AMP-activated protein kinase (AMPK) signaling in endothelial cells
is essential for angiogenesis in response to hypoxic stress

Daisuke Nagata, Masaki Mogi, Kenneth Walsh*

Molecular Cardiology/Whitaker Cardiovascular Institute, Boston University School of Medicine, 715 Albany Street, W611, Boston, MA 02118, USA

Running Title: AMPK signaling and angiogenesis

*Correspondence should be addressed to:
Kenneth Walsh, Ph.D.
Molecular Cardiology/Whitaker Cardiovascular Institute
Boston University School of Medicine
715 Albany Street, W611
Boston, MA 02118, USA
Phone: 617-414-2392
Fax: 617-414-2391
Email: kxwalsh@bu.edu
Summary

AMP-activated protein kinase (AMPK) is a stress-activated protein kinase that is regulated by hypoxia and other cellular stresses that result in diminished cellular ATP levels. Here, we investigated whether AMPK signaling in endothelial cells has a role in regulating angiogenesis. Hypoxia induced the activating phosphorylation of AMPK in human umbilical vein endothelial cells (HUVECs), and AMPK activation was required for the maintenance of pro-angiogenic Akt signaling under these conditions. Suppression of AMPK signaling inhibited both HUVEC migration to VEGF and \textit{in vitro} differentiation into tube-like structures in hypoxic, but not normoxic cultures. Dominant-negative AMPK also inhibited \textit{in vivo} angiogenesis in Matrigel plugs that were implanted subcutaneously in mice. These data identify AMPK signaling as a new regulator of angiogenesis that is specifically required for endothelial cell migration and differentiation under conditions of hypoxia. As such, endothelial AMPK signaling may be a critical determinant of blood vessel recruitment to tissues that are subjected to ischemic stress.

Keywords: angiogenesis, hypoxia, signaling, endothelial cells, protein kinase
Introduction

Angiogenesis is central feature of normal embryonic and post-natal development, and this process plays a critical role in the neovascularization that is associated with tumor growth and occlusive vascular diseases (1-3). A large body of evidence has shown that vascular angiogenic growth factors promote angiogenesis through activation of MAP kinase (4) and phosphatidylinositol 3-kinase (PI3K)-Akt/PKB (5) intracellular signaling pathways. However, the signaling molecules involved in angiogenic cellular responses under conditions of hypoxic stress is incompletely understood.

AMP-activated protein kinase (AMPK\(^1\)) is a metabolite-sensing protein kinase that shares amino acid sequence homology with yeast SNF1 (6,7). In myocytes, AMPK is activated by increases in the AMP:ATP ratio, which is brought about by hypoxia/anoxia (8-10), vigorous exercise and muscle contraction (11-14) or pressure-overload hypertrophy (15). Under these conditions, AMPK is activated by a conformational change after binding AMP (16,17), and by phosphorylation by its upstream kinase AMPK kinase (AMPKK) (17-19). Upon activation, AMPK phosphorylates and downregulates several anabolic enzymes.

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\(^{1}\) The abbreviations used are: AMPK, AMP-activated kinase; AMPKK, AMP-activated kinase kinase, HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; ACC, acetyl-CoA; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; HUVECs, human umbilical vein endothelial cells; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; GFP, green fluorescence protein; EBM, endothelial cell basal medium; EGM, endothelial cell growth medium.
including 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (16,20), acetyl-CoA carboxylase (ACC) (16,21), sn-glycerol-3-phosphate acetyltransferase (22,23) and glycogen synthase (24); thereby diminishing metabolite flux through synthetic pathways that consume ATP. AMPK activation also accelerates β-oxidation of fatty acid, which promotes ATP production (25,26).

Endothelial nitric oxide synthase (eNOS) residue 1177 (in human) is a substrate for both Akt (27,28) and AMPK (29). Phosphorylation of eNOS at this residue leads to enzyme activation and nitric oxide (NO) production. AMPK is reported to phosphorylate eNOS in cardiac myocytes under hypoxic conditions (29), but it is not clear whether NO production by endothelial cells is regulated by this reaction. Recently, AMPK was detected in human umbilical vein endothelial cells (HUVECs) (30). The pharmacological stimulator of AMPK, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), protects HUVECs from apoptosis under hyperglycemic conditions (31). Interestingly, AICAR was found to reverse the inhibitory effect of hyperglycemia on mitogen-stimulated Akt phosphorylation.

