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

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Running Title Page

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Nonstandard Abbreviations:
AMPK, adenosine monophosphate-activated kinase; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; HO, heme oxygenase; HUVEC, human umbilical vein endothelial cell; HAEC, human aortic endothelial cells; SDS, sodium dodecyl sulfate; AdAMPK-CA, adenovirus expressing a constitutively active AMPK mutant; AdGFP, adenovirus expressing green fluorescent protein.
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), 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 following 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 following the phosphorylation of the α-subunit at Thr\(^{172}\) by several upstream kinases (Zou and Wu, 2008). More recently, ADP has also been shown to bind the γ-subunit and stimulate Thr\(^{172}\) 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 via direct phosphorylation of target proteins and by modulating 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 analogue that mimics the effect of AMP on AMPK activation (Corton JM et al., 1995). The thienopyridone compound A-769662 is a
recently identified selective small molecule activator of AMPK that directly binds and activates the kinase in a fashion similar to AMP (Goransson 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 likely 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 (NO) synthase (eNOS) resulting in the liberation of NO, 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-κB and the expression of adhesion molecules and chemokines by endothelial cells (Hattori et al., 2006; Ewart et al., 2008). Moreover, we recently reported 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 to the induction of the vasoprotective protein HO-1 (Gaskin et al., 2007; Gaskin et al, 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.

Methods

Materials

Penicillin, streptomycin, gelatin, heparin, dithiothreitol, sodium dodecyl sulfate (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 Corporation (Carlsbad, CA). A-769662 was from Tocris Biosciences (Ellisville, MO). Endothelial cell growth factor was from Becton Dickinson Biosciences (Bedford, MA), 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 (Santa Cruz, CA). A pan-specific antibody against AMPKα and phosphorylation-specific antibodies against AMPKα, p53, retinoblastoma protein, and acetyl-CoA carboxylate (ACC) were from Cell Signaling Technologies (Beverley, MA).

[^3H]Thymidine (20 Ci/mmol) was from Perkin Elmer (Boston, MA).

Cell Culture

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

Small Interference RNA (siRNA) and Adenovirus Infection Protocol

Gene expression was silenced using siRNAs targeting p21 and p27. The experimental and control, non-targeting siRNAs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and delivered to endothelial cells (100nM) using a commercial transfection reagent (Invitrogen, Carlsbad, CA). A replication-defective adenovirus expressing a constitutively active AMPKα 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 hours prior to treatment.

Cell Proliferation and DNA Synthesis

Endothelial cells were seeded (2 x 10⁴ cells/well) onto 6-well plates in serum-containing media. After 24 hours, 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 Incorporated, Brea, CA). Endothelial cell proliferation was also monitored by measuring DNA synthesis, as previously described (Peyton et al., 2002). Cells were incubated with [³H]thymidine for 4 hours, washed three times with ice-cold PBS, and fixed
with 10% trichloracetic acid for 30 minutes at 4°C. DNA was then extracted with 0.2% SDS/0.2N NaOH and radioactivity 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 70-80% confluence by flow-activated cell sorting, as previously reported (Peyton et al., 2009). Endothelial cells were collected, suspended in PBS, and pelleted by centrifugation at 1000 x g for 5 minutes. Pellets were washed twice with PBS, suspended in 70% ethanol, and fixed overnight at 4°C. Fixed cells were then incubated with propidium iodide (50µg/ml) and RNase A (100µg/ml) for 1 hour at room temperature and DNA fluorescence measured in a Beckman Coulter CyAN™ ADP Cytometer (Brea, CA).

**Western blotting**

Endothelial cells were collected in electrophoresis buffer (125mM Tris [pH 6.8], 12.5% glycerol, 2% SDS, and trace bromphenol blue) and proteins separated by SDS-polyacrylamide gel electrophoresis. Following 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 (1:100), AMPKα (1:500), ACC (1:500), phospho-AMPKα (1:100), phospho-ACC (1:100) or β-actin (1:200). Membranes were washed in PBS, incubated with horseradish peroxidase-conjugated goat anti-rabbit or rabbit anti-goat antibodies and developed with commercial chemoluminescence reagents (Amersham, Arlington Heights, IL). 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 AMPKα or ACC (Liu et al., 2011).

