Impaired Expression of Uncoupling Protein 2 Causes Defective Postischemic Angiogenesis in Mice Deficient in AMP-Activated Protein Kinase α Subunits

Ming-Jiang Xu, Ping Song, Najeeb Shirwany, Bin Liang, Junjie Xing, Benoit Viollet, Xian Wang, Yi Zhu, Ming-Hui Zou

Objective—The aim of the present study was to determine whether mitochondrial uncoupling protein (UCP) 2 is required for AMPK-dependent angiogenesis in ischemia in vivo.

Methods and Results—Angiogenesis was assayed by monitoring endothelial tube formation (a surrogate for angiogenesis) in human umbilical vein endothelial cells (ECs), isolated mouse aortic endothelial cells (MAECs), and pulmonary microvascular endothelial cells or in ischemic thigh adductor muscles from wild-type (WT) mice or mice deficient in either AMPKα1 or AMPKα2. AMPK inhibition with pharmacological inhibitor (compound C) or genetic means (transfection of AMPKα-specific small interfering RNA) significantly lowered the tube formation in human umbilical vein ECs. Consistently, compared with WT mice, tube formation in MAECs isolated from either AMPKα1Δ/− or AMPKα2Δ/− mice, which exhibited oxidative stress and reduced expression of UCP2, was significantly impaired. In addition, adenoviral overexpression of UCP2, but not adenoviruses encoding green fluorescent protein, normalized tube formation in MAECs from either AMPKα1Δ/− or AMPKα2Δ/− mice. Similarly, supplementation with sodium nitroprusside, a nitric oxide (NO) donor, restored tube formation. Furthermore, ischemia significantly increased angiogenesis, serine 1177 phosphorylation of endothelial NO synthase, and UCP2 in ischemic thigh adductor muscles from WT mice but not in those from either AMPKα1Δ/− or AMPKα2Δ/− mice.

Conclusion—We conclude that AMPK-dependent UCP2 expression in ECs promotes angiogenesis in vivo. (Arterioscler Thromb Vasc Biol. 2011;31:1757-1765.)

Key Words: angiogenesis ■ free radicals/free-radical scavengers ■ nitric oxide ■ AMPK

AMP-activated protein kinase (AMPK), an evolutionarily conserved serine/threonine protein kinase, is considered a major metabolic regulator at both the cellular and whole-body levels. AMPK is activated in response to physiological processes that deplete ATP (thereby increasing the AMP:ATP ratio), such as exercise and hypoxia. AMPK is ubiquitously expressed in every cell type in the vascular wall. In vascular endothelial cells (ECs), the AMPKα1β1γ1 heterotrimer is the dominant isoform expressed, whereas AMPKα2 is a minor form.1 However, the AMPKα subunit is essential, and a dual deficiency in AMPKα1 and AMPKα2 causes embryonic lethality in mice.2

Angiogenesis is a central feature of normal embryonic and postnatal development, and this process plays a critical role in the neovascularization that is associated with tumor growth, wound healing, and occlusive vascular diseases.3–5 Angiogenesis is dependent on cell proliferation, migration, and capillary tube formation in ECs.6–8 The most important proangiogenic factor is nitric oxide (NO), and in endothelial NO synthase (eNOS) knockout mice, the capacity for angiogenesis is significantly impaired.9 Increasing evidence suggests that NO is required for vascular endothelial growth factor–induced angiogenesis.10,11 In addition, NO has been shown to assist in the proliferation and migration of ECs during angiogenesis.12,13

Mitochondrial uncoupling proteins (UCP) are mitochondrial transporters that are present in the inner mitochondrial membrane and belong to a family of mitochondrial anion carriers, which includes adenine nucleotide transporters.14 Mild uncoupling of respiration has been reported to diminish mitochondrial reactive oxygen species (ROS) formation.14 Recent evidence implies that the basic role of all UCPs is to prevent oxidative tissue injury by reducing oxidative stress.14 A role for UCP2 in the downregulation of mitochondrial ROS

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production is plausible, because available evidence suggests that this protein is expressed in numerous mammalian tissues.14 For example, we have recently demonstrated that upregulation of UCP2 by AMPK activation in ECs attenuates oxidative stress in diabetes.15