In this study we analyzed whether AMPK signaling plays a role in angiogenesis. Specifically, we tested whether AMPK is essential for endothelial cell migration, differentiation into capillary-like structures, and NO production under both normoxic and hypoxic conditions. We also tested whether AMPK signaling is required for angiogenesis in
Matrigel plugs that were subcutaneously implanted in mice. The results of this study suggest that crosstalk between AMPK and Akt is essential for angiogenesis under conditions of hypoxic stress, but dispensable for angiogenic cellular responses in normoxic endothelial cells.
Experimental Procedures

Materials

Phospho-AMPK (Thr172), pan-α-AMPK and phospho-Akt (Ser473), phospho-eNOS (Ser1177) and phospho-eNOS (Thr495) antibody were purchased from Cell Signaling Technology (Beverly, Massachusetts). Phospho-Acetyl CoA Carboxylase (ACC) (Ser79) and c-Myc tag antibody was purchased from Upstate biotechnology (Lake Placid, New York). ACC protein antibody was from Alpha Diagnostic International (San Antonio, Texas). Akt and eNOS antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Nω-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), and human vascular endothelial growth factor (VEGF) were purchased from Sigma (St. Louis, Missouri).

Adenovirus

A replication-defective adenoviral vector expressing dominant-negative AMPK was a gift from Dr. Birnbaum (University of Pennsylvania). This vector expresses the rat AMPK α2 subunit cDNA, whose lysine 45 residue was changed to arginine (14), fused in-frame with the c-Myc epitope tag. Details of Ad-caAkt and Ad-dnAkt construction were previously described (32,33). The encoded proteins are fused to the hemagglutinin (HA) epitope. Adenoviral vector expressing green fluorescence protein (GFP) was obtained from Qbiogene (Illkirch, France).
Cell culture and adenoviral vectors transduction

Human umbilical vein endothelium cells (HUVECs), endothelial cell basal medium (EBM), and endothelial cell growth medium (EGM) were purchased from Clonetics (San Diego, California). HUVECs were cultured in EGM with 5% Fetal Bovine Serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. All experiments utilized HUVECs at passage 6 or less. HUVECs were transduced with the indicated replication-defective adenoviral vectors at a multiplicity of infection (m.o.i.) of 50 plaque forming units for 1 day. The media was then changed to EBM containing 1% FBS to reduce the effects of stimulation by serum mitogens. After a 1 day incubation in low mitogen media, transduced cells were placed in a hypoxia chamber (GasPak anaerobic system; Becton Dickinson, Franklin Lakes, New Jersey) according to the manufacturer’s instructions for the indicated period of time. This system induces anaerobic conditions of less than 2% of oxygen and more than 4% of carbon dioxide by using carbon dioxide-generating reagents (34). All experiments utilized HUVECs at passage 6 or less.

Western blot analysis

Western blot analysis was carried out as previously described (35). In brief, cell lysates were extracted with NP-40 lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) followed by SDS-PAGE. The membranes were immunoblotted with the indicated
antibodies at a 1:1000 dilution followed by the secondary antibody conjugated with horseradish peroxidase (HRP) at a 1:5000 dilution. ECL-PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech, Piscataway, New Jersey) was used for detection.

**Migration assay**

Migration assays were performed as described previously using a modified Boyden chamber (Neuroprobe, Cabin John, Maryland) (36). HUVECs infected with the indicated adenoviral vectors were serum-starved overnight in serum-free media (EBM). Transduced cells were suspended with 0.05% trypsin-0.53 mM EDTA (Invitrogen, Carlsbad, California), washed with phosphate-buffered saline, and resuspended in EBM. EBM containing VEGF 150 ng/ml was put into the wells of the lower chamber. Polycarbonate filters with 8-µm pores (PVPF; OSMONICS, Kent, Washington) coated with 0.5% gelatin were used to separate the lower chamber and 1 X 10^5 suspended cells in the upper chamber. For some experiments, the indicated concentrations of SNP were added to both chambers. The chambers were then incubated for 8 hours at 37°C under normoxic or hypoxic conditions. Filters were carefully removed and the cells attached to the upper side were removed by wiping. Cells migrating through the filter (cells on the lower side of filter) were fixed with methanol for 15 minutes, stained with Giemsa stain solution (Sigma) and five random microscopic fields per well were quantified. Each experiment was performed in duplicate and three separate experiments were performed.
Tube formation assay

