antiproliferative action of AICAR. The ability of AMPK to block endothelial cell proliferation and DNA synthesis was also substantiated by using the clinically relevant AMPK activator metformin and the more selective AMPK activator A-769662. In addition, the antiproliferative action of the pharmacological activators was confirmed by directly infecting endothelial cells with an adenovirus expressing constitutively active AMPK α . It is noteworthy that

AMPK inhibits endothelial cell proliferation in the absence of cell death, as revealed by trypan blue staining and propidium iodide binding, indicating that AMPK functions as a cytostatic rather than a cytotoxic agent. Although work from our laboratory and others indicates that eNOS and HO-1 contributes to the actions of AMPK on endothelial cell function (Gaskin et al., 2007, 2009; Liu et al., 2011), these proteins are not involved in the antiproliferative effect of AMPK because pharmacological blockade of either enzyme fails to restore endothelial cell growth in response to AMPK activation.

Our finding that AMPK blocks serum-stimulated human endothelial cell growth is consistent with earlier studies in vascular smooth muscle cells (Nagata et al., 2004), fibroblasts (Jones et al., 2005), and various cancer cell lines (Rattan et al., 2005) and a recent report in human aortic endothelial cells (Reihill et al., 2011). In the latter study, AMPK activation was shown to block basal endothelial cell growth, but the underlying mechanism was not explored. It is noteworthy that our results are in contrast with investigations showing that AMPK activation is required in mediating the mitogenic response of endothelial cells to vascular endothelial growth factor and erythropoietin (Reihill et al., 2011; Su et al., 2012). Thus, the capacity of AMPK to regulate endothelial cell growth may be stimulus-dependent. The mechanism by which AMPK differentially regulates endothelial cell proliferation is not known but may reflect the activation of distinct cellular pools of AMPK that are coupled to opposing signaling pathways that modulate cell growth. Consistent with such a notion, AMPK activators have been shown to elicit discrete compartment-specific effects on renal cell physiology (Kodiha et al., 2011). Alternatively, the kinetics of AMPK activation may dictate the nature of the proliferative response. In particular, chronic, sustained activation of AMPK that was observed in our study may favor endothelial cell growth arrest, whereas acute, transient activation of AMPK in response to angiogenic factors promotes a proliferative response (Ouchi et al., 2004; Stahmann et al., 2010; Su et al., 2012). The ability of AMPK to repress endothelial cell growth, an ATP-consuming process, provides another mechanism by which this kinase conserves energy during periods of prolonged metabolic stress.

Endothelial cell proliferation requires the progression of cells through distinct phases of the cell cycle where DNA synthesis and mitosis occurs. Analysis of cell cycle distribution indicates that AMPK activators arrest endothelial cells in the G_0/G_1 phase of the cell cycle. The inhibition of cell cycle progression by AMPK is associated with pronounced decreases in the phosphorylation of retinoblastoma protein and expression of cyclin A and increases in the expression of p21 and p27, which is consistent with the detention of cells in G_0/G_1 . The ability of AMPK to up-regulate the expression of p21 and p27 may reflect AMPK-mediated alterations in gene transcription and/or proteasomal degradation of these cyclin-dependent kinase inhibitors (Imamura et al., 2001; Rattan et al., 2005; Viana et al., 2008; Ishii et al., 2009; Short et al., 2010; Song et al., 2011). In this regard, we found that AMPK increases the expression of the transcription factor p53 in endothelial cells. This protein is a known activator of p21 gene transcription and probably accumulates in endothelial cells because of its phosphorylation by AMPK, which suppresses its degradation (Imamura et al., 2001; Jones et al., 2005).

It is noteworthy that we show that increases in cyclin-dependent kinase inhibitors contribute to the antiproliferative action of AMPK. Whereas individual knockdown of p21 or p27 partially restores endothelial cell growth during AMPK activation, the combined knockdown of both proteins leads to an additive effect in restoring cell growth. However, silencing p21 and p27 expression did not fully return endothelial cell proliferation during AICAR exposure. This may reflect pleiotropic actions evoked by AMPK activators that are independent of AMPK. Indeed, we found that inhibition of AMPK activity by 5'-iodotubercidin does not fully restore endothelial cell growth in AICAR-treated cells. It is noteworthy that silencing p21 and p27 expression is not as effective as 5'-iodotubercidin in reversing the antiproliferative effect of AICAR, suggesting the involvement of other AMPK-dependent growth-suppressive pathways, including inhibition of cyclin A mRNA stability (Wang et al., 2002). The disruption of p21 and p27 expression fails to augment endothelial cell growth in the absence of AICAR, indicating a minor role for these proteins in restricting endothelial cell proliferation under serum-replete conditions.