NO bioactivity is determined by both the synthesis of NO by nitric oxide synthases (NOS) and its inactivation by ROS.16 Specifically, superoxide anions (O2•−) not only reduce the bioavailability of NO, but also inhibit its main target, soluble guanylyl cyclase.17,18 Increasing evidence suggests that AMPK is important in maintaining NO bioactivity. Both AMPKα1 and AMPKα2 have been reported to increase NO release by phosphorylating eNOS at Ser1177 and Ser635.1,2,19–21 In addition, recent data from our group indicate that AMPK functions as a sensor for redox status within a cell and AMPK activation suppresses oxidative stress in endothelial function.22

AMPK has been demonstrated to be required for ischemic angiogenesis.23 However, the molecular mechanisms by which AMPK promotes angiogenesis remains poorly elucidated. Thus, the aim of the present study was to establish the molecular signaling pathways by which AMPK promotes vascular angiogenesis in response to hindlimb hypoxia/ischemia in vivo.

Experimental Procedures

Animal Experiments
Male AMPKα1−/− and AMPKα2−/− mice were generated, as previously described.24 Their genetic controls (C57BL/6 wild-type [WT] mice) were obtained from the Jackson Laboratory (Bar Harbor, ME) and were 10 weeks of age, with a weight of 20 to 25 g. Mice were housed in temperature-controlled cages under a 12-hour light-dark cycle and given free access to water and normal chow. Unilateral hindlimb ischemia was induced by resecting the left femoral arteries and veins of WT mice, AMPKα1−/−, and AMPKα2−/− mice under anesthesia with sodium pentobarbital (50 mg/kg, intraperitoneally), as described previously.25 Briefly, animals were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), and unilateral hindlimb ischemia was induced by resecting the left femoral arteries and veins of WT mice, AMPKα1−/−, and AMPKα2−/− mice. To ensure ischemia efficiency, the proximal portion of femoral artery and vein, and the distal portion of their major branches were ligated, followed by resection of the sections between ligations, and ischemia was confirmed by cyanosis in the distal hindlimb. When hindlimb ischemia was induced, new blood vessels grew into the ischemic limb. After recovery for 2 weeks, animals were euthanized for excision of muscle samples.

Capillary Density Assay
Arteriolar and capillary densities within the ischemic thigh adductor skeletal muscles were analyzed to obtain specific evidence of vascularity at the microcirculation level, similar to a previously described method.25 From each animal, 3 pieces of ischemic muscles were harvested, sliced, and then fixed in 4% formaldehyde. Next, tissues were embedded in paraffin, and multiple 5-mm-thick tissue slices were prepared. Capillary ECs were identified by immunohistochemical staining with anti-mouse von Willebrand factor antibody (Abcam, Boston, MA), and arteriolar smooth muscle cells were identified by an antibody against vascular smooth muscle α-actin. Fifteen random microscopic fields from 3 different sections in each tissue block were examined for the presence of capillary or arteriolar ECs and smooth muscle cells. Capillary density was expressed as the number of capillaries per high-power field (×400).

Cell Culture
Human umbilical vein ECs (HUVECs) were grown in EC basal medium supplemented with EGM SingleQuots from Lonza (Walkersville, MD), penicillin (100 U/mL), and streptomycin (100 μg/mL). In all experiments, cells were between passages 3 and 8. All cells were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Cells were grown to 70% to 80% confluence before being treated with different agents.

A primary mouse aortic EC (MAEC) culture was established according to previously published methods from our group.26 Pulmonary microvascular ECs were isolated as described by Lips et al,27 with Dynabeads sheep anti-rat IgG.

Tube Formation Assay
The formation of vessel-like structures by HUVECs on growth factor-reduced Matrigel (BD Biosciences) was performed as previously described.28 Briefly, 35-mm culture dishes were coated with Matrigel according to the manufacturer’s instructions. The indicated ECs were seeded on coated dishes at 3×105 cells/dish in EC basal medium containing vascular endothelial growth factor (50 ng/mL) and incubated at 37°C for 8 hours under normoxic or hypoxic conditions. In some dishes, sodium nitroprusside (SNP; 1 mmol/L) was also added as indicated. Tube formation was observed using an inverted phase contrast microscope (Nikon, Tokyo, Japan). Images were captured with a videographic system (DEI-750 CE Digital Output camera; Optronics, Goleta, CA). The degree of tube formation was quantified by measuring the number of tubes in 30 randomly chosen low-power fields (×40) from each dish using the National Institutes of Health Image program. Each experiment was repeated 3 times.