The formation of vascular-like structures by HUVECs on growth factor-reduced Matrigel (Becton Dickinson) was performed as previously described (37). Twenty-four-well culture plates were coated with Matrigel according to the manufacturer's instructions. The indicated adenovirus-transduced HUVECs were seeded on coated plates at 5X10^4 cells/well in EBM containing VEGF 50 ng/ml and incubated at 37°C for 18 hours under normoxic or hypoxic conditions. In some wells, SNP (1 µM – 1 mM) was also added. Tube formation was observed using an inverted phase contrast microscope (Nikon, Tokyo, Japan). Images were captured with a video graphic system (DEI-750 CE Digital Output Camera, Optronics, Goleta, California). The degree of tube formation was quantified by measuring the length of tubes in 3 randomly chosen low-power fields (X100) from each well using the National Institutes of Health (NIH) Image Program. Each experiment was repeated for 4 times.

cGMP assay

Intracellular cGMP concentration was measured using cGMP enzyme immunoassay system purchased from Amersham Pharmacia (Piscataway, New Jersey) according to the manufacturer’s instruction. After adenovirus infection, cells were incubated overnight in low serum medium to reduce the effect of mitogens. Then VEGF (50 ng/ml) was added to the cultures in the absence or presence of NO synthase inhibitor, L-NAME (1 mg/ml). Cells were then incubated under
hypoxic or normoxic conditions for 6 hours prior to the cGMP measurement. Each experiment was performed in 4 independent samples, and 2 sets of separate experiments were performed.

**Mouse angiogenesis assay**

The formation of new vessels *in vivo* was evaluated by Matrigel plug assay as described previously (38,39). For these experiments, 400 µl of Matrigel containing bFGF (250 ng/ml) and replication-defective adenoviral vectors encoding GFP, dominant-negative AMPK or dominant-negative Akt (1X10⁸ plaque forming units) was cooled on ice and then injected subcutaneously into the abdomen of C57BL mice. Mice were sacrificed 14 days after the injection. The Matrigel plugs with adjacent subcutaneous tissues were carefully recovered by en bloc resection, fixed in 4% paraformaldehyde, dehydrated with 30% sucrose, and embedded in OCT compound (GTI Microsystems, Tempe, Arizona) in liquid nitrogen. Masson’s trichrome staining and immunohistostaining for CD31 (PECAM-1: Becton Dickinson) were performed on adjacent frozen sections. Primary antibody was used at a 1:50 dilution followed by incubation of secondary antibody (HRP-conjugated anti-rat IgG at a 1:100 dilution). The AEC Substrate Pack (Biogenex, San Ramon, California) was used for detection. CD31-positive capillaries were counted in 4 randomly chosen low-power (X100) microscopic fields. Each experiment was repeated 4 times.
Statistical analyses

Values were expressed as the mean ± SE. Statistical comparisons were performed using analysis of variance (ANOVA) with Scheffe’s F procedure for post hoc analysis.
Results

AMPK activation by hypoxia in endothelial cells

To test whether AMPK is activated in endothelial cells under conditions of hypoxia, HUVEC cultures were incubated in a hypoxia chamber for different lengths of time and Western immunoblot analyses were performed on lysates using an antibody that is specific for AMPK phosphorylation at Thr172 of the α subunit. A basal level of AMPK phosphorylation was detected in cell cultured under normoxic conditions, and hypoxia produced a time-dependent increase in AMPK phosphorylation (Fig. 1A). A representative Western blot of this time course is shown (Fig. 1A). Thr172 phosphorylation leads to AMPK activation, and the induction of AMPK signaling by hypoxia was indicated by the time-dependent phosphorylation of ACC at Ser 79 (Fig. 1B), which is a substrate for AMPK. Hypoxia also led to a time-dependent increase in AMPK activity as assessed by SAMS peptide phosphorylation with immunoprecipitated AMPK (data not shown²). Basal and hypoxia-inducible phosphorylation of ACC was blocked when cells were transduced at 50 m.o.i. with an adenoviral vector expressing a dominant-negative mutant of AMPK (dominant-negative AMPK) that contains a c-Myc epitope tag (Fig. 1A, B). Parallel cultures incubated with an adenoviral vector expressing the GFP marker protein at 50 m.o.i. revealed greater than a 95% transduction efficiency. Transduction

² D.N. and K.W., unpublished observations.
with dominant-negative AMPK also inhibited the hypoxia-induced phosphorylation of endogenous AMPK (Fig. 1A). Hypoxia did not affect the total level of α subunit expression in immunoblot analyses using a pan-α-AMPK antibody (Fig. 1A), nor did it affect the level of ACC protein expression (data not shown).