In the present study, we also found that AMPK regulates endothelial cell migration and tube formation. AICAR attenuates the migration of endothelial cells in a concentration-dependent manner that is largely eliminated after AMPK inhibition. Our results demonstrating that AMPK elicits an antimigratory action is consistent with a recent study showing that metformin blocks the migration of HUVECs (Esfahanian et al., 2012) and previous reports showing that AMPK activators inhibit the migration of monocytes and neurons (Kanellis et al., 2006; Ruscica et al., 2011). However, they differ from other investigations that reported that AMPK enhances the migration of endothelial cells (Nagata et al., 2003; Reihill et al., 2011; Su et al., 2012). The reasons for the disparate effects of AMPK on cell migration are not known but may reflect differences in culture conditions, the activating stimulus, and/or the degree, kinetics, and intracellular location of AMPK activation. In contrast, our finding that AMPK activation by AICAR augments endothelial cell tube formation is in agreement with earlier studies and underscores the critical role of AMPK in promoting endothelial cell differentiation (Nagata et al., 2003; Li et al., 2008; Stahmann et al., 2010; Su et al., 2012).

The finding that AMPK activation blocks the proliferation and migration of endothelial cells is of potential pharmacological significance given that these processes contribute to several pathological disorders including cancer, atherosclerosis, and diabetic retinopathy (Folkman, 2006; Simó et al., 2006; Sluimer and Daemen, 2009). It is noteworthy that retrospective studies conducted in diabetic patients found that metformin is associated with a decreased risk in developing cancer and a better response to chemotherapy (Evans et al., 2005; Li et al., 2009a; Landman et al., 2010). Although several potential mechanisms may explain the antitumorigenic effect of metformin, including direct actions on tumor cells, the ability of metformin to block endothelial cell proliferation and migration may compromise the formation of new blood vessels needed to nourish growing tumors and contribute to the beneficial action of this drug in patients with cancer. In addition, the capacity of AMPK to block endothelial cell growth and movement may restrict angiogenesis-driven atherosclerotic plaque development and promote the

reduction of atherosclerotic lesions that is observed after protracted AMPK activation in mice (Li et al., 2010). Furthermore, the antiproliferative and antimigratory action on endothelial cells by AMPK may, in part, underlie the ability of AICAR to protect against retinopathy in diabetic animals (Kubota et al., 2011).

In conclusion, the present study identifies AMPK as a crucial regulator of endothelial cell function. AMPK activation inhibits endothelial cell proliferation, DNA synthesis, cell cycle progression, and migration and stimulates endothelial cell tube formation. The antiproliferative action of AMPK is eNOS- and HO-independent and mediated, in part, through increased expression of the cyclin-dependent kinase inhibitors p21 and p27. These studies provide novel insight into the mechanism by which AMPK regulates vascular cell growth and identifies this kinase as a potential therapeutic target in treating diseases dependent on endothelial cell proliferation and migration.

Authorship Contributions

Participated in research design: Peyton, Liu, and Durante.

Conducted experiments: Peyton, Liu, Yu, and Yates.

Performed data analysis: Peyton, Yu, Yates, and Durante.

Wrote or contributed to the writing of the manuscript: Peyton and Durante.