**Cell Migration**

Cell migration was determined 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 incubated in serum-containing media in the presence and absence of various test compounds. Cell monolayers were photographed immediately and 24 hours after scratch injury with a digital camera (Q-Imaging, QICAM; Hitschfel Instruments, Incorporated, St. Louis, MO) and the degree of wound closure determined by planimetry.

**Endothelial Cell Tube Formation**

The endothelial cell tube formation assay was performed using growth factor-reduced Matrigel (BD Biosciences, San Jose, CA). Cells (2 x 10⁴ cells/well) were plated in 96-well plates
that had been pre-coated with matrigel (50µL/well). After incubation for 6 hours in serum-containing media, images of tube morphology were taken by an inverted Olympus CKX41 microscope (Olympus America, Incorporated, Center Valley, PA) and the extent of tube formation quantified by counting the number of tubes.

Statistics

Results are expressed as mean ± SEM. 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 HUVEC with AICAR (500µM) resulted in a persistent, time-dependent increase in AMPK activity, as reflected by the phosphorylation of AMPK (Figure 1A). Induction of AMPK activity was detected one hour following AICAR administration and AMPK activity remained elevated during 24 hours of AICAR exposure. Incubation of HUVEC with serum-containing media stimulated a time-dependent increase in cell number that was blocked by AICAR (Figure 1B). The inhibition of HUVEC growth by AICAR was concentration-dependent (Figure 1C). A significant inhibition of cell growth by AICAR was noted at a concentration of 50µM and near-total ablation of proliferation was noted with 500µM. The antiproliferative effect of AICAR was not related to any change in cell viability, as assessed by trypan blue exclusion [control: 95.4±3.7% viable versus AICAR (0.5mM): 94.6 ±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 phosphorylation of the AMPK substrate ACC (Figure 1D), and largely reversed the anti-proliferative action of AICAR (Figure 1E).

The ability of AMPK to inhibit HUVEC growth was also corroborated using two other distinct activators of AMPK. As observed with AICAR, A-769662 (300µM) and metformin (2mM) activated AMPK in HUVEC (Figure 2A) and lead to a significant decline in cell growth (Figure 2B). Furthermore, all three AMPK activators markedly suppressed HUVEC DNA synthesis (Figure 2C). The ability of AICAR to block DNA synthesis was dependent on AMPK activation since it was largely reversed by 5-‘iodotubercidin (Figure 2D). Because we recently reported that eNOS and HO-1 contributes to the biological actions of AMPK on endothelial cells, their role in mediating the antiproliferative action of AICAR was 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) while 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 (Figure 2D). The role of AMPK in regulating HUVEC proliferation was also evaluated using a constitutively active AMPKα mutant (AdAMPK-CA). Infection of HUVEC with AdAMPK-CA resulted in a significant decline in DNA synthesis whereas infection with a control adenovirus had no effect (Figure 2E). Pharmacological activators of AMPK or infection of cells with AdAMPK-CA also blocked DNA synthesis in HAEC (Figure 3A and B).
Subsequently, we determined the effect of AMPK activation on endothelial cell cycle progression. Administration of AICAR, A-769662, or metformin arrested cells in the G0/G1 phase of the cell cycle, as demonstrated by an increase in the percentage of cells in G0/G1 with a corresponding decline in the fraction of cells in S phase and G2/M (Figure 4A and B). In addition, no apparent toxicity was noted by any of the AMPK activators as reflected by the lack of a sub-G0/G1 fraction (Figure 4A).

To determine the mechanism by which AMPK disrupts cell cycle progression in HUVEC, 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 pRb (Figure 5A and B). 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, 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, while the control non-targeting siRNA had no effect on the expression of either protein (Figure 6A 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, while 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 HUVEC with AICAR resulted in a concentration-dependent decrease in cell migration (Figure 7A). An approximate 60% decline in cell migration by AICAR was observed with 500µM, and this was largely reversed in the presence of 5′-iodotubercidin (Figure 7B). Interestingly, AICAR stimulated endothelial cell tube formation and this was completely abolished by 5-iodotubercidin (Figure 7C). In the absence of AICAR, 5-iodotubercidin had no effect on endothelial cell migration or tube formation (Figure 7B and C).

