AMP-Activated Protein Kinase α1 in Macrophages Promotes Collateral Remodeling and Arteriogenesis in Mice In Vivo

Huaiping Zhu, Miao Zhang, Zhaoyu Liu, Junjie Xing, Cate Moriasi, Xiaoyan Dai, Ming-Hui Zou

Objective—AMP-activated protein kinase (AMPK), an energy and redox sensor, is activated in response to various cellular stresses, including hypoxia, nutrient deprivation, oxidative stress, and fluid shear stress at the site of vessel blockade. The activation of AMPK is involved in angiogenesis. However, it is unknown whether AMPK can influence arteriogenesis. Here, we demonstrate the contribution of macrophage AMPK to arteriogenesis and collateral remodeling and their underlying mechanisms in well-characterized in vivo and in vitro models.

Approach and Results—AMPKα1, AMPKα2 knockout and wild-type littermates underwent femoral artery ligation. Collateral arteriogenesis was monitored in wild-type, global AMPKα1 knockout, or macrophage-specific AMPKα1 knockout mice, with or without hindlimb ligation. Compared with wild-type mice with ligation, global AMPKα1 knockout mice displayed significant reduction in blood flow recovery and impaired remodeling of collateral arterioles. Similar impairments were observed in macrophage-specific AMPK α1 knockout mice after hindlimb ligation. Mechanistically, we found that AMPKα1 promotes the production of growth factors, such as transforming growth factor β, by directly phosphorylating the inhibitor of nuclear factor κB kinase alpha, resulting in an nuclear factor κB–dependent production of growth factors

Conclusions—Our findings suggest a novel role for macrophage AMPKα1 in arteriogenesis and collateral remodeling and indicate that AMPKα1 activation might be beneficial for recovery from occlusive vascular disorders.

Key Words: AMP-activated protein kinases ▪ macrophages ▪ mice, knockout ▪ NF-kappa B ▪ transforming growth factor beta

Arteriogenesis is a process of developing collateral circulation through the remodeling and growth of pre-existing collateral arteries after elevated shear stress induced by occlusion.1–3 Arteriogenesis takes place both during embryogenesis and in adult tissues. In the latter case, arteriogenesis, which usually occurs at sites of occlusion or physical disruption of pre-existing arterial conduits such as coronary artery occlusion or femoral artery ligation, plays a vital role in recovery from ischemic insults.4,5 Understanding the biological factors that affect arteriogenesis will aid in the development of new treatments for patients with arterial stenosis and occlusions.

There are 2 commonly considered mechanisms for arteriogenesis: expansion of pre-existing collaterals and de novo arteriogenesis.6,7 Inflammation caused by mechanical hemodynamic forces, such as shear stress and circumferential wall tension, is considered a pivotal trigger and driver for arteriogenesis.8–11 Previous studies have shown that monocytes accumulated in the surrounding tissues of collateral vessels alter arterial occlusion.7,11–13 These macrophages are potent sources of cytokines and growth factors, which are required for natural adaptive arteriogenesis. In spite of a mounting number of putative arteriogenic factors, the exact mechanisms that regulate collateral remodeling are poorly characterized. Furthermore, the processes responsible for arteriogenesis, and its associated molecular signals are poorly understood.

AMP-activated protein kinase (AMPK) is a serine/threonine kinase composed of α, β, and γ subunits.14,15 The α subunit containing the α1 and α2 isomorph is the catalytic subunit, whereas the β and γ are regulatory subunits that maintain the stability of the heterotrimer complex. As an energy sensor, AMPK is activated by various cellular stresses, such as hypoxia, nutrient deprivation, and oxidative stress.16–18 Once activated, AMPK phosphorylates and regulates several downstream kinases that reduce energy demand and increase energy supply to maintain whole-body energy homeostasis.19 In addition, AMPK also regulates many other cellular processes, including cell polarity, cell growth, and proliferation.20–22

Emerging studies have demonstrated that AMPK is activated in response to shear or ischemic stress.23,24 AMPK signaling is required for angiogenesis in vivo and in vitro.25–28 However, there is no information on the consequences of AMPK deletion in arteriogenesis. In this study, we sought to examine the role.