Transfection of Small Interfering RNA Into ECs
Transient transfection of small interfering RNA (siRNA) was performed according to the manufacturer’s protocol (Santa Cruz Biotechnology, Santa Cruz, CA). Briefly, siRNAs were dissolved in siRNA buffer (20 mmol/L KCl, 6 mmol/L HEPES [pH 7.5], 0.2 mmol/L MgCl2) to prepare a 10 μM stock solution. HUVECs grown in 6-well plates were transfected with siRNA in transfection medium ( Gibco/Invitrogen) containing liposomal transfection reagent (Lipofectamine RNAmax, Invitrogen). For each transfection, 100 μL of transfection medium containing 4 μL of siRNA stock solution was gently mixed with 100 μL of transfection medium containing 4 μL of transfection reagents. After a 30-minute incubation at room temperature, siRNA-lipid complexes were added to the cells in 1.0 mL of transfection medium, and then cells were incubated with this mixture for 6 hours at 37°C. The final concentration of control or
AMPK-specific siRNA was 40 nmol/L. The transfection medium was then replaced with normal medium, and cells were cultured for 24 hours.29

**Adenoviral Infection**

Adenovirus encoding green fluorescent protein (Ad-GFP), a replication-defective adenoviral vector expressing GFP, served as control. The Ad-UCP2 adenoviral vector expresses the full-length UCP2 gene. MAECs were infected with Ad-GFP and Ad-UCP2 overnight in medium supplemented with 2% fetal bovine serum. Cells were then washed and incubated in fresh medium for an additional 12 hours before experimentation. These conditions typically produced an infection efficiency of >80%, as determined by GFP expression.

**Western Blot Analysis**

Cell lysates were subjected to Western blot analysis. The protein content was assayed using the BCA protein assay reagent (Pierce, Rockford, IL). Proteins were subjected to SDS-PAGE and then transferred to membranes. Membranes were incubated with a 1:1000 dilution of primary antibody, followed by a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by enhanced chemiluminescence (GE Healthcare, Barrington, IN).

**Assays of AMPK Activity**

AMPK activity was measured in immunoprecipitates from 200 μg of cell lysate protein using antibodies bound to protein A/G-Sepharose, as described previously.30,31 Briefly, immunocomplexes were collected by centrifugation at 8000g for 1 minute. After being washed extensively with immunoprecipitation buffer, the immunoprecipitates were divided equally for further assays. AMPK activity assays were performed at 30°C in a total volume of 50 μL containing Na-HEPES (40 mmol/L), pH 7.4, NaCl (80 mmol/L), dithiothreitol (1 mmol/L), and SAMs peptide (HMRSAMSGLHLVKRR, 200 μmol/L).30 Variable concentrations of 5'-AMP (0 or 200 μmol/L), 32P-ATP (200 μmol/L), and magnesium acetate (5 mmol/L) were used. Reactions were initiated by the addition of 32P-ATP. For each analysis, blanks were included without the immunoprecipitates and peptide. At the conclusion of the assay (10 minutes), an aliquot of the reaction mixture was spotted on a 1×1-cm square of P81 paper (Whatman), followed by immersion in a perforated plastic beaker inside a glass beaker containing ice-cold phosphoric acid (150 mmol/L).

**Immunohistochemistry**

The thigh adductor skeletal muscles were fixed in 4% paraformaldehyde overnight and were then processed, embedded in paraffin, and sectioned as 5-μm slices. The deparaffinized, rehydrated sections were microwaved in citrate buffer for antigen retrieval. Sections were incubated in endogenous peroxidase (Dako) and protein block buffer, and then with primary antibodies overnight at 4°C. Slides were rinsed with washing buffer and incubated with labeled polymer-horseradish peroxidase-anti-mouse or rabbit antibodies followed by DAB+ chromogen detection (Dako). After final washes, sections were counterstained with hematoxylin. All positive staining was confirmed by ensuring that no staining occurred under the same conditions using nonimmune rabbit or mouse control IgG. Semiquantitative analysis of tissue immunoreactivity was performed by 4 observers blinded to the identity of the samples using an arbitrary grading system to estimate the degree of positive staining for each individual marker.