Hypoxia led to a modest reduction in basal Akt phosphorylation at Ser 473, which is required for kinase activity (40,41) (Fig. 1A). Transduction with dominant-negative AMPK further suppressed Akt phosphorylation, inhibiting Akt phosphorylation at every time point examined (Fig. 1C). The relative magnitude of the inhibition by dominant-negative AMPK increased as the length of exposure to hypoxia increased.

**Regulation of eNOS Ser1177 phosphorylation and NO production by AMPK**

The production of endothelial NO is important for vascular homeostasis and angiogenesis (42-46). VEGF stimulation promotes Akt signaling which, in turn, produces NO through the activating phosphorylation of eNOS at residue 1177 (27). Because AMPK is also reported to phosphorylate eNOS at residue 1177 in cardiac myocytes (29), the contribution of AMPK to VEGF-stimulated eNOS phosphorylation was analyzed in HUVEC under normoxic and hypoxic conditions (Fig. 2A). In our assays, VEGF did not affect the level of AMPK expression on phosphorylation (data not shown). However, transduction with dominant-negative AMPK effectively blocked eNOS phosphorylation under conditions of hypoxia, whereas it had
little effect in normoxic HUVEC cultures (Fig. 2B). While basal levels of AMPK in normoxic endothelial cells has little effect on the status of eNOS 1177 phosphorylation, AMPK signaling was essential for ACC phosphorylation under these conditions (Fig. 2A). A vector expressing dominant-negative Akt was used as a positive control in these experiments because it is known to inhibit VEGF-stimulated eNOS phosphorylation in normoxic endothelial cell cultures (27). In contrast to dominant-negative AMPK, transduction with dominant-negative Akt significantly inhibited eNOS phosphorylation at Ser1177 in normoxic cultures. Transduction with dominant-negative Akt also inhibited eNOS phosphorylation in hypoxic cultures. However, it had no effect on ACC phosphorylation under any condition.

Recently, it has been shown that eNOS activity is negatively regulated through phosphorylation of Thr-495 (29,47,48). In normoxic cultures, VEGF stimulation led to a decrease in Thr-495 phosphorylation (Fig. 2A, C), consistent with a previous report (47). The reduction in Thr-495 phosphorylation by VEGF was reversed by transduction with dominant-negative Akt, while dominant-negative AMPK had no effect. Similar trends in Thr-495 phosphorylation were also seen in hypoxic cultures, but the overall magnitude of the VEGF effect was less than that seen in normoxic cultures. Furthermore, dominant-negative AMPK had no detectable effect on decline in Thr-495 under conditions of hypoxia.
The production of NO by eNOS was measured in intact HUVEC cultures by monitoring the accumulation of cGMP (39). Under the conditions of these assays, VEGF-induced cGMP accumulation was similar between HUVEC cultures incubated under normoxic and hypoxic conditions (Fig. 3). Under both conditions, cGMP accumulation was largely inhibited by the eNOS inhibitor L-NAME. Transduction with dominant-negative AMPK significantly inhibited NO output in hypoxic cultures. There was a trend toward lower NO output in normoxic cultures transduced with dominant-negative AMPK, but this was not statistically significant (P = 0.1). In contrast, transduction with dominant-negative Akt significantly inhibited NO output in both hypoxic and normoxic HUVEC cultures. Transduction with dominant-negative Akt or dominant-negative AMPK did not affect basal NO production in the absence of VEGF in either hypoxic or normoxic cultures.