References

- Colombo SL and Moncada S (2009) AMPK α 1 regulates the antioxidant status of vascular endothelial cells. *Biochem J* **421**:163–169.
- Corton JM, Gillespie JG, Hawley SA, and Hardie DG (1995) 5-Aminoimidazole-4-carboxamide ribonucleoside. A specific method for activating AMP-activated protein kinase in intact cells? *Eur J Biochem* **229**:558–565.
- Davis BJ, Xie Z, Viollet B, and Zou MH (2006) Activation of the AMP-activated kinase by antidiabetes drug metformin stimulate nitric oxide synthesis in vivo by promoting the association of heat shock protein 90 and endothelial nitric oxide synthase. *Diabetes* **55**:496–505.
- Drummond GS and Kappas A (1981) Prevention of neonatal hyperbilirubinemia by tin protoporphyrin IX, a potent competitive inhibitor of heme oxidation. *Proc Natl Acad Sci U S A* **78**:6466–6470.
- El-Mir MY, Nogueira V, Fontaine E, Avéret N, Rigoulet M, and Leverve X (2000) Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem* **275**:223–228.
- Esfahanian N, Shakiba Y, Nikbin B, Soraya H, Maleki-Dizaji N, Ghazi-Khansari M, and Garjani A (2012) Effect of metformin on the proliferation, migration, and MMP-2 and -9 expression of human umbilical vein endothelial cells. *Mol Med Report* **5**:1068–1074.
- Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, and Morris AD (2005) Metformin and reduced risk of cancer in diabetic patients. *BMJ* **330**:1304–1305.
- Ewart MA, Kohlhaas CF, and Salt IP (2008) Inhibition of tumor necrosis factor α -stimulated monocyte adhesion to human aortic endothelial cells by AMP-activated protein kinase. *Arterioscler Thromb Vasc Biol* **28**:2255–2257.
- Folkman J (2006) Angiogenesis. *Annu Rev Med* **57**:1–18.
- Gaskin FS, Kamada K, Yusof M, Durante W, Gross G, and Korthuis RJ (2009) AICAR preconditioning prevents postischemic leukocyte rolling and adhesion: role of K_{ATP} channels and heme oxygenase. *Microcirculation* **16**:167–176.
- Gaskin FS, Kamada K, Yusof M, and Korthuis RJ (2007) 5'-AMP-activated protein kinase activation prevents postischemic leukocyte-endothelial cell adhesive interactions. *Am J Physiol Heart Circ Physiol* **292**:H326–H332.
- Göransson O, McBride A, Hawley SA, Ross FA, Shpiro N, Foretz M, Viollet B, Hardie DG, and Sakamoto K (2007) Mechanism of action of A-769662, a valuable tool for activation of AMP-activated protein kinase. *J Biol Chem* **282**:32549–32560.
- Hardie DG, Hawley SA, and Scott JW (2006) AMP-activated protein kinase—development of the energy sensor concept. *J Physiol* **574**:7–15.
- Hattori Y, Suzuki K, Hattori S, and Kasai K (2006) Metformin inhibits cytokine-induced nuclear factor κ B activation via AMP-activated protein kinase activation in vascular endothelial cells. *Hypertension* **47**:1183–1188.
- Hawley SA, Ross FA, Chevtzoff C, Green KA, Evans A, Fogarty S, Towler MC, Brown LJ, Ogunbayo OA, Evans AM, et al. (2010) Use of cells expressing γ subunit variants to identify diverse mechanisms of AMPK activation. *Cell Metab* **11**:554–565.
- Henderson JF, Paterson AR, Caldwell IC, Paul B, Chan MC, and Lau KF (1972) Inhibitors of nucleoside and nucleotide metabolism. *Cancer Chemother Rep* **2**:371–85.
- Ido Y, Carling D, and Ruderman N (2002) Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation. *Diabetes* **51**:159–167.
- Imamura K, Ogura T, Kishimoto A, Kaminishi M, and Esumi H (2001) Cell cycle regulation via p53 phosphorylation by a 5'-AMP activated protein kinase activator,