**Results**

**Functional AMPK Is Essential for Tube Formation In HUVECs In Vitro**

To demonstrate that vascular tube formation is dependent on a functional AMPK signaling system, we evaluated the contribution of AMPK in the formation of vessel-like tubes (EC tubes) in cultured HUVECs. HUVECs were cultured on Matrigel-coated plates with or without compound C (10 μmol/L), a potent inhibitor of AMPK. As depicted in Figure 1A, compound C significantly suppressed tube formation in HUVECs, suggesting that AMPK might be important for tube formation in HUVECs.

To exclude potential off-target effects of compound C, HUVECs were transfected with control siRNA, AMPKα-specific siRNA, AMPKα1-, or AMPKα2-specific siRNA. As depicted in Figure 1B, compared with cells transfected with control siRNA, transfection of nonselective AMPKα siRNA suppressed tube formation in HUVECs. Transfection of AMPKα1- or AMPKα2-specific siRNA consistently attenuated tube formation, but to a lesser degree than nonselective siRNA (Figure 1B). Together, our results suggest that AMPKα is required for optimal tube formation.

We further evaluated the effects of compound C and AMPK siRNA on AMPK activity. As shown in Figure 1C, transfection of AMPKα-specific siRNA in HUVECs caused a reduction of ~70% of AMPK, whereas compound C inhibited AMPK activity by more than 90%. The greater reduction of AMPK activity by compound C compared with AMPKα-specific siRNA might help explain the greater effects of compound C on tube formation. Interestingly, silencing AMPKα1 caused a greater reduction of AMPK activity compared with the transfection of AMPKα2 siRNA in HUVECs (Figure 1C), indicating that AMPKα1 is a predominant isoform in HUVECs.

**AMPKα1 or α2 Deletion Impairs Tube Formation In ECs In Vitro**

To ensure that the effects of AMPKα deletion we observed in HUVECs were not limited to this cell type, we further tested the contribution of AMPKα1 and α2 in tube formation in cultured MAECs and pulmonary microvascular ECs derived...
AMPKα Deletion in Mice Causes Defective Angiogenesis In Vivo

Next, we determined whether AMPKα was critically involved in the angiogenic response in vivo. To this end, WT mice and mice deficient in either AMPKα1 or AMPKα2 were subjected to hindlimb ischemia for 2 weeks. Ischemic muscle was harvested from the affected area and stained for various immunohistochemical markers on postoperative day 14. Arteriolar and capillary densities were assessed by staining with antibodies against vascular smooth muscle α-actin and von Willebrand factor (vWF), respectively. Vessel density under basal conditions was decreased in tissues obtained from AMPKα1−/− and AMPKα2−/− animals when compared with WT controls (Figure 2B and 2D). As expected, microvessel density exhibited a robust increase in ischemic WT muscles (Figure 2B and 2D). By contrast, only a modest increase was found in AMPKα1−/− or AMPKα2−/− mice (Figure 2B and 2D), implying that

from AMPKα1−/−, AMPKα2−/−, and WT control animals. As depicted in Figure 1D and 1E, tube formation was also dramatically reduced in MAECs and pulmonary microvascular ECs cultured from AMPKα1- and AMPKα2-deficient animals compared with WT controls.

**AMPKα Deletion in Mice Causes Defective Angiogenesis In Vivo**

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Figure 1. AMPKα subunit deletion suppresses endothelial tube formation in vitro. A, HUVECs on Matrigel-coated plates were incubated with AMPK inhibitor compound C (10 μmol/L) for 3 hours. Number of tubes per visual field (×40) was counted as described in Methods and Materials. B, HUVECs were subjected to siRNA transfection targeting AMPKα1, AMPKα2, or AMPKα2 for 24 hours and then were incubated on Matrigel-coated plates for 3 hours to detect tube formation. C, Assays of AMPK activity in HUVECs treated for 24 hours with compound C (3 hours); siRNA specific for total AMPKα1, AMPKα2, or control siRNA. *P<0.05 vs control siRNA, n=5. D and E, Assays of tube formation in MAECs and pulmonary microvascular ECs (PMECs) from WT, AMPKα1−/−, and AMPKα2−/− mice. *P<0.05 vs WT, #P<0.05 vs AMPKα1−/−, n=3 in each group.