**AMPK signaling is essential for endothelial cell migration under hypoxic conditions**

To evaluate the role of AMPK signaling on endothelial cell migration, HUVEC were transduced with Ad-dnAMPK and tested for their ability to migrate toward VEGF (150 ng/ml) in a Boyden chamber apparatus. Under hypoxic conditions, transduction with dominant-negative AMPK significantly suppressed cell migration relative to cells transduced with Ad-GFP control (Fig. 4). No differences in cell migration were detected between Ad-GFP-treated and non-transduced cells. Furthermore, the reduction in cell migration did not appear to be due to
decreased cell viability. Under these cell culture conditions dominant-negative AMPK-transduced cells did not display detectable increases in the frequencies of TUNEL- or pyknotic nuclei-positive cells, nor was there a detectable decrease in mitochondrial function (data not shown\(^2\)). Addition of 10\(\mu\)M sodium nitroprusside (SNP), an NO donor, partially reversed the inhibitory action of dominant-negative AMPK on HUVEC migration to VEGF in the hypoxic cultures (Fig. 4). Similar effects were also observed at 1 and 100 \(\mu\)M SNP (data not shown\(^2\)). However, co-transfection with an adenoviral vector expressing a constitutively-active form of Akt was more effective than the NO donor at reversing the inhibition by dominant-negative AMPK in hypoxic cultures (\(P<0.05\) relative to dominant-negative AMPK plus SNP, and not significant relative to control). Transduction with dominant-negative AMPK had no effect on HUVEC migration to VEGF under normoxic conditions. In contrast, transduction with dominant-negative Akt suppressed migration under normoxic conditions, consistent with previous findings (49), and also exhibited this activity in hypoxic cultures.

**AMPK regulates endothelial cell tube formation under hypoxic conditions**

To examine the role of AMPK signaling on endothelial cell differentiation into vascular structures \textit{in vitro}, HUVECs were plated on matrigel in the presence of VEGF after transduction with GFP, dominant-negative AMPK or dominant-negative Akt, and tube formation was assessed under normoxic and hypoxic conditions (Fig. 5A). Tube structure length was quantified
after 18 hours using NIH imaging software (Fig. 5B). Transduction with dominant-negative AMPK suppressed tube formation to a similar extent as dominant-negative Akt under hypoxic conditions. The inhibition of tube formation by dominant-negative AMPK was partially reversed by the inclusion of 10 µM SNP in the culture media. Similar effects were observed at 1 and 100 µM SNP (data not shown\(^2\)). Co-transfection with an adenoviral vector expressing constitutively-active Akt was more effective than the NO donor in reversing the inhibition of tube formation by dominant-negative AMPK (P<0.05 relative to dominant-negative AMPK plus SNP, and not significant relative to control). In contrast, transduction with dominant-negative Akt, but not dominant-negative AMPK, suppressed on tube formation in normoxic cultures (Fig. 5).

**AMPK regulates capillary formation in Matrigel plugs in vivo**

To assess the role of AMPK signaling on angiogenesis in an *in vivo* model, matrigel plugs containing adenoviral vectors were subcutaneously implanted on the abdomen of mice. In this assay the matrigel plug serves as a reservoir for the adenoviral vector, and endothelial cells that infiltrate the plug become transduced and express transgene (39). Two weeks after implantation, plugs were harvested and subjected to histochemical analysis using Masson’s trichrome staining to identify capillary structures and immunohistochemical analysis using the endothelial cell marker CD31. Matrigel plugs formulated with Ad-dnAMPK showed fewer
capillary structures than control (Fig. 6A). Plugs were predominantly comprised of CD31-positive endothelial cells, and plugs containing Ad-dnAMPK revealed fewer CD31-positive cells. Quantitative analyses of histological sections revealed a significantly lower density of CD31-positive cells in the plugs cast with Ad-dnAMPK (Fig. 6B). Similarly, matrigel plugs cast with Ad-dnAkt showed fewer capillary structures and a lower density of CD31-positive cells.
Discussion

AMPK is widely recognized as a regulator of cellular metabolism in response to hypoxia or vigorous contraction in muscle (8-13). Here, a new regulatory function for AMPK in the vascular endothelium is described. AMPK signaling is induced by oxygen-deprivation in endothelial cells, and this induction is essential for angiogenic cellular responses under these conditions. Inhibition of AMPK signaling through overexpression of a dominant-negative mutant of the α2 subunit inhibited endothelial cell migration toward VEGF and impaired *in vitro* endothelial cell differentiation into tube-like structures in hypoxic cultures. Because migration and differentiation of endothelial cells are critical features of vessel formation, a mouse Matrigel angiogenesis assay was employed to examine the role of endothelial AMPK signaling in capillary formation *in vivo*. In this assay subcutaneous Matrigel serves as a reservoir for viral vectors that transduce endothelial cells as they infiltrate the plug, permitting the assessment of intracellular protein function on capillary formation (39). Dominant-negative AMPK significantly inhibited the vascularization of Matrigel plugs, providing evidence that this signaling step is essential for angiogenesis *in vivo*.