- 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside, in a human hepatocellular carcinoma cell line. *Biochem Biophys Res Commun* **287**:562–567.
- Ishii N, Matsumura T, Kinoshita H, Motoshima H, Kojima K, Tsutsumi A, Kawasaki S, Yano M, Senokuchi T, Asano T, et al. (2009) Activation of AMPK-activated protein kinase suppresses oxidized low-density lipoprotein-induced macrophage proliferation. *J Biol Chem* **284**:34561–34569.
- Jones RG, Plas DR, Kubek S, Buzzai M, Mu J, Xu Y, Birnbaum MJ, and Thompson CB (2005) AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol Cell* **18**:283–293.
- Kanellis J, Kandane RK, Etemadmoghadam D, Fraser SA, Mount PF, Levidiotis V, Kemp BE, and Power DA (2006) Activators of the energy sensing kinase AMPK inhibit random cell movement and chemotaxis in U937 cells. *Immunol Cell Biol* **84**:6–12.
- Kodiha M, Ho-Wo-Cheong D, and Stochaj U (2011) Pharmacological AMP-kinase activators have compartment-specific effects on cell physiology. *Am J Physiol Cell Physiol* **301**:C1307–C1315.
- Kubota S, Ozawa Y, Kurihara T, Sasaki M, Yuki K, Miyake S, Noda K, Ishida S, and Tsubota K (2011) Roles of AMP-activated protein kinase in diabetes-induced retinal inflammation. *Invest Ophthalmol Vis Sci* **52**:9142–9148.
- Landman GW, Kleefstra N, van Hateren KJ, Groenier KH, Gans RO, and Bilo HJ (2010) Metformin associated with lower cancer mortality in type 2 diabetes: ZODIAC-16. *Diabetes Care* **33**:322–326.
- Li D, Wang D, Wang Y, Ling W, Feng X, and Xia M (2010) Adenosine monophosphate-activated protein kinase induces cholesterol efflux from macrophage-derived foam cells and alleviates atherosclerosis in apolipoprotein E-deficient mice. *J Biol Chem* **285**:33499–33509.
- Li D, Yeung SC, Hassan MM, Konopleva M, and Abbruzzese JL (2009a) Antidiabetic therapies affect risk of pancreatic cancer. *Gastroenterology* **137**:482–488.
- Li X, Han Y, Pang W, Li C, Xie X, Shyy JY, and Zhu Y (2008) AMP-activated protein kinase promotes the differentiation of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* **28**:1789–1795.
- Li XN, Song J, Zhang L, LeMaire SA, Hou X, Zhang C, Coselli JS, Chen L, Wang XL, Zhang Y, et al. (2009b) Activation of the AMPK-FOXO3 pathway reduces fatty acid-induced increase in intracellular reactive oxygen species by upregulating thioredoxin. *Diabetes* **58**:2246–2257.
- Liu XM, Peyton KJ, Shebib AR, Wang H, Korthuis RJ, and Durante W (2011) Activation of AMPK stimulates heme oxygenase-1 gene expression and human endothelial cell survival. *Am J Physiol Heart Circ Physiol* **300**:H84–H93.
- Morrow VA, Fougelle F, Connell JM, Petrie JR, Gould GW, and Salt IP (2003) Direct activation of AMP-activated protein kinase stimulates nitric-oxide synthesis in human aortic endothelial cells. *J Biol Chem* **278**:31629–31639.
- Nagata D, Mogi M, and Walsh K (2003) AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress. *J Biol Chem* **278**:31000–31006.
- Nagata D, Takeda R, Sata M, Satonaka H, Suzuki E, Nagano T, and Hirata Y (2004) AMP-activated protein kinase inhibits angiotensin II-stimulated vascular smooth muscle cell proliferation. *Circulation* **110**:444–451.
- Ouchi N, Kobayashi H, Kihara S, Kumada M, Sato K, Inoue T, Funahashi T, and Walsh K (2004) Adiponectin stimulates angiogenesis by promoting cross-talk between AMP-activated protein kinase and Akt signaling in endothelial cells. *J Biol Chem* **279**:1304–1309.
- Palmer RM, Rees DD, Ashton DS, and Moncada S (1988) L-arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem Biophys Res Commun* **153**:1251–1256.
- Peyton KJ, Ensenat D, Azam MA, Keswani AN, Kannan S, Liu XM, Wang H, Tulis DA, and Durante W (2009) Arginase promotes neointima formation in rat injured carotid arteries. *Arterioscler Thromb Vasc Biol* **29**:488–494.