Discussion

The present study demonstrates that AMPK is a potent inhibitor of human endothelial cell proliferation. The inhibition of endothelial cell proliferation is observed after pharmacological or molecular activation of AMPK and is seen in endothelial cells obtained from both the arterial and venous circulation. In addition, AMPK arrests endothelial cells in the G0/G1 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 activity since 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 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 AMPKα. Of note, 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 cytostatic agent. Although recent work from our laboratory and others indicate that eNOS and HO-1 contributes to the actions of AMPK on endothelial cell function (Gaskin et al., 2007; Gaskin et al, 2009; Liu et al., 2011), these proteins are not involved in the antiproliferative effect of AMPK since 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 to a recent report in human aortic endothelial cells (Reihill et al., 2011). In this latter study, AMPK activation was shown to block basal endothelial cell growth but the underlying mechanism was not explored. Interestingly, 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 is observed in our study may favor endothelial cell growth arrest while acute, transient activation of AMPK in response to angiogenic factors promote 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 G0/G1 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, consistent with detention of cells in G0/G1. The ability of AMPK to upregulate 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 likely accumulates in endothelial cells due to its phosphorylation by AMPK, which suppresses its degradation (Imamura et al., 2001; Jones et al., 2005).

Importantly, we show that increases in cyclin-dependent kinase inhibitors contribute to the anti-proliferative action of AMPK. While 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. Interestingly, 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 following AMPK inhibition. Our results demonstrating that AMPK elicits an antimigratory action is consistent with a recent study showing that metformin blocks the migration of HUVEC (Esfahanian et al., 2012) and with 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 which reported that AMPK enhances the migration of endothelial cells (Nagata et al., 2003; Rehill et
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; Sluimer and Daemen, 2009; Simo et al., 2006). Interestingly, recent retrospective studies conducted in diabetic patients found that metformin is associated with a decreased risk in developing cancer and with a better response to chemotherapy (Evans et al., 2005; Landman et al., 2010; Li et al., 2009a). While 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 cancer patients. 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

Participation in research design: Peyton, Liu, Durante
Conducted experiments: Peyton, Liu, Yu, Yates
Performed data analysis: Peyton, Yu, Yates, Durante
Wrote or contributed to writing of the manuscript: Peyton, Durante
References


Footnote

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Figure Legends

1. AICAR inhibits the proliferation of human umbilical vein endothelial cells (HUVEC) in an AMPK-dependent manner. A. AICAR (500µM) induced a sustained increase in AMPK activity, as reflected by the phosphorylation of AMPK. B. Time-dependent increase in HUVEC number was blocked by AICAR. Cells were incubated in the absence and presence of AICAR (500µM) for 3 days. C. AICAR inhibited the proliferation of HUVEC in a concentration-dependent manner. HUVEC were incubated in the absence or presence of different concentrations of AICAR (5-500µM) for 3 days. D. Inhibition of AICAR (500µM for 24 hours)-mediated AMPK activation by 5'-iodotubercidin (Iodo; 0.3µM), as reflected by inhibition of the phosphorylation of the AMPK substrate, ACC. E. Effect of AMPK inhibition on AICAR-mediated inhibition of HUVEC proliferation. Cells were treated with AICAR (500µM) in the absence or presence of Iodo (0.3µM) for 3 days. Results are means ± SEM (n=4-6). *Statistically significant effect of AICAR. †Statistically significant effect of Iodo.