Figure 2. Impaired angiogenesis in AMPKα-deficient mice. A, WT, AMPKα1−/−, and AMPKα2−/− mice were subjected to hindlimb ischemia (IS) for 2 weeks as described in Methods. Three skeletal muscle samples were taken from the ischemic left hindlimb and the sham-operated right hindlimb of each mouse. Three slices of each sample were used for immunohistochemical staining. Arteriole and capillary densities were assessed by staining with antibodies against vascular smooth muscle α-actin (SM-α-actin) or EC marker (vWF), respectively. Representative immunohistochemical staining for SM-α-actin (×400) (C) and quantification for capillary number per visual field (D) are shown. *P<0.05 vs WT/sham group, #P<0.05 vs WT/ischemia group, n=3 (WT) or n=5 (AMPKα1−/− and AMPKα2−/−).
AMPKα was required for the angiogenic response in ischemia.

**AMPKα Deletion in Mice Increases Reactive Nitrogen Species Stress in ECs Postischemia**

Increasing evidence suggests that hypoxia increases superoxide anions (O$_2^-$) and peroxynitrite (ONOO$^-$) on its reaction with NO.$^{32}$ ONOO$^-$ can nitrate protein tyrosine residues, forming 3-nitrotyrosine (3-NT).$^{33}$ To assess reactive nitrogen species stress in ECs following ischemia, we analyzed 3-NT immunostaining in ischemic and sham-treated tissues in WT, AMPKα1$^{-/-}$, and AMPKα2$^{-/-}$ mice. As expected, ischemia significantly increased 3-NT staining in WT mice. Compared with WT mice, the levels of 3-NT staining were significantly higher in ischemic tissues from both AMPKα1$^{-/-}$ and AMPKα2$^{-/-}$ mice (Figure 3A).

To further confirm the increase in 3-NT-positive proteins in ischemic tissues, we first immunoprecipitated 3-NT-positive proteins from tissues of WT, AMPKα1$^{-/-}$, and AMPKα2$^{-/-}$ mice, followed by Western blot detection of 3-NT-positive protein. As shown in Figure 3B and 3C, the levels of 3-NT proteins were markedly elevated in tissues from either AMPKα1$^{-/-}$ mice or AMPKα2$^{-/-}$ mice when compared with those from WT. As expected, ischemia significantly increased the levels of 3-NT-positive proteins in tissues from AMPKα1$^{-/-}$ and AMPKα2$^{-/-}$ mice (Figure 3B and 3C). Interestingly, ischemia caused a greater increase in 3-NT-positive proteins in tissues from AMPKα1$^{-/-}$ and AMPK α2$^{-/-}$ mice than those from WT mice, suggesting that AMPKα deficiency might enhance the formation of reactive oxygen and nitrogen species in response to ischemic insults.
4-Hydroxynonenal (4-HNE), a lipid oxidation product, is a well-characterized marker for oxidative stress in tissues.\(^3\) Ischemia increases the formation of 4-HNE-modified proteins in tissues.\(^3\) Therefore, we next determined 4-HNE-modified proteins in ischemia- and sham-treated tissues in WT, AMPK\(^{-/-}\), and AMPK\(^{2/-}\) mice. As depicted in Figure 3D, there was no marked difference among sham-treated tissues in WT, AMPK\(^{-/-}\), and AMPK\(^{2/-}\) mice. As expected, ischemia markedly increased 4-HNE-modified protein staining in WT. A greater increase of 4-HNE-modified proteins were observed in tissues from ischemic AMPK\(^{-/-}\) mice compared with ischemic WT (Figure 3D). In addition, 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress to DNA,\(^3\) was dramatically increased in the tissues from AMPK\(^{1/-}\) and AMPK\(^{2/-}\) mice either sham or ischemia treated compared with WT mice (Figure 3E).