- Peyton KJ, Reyna SV, Chapman GB, Ensenat D, Liu XM, Wang H, Schafer AI, and Durante W (2002) Heme oxygenase-1-derived carbon monoxide is an autocrine inhibitor of vascular smooth muscle cell growth. *Blood* **99**:4443–4448.
- Peyton KJ, Yu Y, Yates B, Shebib AR, Liu XM, Wang H, and Durante W (2011) Compound C inhibits vascular smooth muscle cell proliferation and migration in an AMP-activated protein kinase-independent fashion. *J Pharmacol Exp Ther* **338**:476–484.
- Rattan R, Giri S, Singh AK, and Singh I (2005) 5-Aminoimidazole-4-carboxamide-1- β -D-ribofuranoside inhibits cancer cell proliferation in vitro and in vivo via AMP-activated protein kinase. *J Biol Chem* **280**:39582–39593.
- Reihill JA, Ewart MA, and Salt IP (2011) The role of AMP-activated protein kinase in the functional effects of vascular endothelial growth factor-A and -B in human aortic endothelial cells. *Vasc Cell* **3**:9.
- Ruscica M, Dozio E, Steffani L, Passafaro L, Mazzer M, Castellano JM, Motta M, Tena-Sempere M, and Magni P (2011) Role of the energy sensor adenosine monophosphate-activated protein kinase in the regulation of immature gonadotropin-releasing hormone neuron migration. *J Endocrinol Invest* **34**:e362–e368.
- Short JD, Dere R, Houston KD, Cai SL, Kim J, Bergeron JM, Shen J, Liang J, Bedford MT, Mills GB, et al. (2010) AMPK-mediated phosphorylation of murine p27 at T197 promotes binding of 14–3–3 proteins and increases p27 stability. *Mol Carcinog* **49**:429–439.
- Simó R, Carrasco E, García-Ramírez M, and Hernández C (2006) Angiogenic and antiangiogenic factors in proliferative diabetic retinopathy. *Curr Diabetes Rev* **2**:71–98.
- Sluimer JC and Daemen MJ (2009) Novel concepts in atherogenesis: angiogenesis and hypoxia in atherosclerosis. *J Pathol* **218**:7–29.
- Song P, Wang S, He C, Wang S, Liang B, Viollet B, and Zou MH (2011) AMPK α deletion exacerbates neointima formation by upregulating Skp2 in vascular smooth muscle cells. *Circ Res* **109**:1230–1239.
- Stahmann N, Woods A, Spengler K, Heslegrave A, Bauer R, Krause S, Viollet B, Carling D, and Heller R (2010) Activation of AMP-activated protein kinase by vascular endothelial growth factor mediates endothelial angiogenesis independently of nitric-oxide synthase. *J Biol Chem* **285**:10638–10652.
- Su KH, Yu YB, Hou HH, Zhao JF, Kou YR, Cheng LC, Shyue SK, and Lee TS (2012) AMP-activated protein kinase mediates erythropoietin-induced activation of endothelial nitric oxide synthase. *J Cell Physiol* **227**:3053–3062.
- Viana R, Aguado C, Esteban I, Moreno D, Viollet B, Knecht E, and Sanz P (2008) Role of AMP-activated protein kinase in autophagy and proteasome function. *Biochem Biophys Res Commun* **369**:964–968.
- Wang W, Fan J, Yang X, Fürer-Galban S, Lopez de Silanes I, von Kobbe C, Guo J, Georas SN, Fougelle F, Hardie DG, et al. (2002) AMP-activated kinase regulates cytoplasmic HuR. *Mol Cell Biol* **22**:3425–3436.
- Wei Y, Liu XM, Peyton KJ, Wang H, Johnson FK, Johnson RA, and Durante W (2009) Hypochlorous acid-induced heme oxygenase-1 gene expression promotes human endothelial cell survival. *Am J Physiol Cell Physiol* **297**:C907–C915.
- Xiao B, Sanders MJ, Underwood E, Heath R, Mayer FV, Carmena D, Jing C, Walker PA, Eccleston JF, Haire LF, et al. (2011) Structure of mammalian AMPK and its regulation by ADP. *Nature* **472**:230–233.
- Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doeber T, Fujii N, et al. (2001) Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* **108**:1167–1174.
- Zou MH and Wu Y (2008) AMP-activated protein kinase activation as a strategy for protecting vascular endothelial function. *Clin Exp Pharmacol Physiol* **35**:535–545.

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