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of monocytes/macrophage AMPK in adult arteriogenesis. We report here that AMPKα1 deletion results in impaired collateral formation, blood flow recovery, and foot movement, as well as necrosis after ligation of the femoral artery.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results

AMPKα1 Regulates Plantar Perfusion Recovery After Hindlimb Ischemia
To investigate the role of AMPKα in arteriogenesis, AMPKα1−/−, AMPKα2−/− mice, and wild-type (WT) littersmates were subjected to hindlimb ischemia brought about by femoral artery ligation, which reduces perfusion to the lower limb, resulting in ischemia in the calf (crural muscle). Blood perfusion in the hind paws (plantar perfusion) was quantified and normalized to sham control to compare various time points (Figure 1A and 1B). The quantitation of perfusion recovery revealed that AMPKα1−/− mice had a greater drop in perfusion than did AMPKα2−/− mice, from 1 to 4 weeks after surgery, P value <0.05 (Figure 1A). However, there was no difference in perfusion recovery among the 3 groups of mice from 0 to 3 days (Figure 1A), implying that AMPKα1 deletion did not affect pre-existing collaterals, and that the impaired recovery in AMPKα1-deleted mice occurred only after 3 days.

AMPKα1 Deletion Impairs Adult Arteriogenesis and Angiogenesis
Next, we measured the diameter of collaterals in adductor muscle by histomorphometry. There was no difference in the baseline (de novo) diameter of collaterals between the 3 genotypes (Figure 1C; Figure IA in the online-only Data Supplement). However, the diameters of AMPKα1−/− collaterals (46.25±2.09 μm) were markedly smaller than those of WT collaterals (78.67±3.82 μm) at 1 week post ligation (Figure 1C; Figure IA in the online-only Data Supplement), indicating that impaired perfusion recovery in the absence of AMPKα1 was likely ascribed to poor arteriogenesis.

During arteriogenesis, collateral vessels undergo extensive remodeling involving thickening of the tunica media, which is composed of α-smooth muscle actin-positive smooth muscle cells (SMCs), and extension of vessel diameter. Numbers and total areas of α-smooth muscle actin-positive collateral vessels were comparable in both genotypes at baseline, but lower in AMPKα1−/− adductors after ischemia, compared with WT (Figure 1D; Figure IB and IC in the online-only Data Supplement). Based on the protocol, significant growth and the characteristic changes of collateral vessels occur at day 7 after artery occlusion. Therefore, we observe arteriogenesis at this time point. Whole-mount visualization of hindlimb arteries by pigment particle perfusion showed that the number of collaterals that grew to large caliber conduction vessels with a corkscrew pattern was significantly reduced (8.2±0.86) in the AMPKα1−/− hindlimb 7 days after femoral artery ligation, compared with WT mice (14.2±1.43; Figure 1E and 1F). Compared with baseline (de novo) collateral vessel, femoral artery ligation induces expansion of pre-existing collateral vessels in both genotype mice (Figure 1C and 1E). The collateral remodeling involves the expansion of pre-existing collaterals and de novo arteriogenesis (Figure 1C and 1F). These data show that the collateral vessels of AMPKα1−/− mice at baseline were similar to those of WT mice, but the capacity for arteriogenesis (expansion of pre-existing collaterals and de novo arteriogenesis) in AMPKα1−/− mice is decreased after femoral artery ligation.

In addition, we also observed capillary growth (angiogenesis) in ischemic calf tissues by endothelial immunofluorescent staining of the cross sections with anti-CD31 antibody (Figure II A in the online-only Data Supplement). Consistent with our earlier study,26 we found that capillary density was lower in calf tissues from AMPKα1−/− mice than in those from AMPKα2−/− or WT mice at day 7 after femoral artery ligation (Figure IIB in the online-only Data Supplement). As expected, AMPKα1 deletion significantly reduced levels of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β (Figure IIC and IID in the online-only Data Supplement). To determine the contributions of endothelial progenitor cells, we detected endothelial progenitor cell numbers by flow cytometry in circulating blood of mice with hindlimb ischemia. Global deletion of AMPK α1 did not alter the number of endothelial progenitor cells (Figure IIE in the online-only Data Supplement). Taken together, these results suggest that macrophage AMPKα1 is important in arteriogenesis.

Because of impaired collateral vessel remodeling and angiogenesis, muscles from ligated limbs in AMPKα1−/− mice exhibited more extensive areas of necrosis than those in control mice at day 7 after surgery (Figure IIIA and IIIB in the online-only Data Supplement). By using a scoring system based on active foot movements, the functional outcomes of impaired revascularization in AMPKα1−/− mice were further observed. Foot movements in the ligated limb were markedly reduced, P<0.05 at days 7, 14, 21, and 28 in AMPKα1−/− mice compared with control mice (Figure IIIC in the online-only Data Supplement), indicating that AMPK activity is required for proper vascular recovery after femoral artery ligation.