A key finding of this study is that AMPK signaling has little or no effect on endothelial cell migration, tube formation or NO production when cultures are exposed to normoxia. These findings indicate the specificity of the dominant-negative AMPK vector since this reagent only
affects angiogenic cellular responses under cell culture conditions associated with robust AMPK induction. Furthermore, these data show that the basal level of AMPK signaling, detected in normoxic cells, contributes minimally to the angiogenic phenotype. Presumably, these relatively low levels of AMPK function in normoxic cells to regulate fatty acid oxidation and glucose metabolism in the normal endothelium (50). Consistent with this hypothesis, transduction of normoxic endothelial cells with dominant-negative AMPK significantly reduced the phosphorylation of ACC, a regulator of fatty acid metabolism (16,21).

A vector expressing dominant-negative Akt was used as a positive control in this study because Akt signaling is recognized as an essential regulator of NO production (27,28,51), survival (32,52), migration (49,53,54), and differentiation (37,55) in normoxic endothelial cells. However, the role of Akt signaling in hypoxic endothelial cells has not been examined previously. Thus, a secondary finding of this study is that Akt signaling is essential for angiogenic cellular responses in hypoxic endothelial cells. Hypoxia produced a modest time-dependent reduction in Akt phosphorylation at Ser 473. Despite this reduction in Akt activation, signaling through this signaling step was essential for VEGF-mediated endothelial cell differentiation, migration and NO output under conditions of low oxygen tension. Transduction with dominant-negative Akt also inhibited capillary formation in Matrigel plugs implanted in mice. While a number of studies have shown that constitutive activation of Akt signaling is sufficient to promote
angiogenesis in vivo (37,56,57), the data presented here provide the first direct evidence showing
that Akt signaling is essential for neovascularization in an animal model.

Several lines of evidence suggest that crosstalk between AMPK and Akt signaling is an
integral component of the pro-angiogenic cellular response to hypoxic stress (Fig. 7). It was
found that inhibition of AMPK signaling suppresses the activating phosphorylation of Akt at Ser
473 in hypoxic endothelial cells. The observation that the inhibition of endothelial cell migration
and differentiation by dominant-negative AMPK can be reversed by co-transduction with
constitutively-active Akt is also consistent with an AMPK-Akt crosstalk mechanism. AMPK-Akt
crosstalk may also participate in the regulation of eNOS activation by phosphorylation, because
transduction with either dominant-negative Akt or dominant-negative AMPK effectively
suppressed eNOS Ser1177 phosphorylation and NO output from hypoxic endothelial cells.
Collectively, these data suggest that eNOS regulation is complex in hypoxic endothelial cells and
depends upon the relative phosphorylation status of eNOS at Ser1177 and Thr-495, the relative
activities of Akt and AMPK, and the length of time that cells are exposed to low oxygen tension
which causes relatively slow changes in the activities of both of these signaling steps. Under the
conditions of our assays, the direct phosphorylation of eNOS by AMPK appeared to contribute
minimally to eNOS phosphorylation or endothelial NO output (Fig. 7). However, direct
AMPK-mediated eNOS phosphorylation at Ser1177 may become significant when cultures are
incubated under conditions of prolonged hypoxia or when peroxynitrite is produced during ischemia followed by reperfusion (58). Therefore, further analyses of AMPK and Akt signaling, and potential crosstalk between the molecules, may shed additional light on the process that controls angiogenesis in ischemic tissues.