2. Activation of AMPK inhibits the proliferation of human umbilical vein endothelial cells (HUVEC). A. Treatment of HUVEC with AICAR (500µM), A769662 (300µM), or metformin (2mM) for 24 hours stimulated AMPK activity, as reflected by the phosphorylation of AMPK. B. AMPK activators inhibited the proliferation of HUVEC. Cells were incubated in the absence or presence of AICAR (500µM), A769662 (300µM), or metformin (2mM) for 3 days. C. AMPK activators inhibited HUVEC DNA synthesis. Cells were incubated in the absence or presence of AICAR (500µM), A769662 (300µM),
or metformin (2mM) for 24 hours. D. AICAR-mediated inhibition of DNA synthesis is dependent on AMPK activity but independent of heme oxygenase or endothelial nitric oxide synthase activity. Cells were treated with AICAR (500µM) in the absence or presence of 5'-iodotubercidin (Iodo; 0.3µM), methyl-L-arginine (L-NMA; 1mM), or tin protoporphyrin-IX (SnPP; 5µM). E. Infection of HUVEC with an adenovirus expressing constitutively active AMPK (AdAMPK-CA) but not green fluorescent protein (AdGFP) inhibited DNA synthesis. Results are means ± SEM (n=4-6). *Statistically significant effect of AMPK activators or AdAMPK-CA. †Statistically significant effect of Iodo.

3. Activation of AMPK inhibits the proliferation of human aortic endothelial cells (HAEC).
A. AMPK activators inhibited the proliferation of HAEC. Cells were incubated in the absence or presence of AICAR (500µM), A769662 (300µM), or metformin (2mM) for 3 days. B. Infection of HAEC with an adenovirus expressing constitutively active AMPK (AdAMPK-CA) but not green fluorescent protein (AdGFP) inhibited DNA synthesis. Results are means ± SEM (n=4). *Statistically significant effect of AMPK activators or AdAMPK-CA.

4. Activation of AMPK inhibits cell cycle progression by human umbilical vein endothelial cells (HUVEC). A. Representative histograms of HUVEC incubated in the absence or presence of AICAR (500µM), A-769662 (300µM), or metformin (2mM) for 24 hours. B. Effect of AICAR (500µM), A-769662 (300µM), or metformin (2mM) exposure for 24 hours on the distribution of HUVEC in the cell cycle. Results are means ± SEM (n=3). *Statistically significant effect of AMPK activators.
5. Effect of AICAR on the expression and phosphorylation of cell cycle regulatory proteins in human umbilical vein endothelial cells (HUVEC). A. Cells were incubated in the absence or presence of AICAR (500µM) for 24 hours and protein expression and phosphorylation determined by western blotting. B. Quantification of protein expression or phosphorylation relative to control, untreated cells. Results are means ± SEM (n=4). *Statistically significant effect of AICAR.

6. AMPK-mediated inhibition of human umbilical vein endothelial cell (HUVEC) proliferation is dependent on p21 and p27 protein expression. A. Silencing p21 protein expression in HUVEC. B. Silencing p27 protein expression in HUVEC. 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 small interfering RNA (siRNA) to p21 (p21 siRNA; 100nM), p27 (p27 siRNA; 100nM) and/or non-targeting siRNA (NT siRNA; 100nM) for 48 hours and then treated with AICAR (500µM) in the absence or presence of 5′-iodotubercidin (Iodo; 0.3µM) for 24 hours, at which point cells were collected for western blotting or pulsed with [³H]thymidine for DNA synthesis determination. Results are means ± SEM (n=4). *Statistically significant effect of AICAR. †Statistically significant effect of siRNA transfection or Iodo.

7. Effect of AMPK activation on migration and tube formation by human umbilical vein endothelial cells (HUVEC). A. AICAR inhibited the migration of HUVEC in a concentration-dependent manner. Cells were incubated in the absence or presence of different concentrations of AICAR (5-500µM) for 24 hours after wound injury. B. Effect
of AMPK inhibition on AICAR-mediated inhibition of HUVEC migration. Cells were treated with AICAR (500µM) for 24 hours after wound injury in the absence or presence of 5’-iodotubericidin (Iodo; 0.3µM). C. AMPK activation stimulated tube formation by HUVEC. Cells were treated with AICAR (500µM) for 6 hours after plating onto matrigel in the absence or presence of Iodo (0.3µM). Results are means ± SEM (n=4).

*Statistically significant effect of AICAR. †Statistically significant effect of Iodo.
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