**UCP2 Levels Are Lower in AMPK\(^{1/-}\) or AMPK\(^{2/-}\) Mice Postischemia**

UCP2 has been reported to reduce mitochondrial ROS in several cell types, including ECs, as well as in various organs.\(^3\)–\(^4\) It was interesting to determine whether AMPK deficiency altered UCP2 expression under ischemic conditions. Under normoxia, UCP2 expression was significantly lower in tissues from AMPK\(^{1/-}\) and AMPK\(^{2/-}\) mice compared with those from WT mice (Figure 4A and 4B). As expected, ischemia markedly upregulated the UCP2 levels in WT mice. However, ischemia did not significantly alter the UCP2 levels in tissues from either AMPK\(^{1/-}\) or AMPK\(^{2/-}\) mice (Figure 4A and 4B).

Because the amount of ECs was not sufficient for Western blot analysis of UCP2 protein levels in ischemic tissues, we also probed for UCP2 in subcultured MAECs from WT, AMPK\(^{1/-}\), and AMPK\(^{2/-}\) mice by Western blot analysis of UCP2 protein levels in ischemic tissues, we confirmed that it was significantly suppressed in both AMPK\(^{1/-}\) and AMPK\(^{2/-}\) mice compared with WT mice (Figure 4C).

We next determined the phosphorylation (p) of eNOS at Ser1177 (p-eNOS) in WT, AMPK\(^{1/-}\), and AMPK\(^{2/-}\) mice. Compared with WT, the basal levels of p-eNOS in either AMPK\(^{1/-}\) or AMPK\(^{2/-}\) mice were significantly reduced (Figure 4D and 4E). As expected, ischemia markedly increased the levels of p-eNOS in WT mice (Figure 4D and 4E). Importantly, ischemia-enhanced p-eNOS in either AMPK\(^{1/-}\) or AMPK\(^{2/-}\) mice was markedly attenuated compared with that in ischemic WT mice (Figure 4D and 4E).

**Effects of UCP2 Gene Silencing on the Phosphorylation of Both AMPK and eNOS in HUVECs**

We next detected whether endogenous UCP2 altered the phosphorylation of both AMPK and eNOS. As depicted in Figure 5A, the transfection of UCP2-specific siRNA but not control siRNA reduced the levels of UCP2 by \(\approx 60\%\) in HUVECs. In line with an earlier report, gene silencing of UCP2 had no effect on either p-AMPK or p-eNOS (Figure 5B). As expected, adenoviral overexpression of UCP2 but not Ad-GFP slightly increased the phosphorylation of both p-AMPK and p-eNOS (Figure 5B).

It was interesting to determine whether UCP2 knockdown altered 5-aminomidazole-4-carboxamide ribonucleoside-induced phosphorylation of eNOS and Akt. As expected, 5-aminomidazole-4-carboxamide ribonucleoside markedly increased the levels of p-AMPK, p-eNOS, and UCP2 in HUVECs (Figure 5C and 5D). Interestingly, neither UCP2 siRNA nor control siRNA altered 5-aminomidazole-4-carboxamide ribonucleoside-enhanced phosphorylation of
AMPK, eNOS, and Akt (Figure 5C and 5D). Taken together, these results suggest that AMPK is an upstream kinase for eNOS and UCP2.

Inhibition of UCP2 or eNOS Attenuates the Tube Formation in HUVECs

We reasoned that UCP2 would be critically involved in tube formation under ischemic conditions by increasing NO bioactivity in ECs. To this end, we examined tube formation in HUVECs transfected with UCP2 siRNA or with the NOS inhibitor L-NG-nitro-L-arginine methyl ester. As shown in Figure 5E, UCP2 siRNA inhibited HUVECs tube formation under either normoxia or hypoxia condition. Inhibition of NOS also dramatically abrogated tube formation in HUVECs (Figure 5F).

Supplementation of UCP2 or Exogenous NO Normalizes the Tube Formation in MAECs From Mice Deficient in AMPKα1 or AMPKα2

We determined whether supplementation of UCP2 or exogenous NO restored defective tube formation in MAECs from AMPKα1−/− and AMPKα2−/−. Consistent with our earlier report, UCP2 was markedly reduced in MAECs from AMPKα1−/− and AMPKα2−/− compared with those from WT (Figure 6A). As expected, adenoviral overexpression of UCP2 markedly increased the levels of UCP2 in MAECs from AMPKα1−/− and AMPKα2−/− compared with those from WT (Figure 6A). More compellingly, when MAECs derived from AMPKα1−/− and AMPKα2−/− mice were infected with either adenoviral overexpression of UCP2 or treated with the NO donor SNP, robust recovery of the tube forming response was observed (Figure 6B and 6C). These findings strongly suggest that NO and UCP2 are both critically required by ECs to mount a normal angiogenic response.