The maintenance of NO output under hypoxic conditions may contribute partially to the pro-angiogenic activity of AMPK. Endothelium-derived NO is a positive modulator of angiogenesis (42-46), although the mechanism by which NO exerts this effect is unknown. In the study reported here, dominant-negative AMPK diminished eNOS phosphorylation on Ser 1177 and NO output under conditions of low oxygen tension, whereas AMPK-inhibition had little if any effect on these parameters in normoxic cultures. Furthermore, the inclusion of an NO donor to the media partially reversed the negative effects of dominant-negative AMPK on endothelial cell differentiation and migration to VEGF in hypoxic cultures. However, co-transduction with constitutively-active Akt was more effective than the NO donor at reversing the inhibitory effects of dominant-negative AMPK. These data suggest that while NO has a stimulatory effect on angiogenic cellular processes, AMPK signaling can regulate angiogenesis independent of NO production (Fig. 7). Similar conclusions have recently been drawn from analyses of the NO-independent regulation of angiogenesis by glycogen synthase kinase-3β (39).
In summary, we report that the activation of a pro-angiogenic phenotype in endothelial cells exposed to hypoxia is a new stress-related function for AMPK signaling. The finding that dominant-negative AMPK inhibited the vascularization of implanted Matrigel plugs suggests the physiological importance of AMPK in angiogenesis in vivo. Stimuli that may activate endothelial AMPK in vivo include decreases in oxygen tension that are associated with tumor growth or occlusive vessel diseases (29,59). In contrast, angiogenesis associated with normal tissue growth and organ enlargement during development can be subject to regulatory controls that function independently of tissue oxygen gradients (56,60). Collectively, these data suggest endothelial AMPK signaling may be an essential feature of the angiogenic response to ischemic stress, while having minimal effects on angiogenic endothelial cell responses during development or normal post-natal tissue growth. Therefore, endothelial AMPK may represent a pharmacological target for the selective inhibition of angiogenesis associated with pathological tissue growth.
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Figure Legends

Figure 1. Regulation of AMPK phosphorylation by hypoxic and dominant-negative AMPK in endothelial cells.

A) Western blot analyses were performed on seventy-five µg protein in each lane. Hypoxia was induced for the indicated lengths of time and AMPK phosphorylation at Thr 172 (p-AMPK) and ACC phosphorylation of Ser 79 (p-ACC) was assessed. Parallel cultures were transduced with an adenoviral vector expressing dominant-negative AMPK tagged with c-Myc (dominant-negative AMPK), two days prior to the initiation of hypoxia. Control cultures were transduced with an adenoviral vector expressing GFP under identical conditions. Representative blots of three to four experiments each are shown. B) Phosphorylation levels of ACC in the presence (dashed lines, empty symbols) or absence (solid line, filled symbols) of dominant-negative AMPK were quantified using NIH image program. Immunoblots were normalized to total loaded protein. *, P<0.01; #, P<0.05 vs. control.

Figure 2. AMPK-mediated regulation of eNOS Ser1177 phosphorylation in hypoxic endothelial cells.

Relative levels of eNOS phosphorylation at Ser1177 and Thr495 and ACC phosphorylation were assessed by Western blot analyses. Parallel cultures were maintained under normoxic conditions
or exposed to hypoxia for 6 hours. Some cultures were exposed to 50 ng/ml VEGF for 6 hours
prior to lysate preparation. Cultures were also transduced with adenoviral vectors expressing
dominant-negative AMPK (dominant-negative AMPK), dominant-negative Akt (dominant-negative Akt), constitutively-active Akt (caAkt) or GFP (control). A) Western
immunoblots were performed by loading 100 µg of lysate in each lane. Expression from the
AMPK and Akt adenoviral vectors was checked by anti-c-Myc and anti-HA immunoblot,
respectively. B) Relative phosphorylation levels of eNOS Ser1177 in the presence or absence of
VEGF, dominant-negative AMPK or dominant-negative Akt were quantified using NIH image
program. Immunoblots were normalized to total loaded protein. C) Relative phosphorylation
levels of eNOS Thr495 *, P<0.01 relative to VEGF alone.

Figure 3. AMPK-mediated regulation of NO production in hypoxic endothelial cells.

Intracellular cGMP was measured to evaluate the regulation of VEGF-induced endothelial NO
production by AMPK and Akt under normoxic or hypoxic conditions. Parallel cultures were
maintained under conditions of normoxia or exposed to hypoxia for 6 hours in the absence of
VEGF (crosshatch). Other cultures were stimulated with VEGF (50 ng/ml) in the presence (dots)
or absence (solid) of the eNOS inhibitor L-NAME. VEGF-stimulated cultures were transduced
with dominant-negative Akt (dnAkt, open) or dominant-negative AMPK (dnAMPK, hatch) two
days prior to the experiment. Control, VEGF alone and VEGF + L-NAME cultures were
transduced with an adenoviral vector expressing GFP. Four independent experiment were
performed. *, P<0.01 vs. control.