Discussion

The present study has demonstrated that intact AMPK signaling is critical in the angiogenic response to ischemia in vitro as well as in vivo. Most importantly, we have provided convincing evidence that implicates UCP2 in mitochondrial uncoupling under ischemic conditions resulting in the suppression of ROS. Mitigation of oxidative stress by UCP2 in turn increases NO bioavailability and this ultimately integrates endothelial tube formation and microvascular angiogenesis in ischemia. Our novel observations might help explain how hypoxia/ischemia, via ROS suppression by AMPK and UCP2, underlies the angiogenic response in vivo (Figure 6D).

The most important finding of the present study is AMPK-regulated UCP2 expression in hypoxic angiogenesis. In our experiments, we found that in AMPKα1−/− and AMPKα2−/− mice, UCP2 expression was markedly reduced compared with that in WT control mice. In addition, when HUVECs were transfected with siRNA specific to UCP2, they exhibited significant abrogation of endothelial tube formation. When these cells were treated with an NOS inhibitor, tube formation was also suppressed significantly. These findings were validated in MAECs derived from AMPKα1−/− and AMPKα2−/− mice that were treated with SNP or adenoviral overexpression of UCP2. Both interven-
tions partly but significantly mitigated the tube formation defect. Our findings likely represent an early report of a possible physiological role of UCP2, which is usually classified as an atypical or novel UCP, in part because its exact function is uncertain. Our findings also appear to be consistent with previous reports suggesting a critical role for AMPK in compensatory new vessel formation when tissues become ischemic/hypoxic.

Another important finding of this study is that UCP2 increases angiogenesis by reducing reactive oxygen and nitrogen species in vivo. Hypothetically, UCP2 can lead to more rapid electron flux through the respiratory chain, thereby dampening mitochondrial ROS synthesis. Down-regulation of mitochondrial ROS production may be the most plausible role for UCP2, because its expression has been confirmed in numerous mammalian tissues. The ability of UCP2 to reduce ROS not only in mitochondria but within the cytosol or even in the extracellular space has also been documented. Duval et al have recently shown that UCP2-mediated uncoupling in ECs may decrease extracellular ROS in coinubated low-density lipoproteins. Furthermore, mice with deleted low-density lipoprotein receptors exhibit extensive diet-induced atherosclerotic plaques when they receive bone marrow transplants from UCP2−/− mice, and appearance of these plaques is prevented when they receive bone marrow transplants from UCP2+/+ mice, suggesting that the basic role of all UCP2 is to reduce oxidant stress to avoid oxidative tissue injury. In a previously published report from our laboratory, we have laid a foundation for the mechanism we are proposing in the present study. In that study, our group demonstrated that when HUVECs or mice are treated with 5-aminoimidazole-4-carboxamide ribonucleoside (a potent activator of AMPK), UCP2 expression is upregulated and oxidative stress is attenuated. Overall, our results, together with previous reports, suggest that hypoxia/ischemia can activate AMPK. In turn, AMPK can suppress ROS synthesis via mitochondrial uncoupling mediated through UCP2. The significant attenuation of ROS that ensues can then enhance the bioavailability of NO. NO, through its stimulatory impact in vascular endothelial growth factor, as well as its role in promoting endothelial proliferation and migration, can then promote new vessel formation.10,11

In summary, the present study proposes a role for UCP2 in the tissue response of angiogenesis to ischemic stress. Specifically, when ischemia is induced in tissues, AMPK is activated via multiple stimuli, such as increased AMP/ATP ratios, vascular endothelial growth factor, NO, and the formation of ROS or reactive nitrogen species, such as H2O2 and ONOO−. AMPK activation appears to enhance the expression of UCP2, which then blocks ROS synthesis by the mitochondrial respiratory chain. ROS suppression serves to maintain high levels of NO bioavailability ultimately necessary for a normal angiogenic response to ischemia or hypoxia (Figure 6D). Further delineation of these proposed mechanisms will be necessary before a complete understanding of this process is achieved.

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Disclosures
None.

References
AMPK, UCP2, and Ischemic Angiogenesis

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