Figure 4. AMPK-mediated regulation of endothelial cell migration to VEGF under hypoxic
and normoxic conditions

Cell migration was evaluated with a Boyden chamber assay. Parallel HUVEC cultures were
evaluated for migration toward VEGF under normoxic and hypoxic conditions. Cultures were
transduced with dominant-negative Akt (dominant-negative Akt), constitutively-active Akt
(caAkt), dominant-negative AMPK (dominant-negative AMPK) or GFP (control) two days prior
to the experiment. Sodium nitroprusside (SNP, 10 µM) was added to some cultures. Quantitative
analysis of migration under the different experimental conditions were performed by counting
cells on membranes that were stained with Giemsa solution. Each experiment was performed in
duplicate, and four independent experiments were performed. Cells were counted in four
randomly-selected microscopic field from each membrane. Results are shown as the mean ±
SE. Results are expressed relative to the values compared to the normoxic control condition.
Solid, control; open, dominant-negative Akt; hatch, dominant-negative AMPK; crosshatch,
dominant-negative AMPK plus 10 µM SNP; dots, dominant-negative AMPK plus caAkt. *,
P<0.0001 vs. control; #, P<0.01 vs. dominant-negative AMPK alone.
Figure 5. AMPK-mediated regulation of endothelial cell differentiation into capillary structures \textit{in vitro} under normoxic and hypoxic conditions

Parallel HUVEC cultures were evaluated for differentiation under normoxic and hypoxic conditions. Cultures were transduced with dominant-negative Akt (dominant-negative Akt), constitutively-active Akt (caAkt), dominant-negative AMPK (dominant-negative AMPK) or GFP (control) two days prior to plating on Matrigel-coated culture dishes. Sodium nitroprusside (SNP, 10 µM) was added to some cultures. A) Representative cultures for some experimental conditions are shown. B) Quantitative analysis of tube formation under the different experimental conditions using NIH image software. Four independent experiments were performed. Results are show as the mean ± SE. Results are expressed relative to the values compared to the normoxic control condition. Solid, control; open, dominant-negative Akt; hatch, dominant-negative AMPK; crosshatch, dominant-negative AMPK plus 10 µM SNP; dots, dominant-negative AMPK plus caAkt. *, P<0.0001 vs. control; #, P<0.01 vs. dominant-negative AMPK alone.

Figure 6. AMPK-mediated regulation of angiogenesis \textit{in vivo}.

An \textit{in vivo} Matrigel plug assay was performed to evaluate the role of AMPK and Akt signaling
in angiogenesis. Matrigel plugs containing bFGF and adenoviral vectors expression
dominant-negative AMPK (dominant-negative AMPK), dominant-negative Akt or GFP (control)
were injected subcutaneously into mice. A) After sacrifice, plugs were excised and stained with
Masson’s trichrome, to reveal cellularity, and the endothelial cell marker CD31. B) The
frequency of CD31-positive cells in five low power fields (lpf) was determined for each Matrigel
plug. This experiment was repeated four times. Results are expressed as the mean ± SE. *,
P<0.01.

**Figure 7. Proposed scheme for endothelial AMPK signaling in the control of angiogenesis.**

Hypoxia activates AMPK which, in turn, promotes mitogen-stimulated Akt activation and
angiogenesis. Akt can be the predominant kinase involved in eNOS phosphorylation at Ser1177
in hypoxic endothelial cells. However, direct eNOS phosphorylation by AMPK may become
appreciable under conditions of prolonged hypoxia or peroxynitrite production (58).
Figure 1 (Nagata)
Figure 2 (Nagata)

(A) Western blot analysis of p-eNOS (Ser1177), p-eNOS (Thr495), total eNOS, p-ACC (Ser79), cMyc, and HA under normoxic and hypoxic conditions with or without dnAMPK and dnAkt.

(B) Graph showing relative phosphorylation (%) of Ser1177 in response to VEGF treatment with or without dnAMPK and dnAkt under normoxic and hypoxic conditions.

(C) Graph showing relative phosphorylation (%) of Thr495 in response to VEGF treatment with or without dnAMPK and dnAkt under normoxic and hypoxic conditions.
Figure 5 (Nagata)
Figure 6 (Nagata)
Hypoxia → AMPK → eNOS → NO → Akt → Angiogenesis → VEGF

Figure 7 (Nagata)
AMP-activated protein kinase (AMPK) signaling in endothelial cells is essential for angiogenesis in response to hypoxic stress
Daisuke Nagata, Masaki Mogi and Kenneth Walsh

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