AMPKα1 Deletion Reduces Accumulation of Macrophages in Ischemic Hindlimb
Because inflammatory cells, particularly macrophages, are mainly responsible for collateral vessel remodeling,
macrophage accumulation in the adjacent tissues of collateral vessels peak at 3 to 4 day after ligation; therefore, we first conducted immunostaining to analyze a macrophage marker (F4/80) in adductor muscle sections from AMPKα1−/−, AMPKα2−/− and WT mice 3 days after femoral artery ligation. There was significant accumulation of macrophage in perivessel of adductor tissues in all 3 genotype mice after hindlimb ischemia (Figure 2A and 2B). However, the numbers of accumulated macrophage in AMPKα1−/− mice were significantly lower than those in WT and AMPKα2−/− mice. To further test whether AMPKα1−/− influences macrophage accumulation, we stained for smooth muscle α-actin to analyze collateral lumen wall in WT and AMPKα2−/− mice. Whole-mount visualization of hindlimb arteries through pigment particle perfusion revealed a significantly lower number of enlarged collateral arteries in AMPKα1−/− mice than in WT mice (*P<0.05 vs WT, n=7).

Figure 1. Deletion of AMPKα1 affects blood flow and collateral remodeling after ligation of the femoral artery (FAL). Blood flow and collateral remodeling were assessed in wild-type (WT) control and knockout (KO) mice. A, Quantitative Laser Doppler analyses showing the ratio of left plantar perfusion:right plantar perfusion after occlusion of the femoral artery. AMPKα1−/−, but not AMPKα2 deletion, mice displayed delayed restoration of perfusion compared with WT control mice. B, Representative images obtained using Laser Doppler Imaging, showing the efficiency of the surgery distal to the occlusion side and the recovery of perfusion at the indicated time points. C, Representative hematoxylin and eosin staining of adductor muscle sections from ligated and nonligated limbs in KO and WT mice (scale bar, 50 μm). D, Staining for smooth muscle α-actin to analyze collateral lumen wall (scale bar, 50 μm). E, Whole-mount visualization of hindlimb arteries through pigment particle perfusion (scale bar, 1 mm). F, Transparent hindlimb muscle of the ligated side of the mice at 7 d post surgery showed a significantly lower number of enlarged collateral arteries in AMPKα1−/− mice than in WT mice (*P<0.05 vs WT, n=7).

Figure 2. Deletion of AMPKα1 decreases the accumulation of macrophages in the muscle from ischemic hind limbs in vivo. A, Representative immunostaining with an antibody against F4/80, a macrophage marker, of adductor muscle sections from wild-type (WT), AMPKα1−/− and AMPKα2−/− mice 3 d after femoral artery ligation (scale bar, 50 μm). B, Leica Application Suite Advanced Fluorescence Lite (LAS AF Lite) quantification of F4/80 signal mean gray value (MGV) in 1259.57 μm2 region of interest in adductor sections from mice 3 d after ligation of the femoral artery. *P<0.05 versus WT, n=5. C and D, Representative flow cytometric contour plots for CD45+ cells in adductor (C) and calf tissue (D). E and F, Quantification of the ratio of macrophages in blood-derived mononuclear cells by flow cytometry in adductor muscle (E) and calf muscle (F) from WT and AMPKα1−/− mice 3 d after femoral artery ligation. *P<0.05 vs WT, n=7. M&M indicates monocyte and macrophage.
femoral artery (FAL)–triggered arteriogenesis, we analyzed the accumulation of various leukocyte subsets in the ischemic hindlimb. Flow cytometry analysis showed that neither the number of CD45+ leukocytes in tissues (Figure 2C and 2D; Figure IVA and IVB in the online-only Data Supplement) nor the ratios of monocytes and macrophages in CD45+ leukocytes (Figure IVC and IVD in the online-only Data Supplement) were different between WT and AMPKα1 deletion mice 3 days after surgery. In contrast, the percentage of macrophages was more significantly reduced in adductor and calf tissues from AMPKα1−/− mice than in their counterparts from control mice (Figure 2E and 2F), and the ratios of monocytes to macrophages were significantly higher in adductor and calf tissues from AMPKα1−/− mice than ratios from control tissues (Figure IVE and IVF in the online-only Data Supplement). Altogether, these results suggest that AMPKα1 deletion impairs monocyte to macrophage terminal differentiation in ischemic tissues.

**AMPKα1 Deletion Inhibits the Expression of Growth Factors in Macrophages**

To further explore whether AMPKα1 deletion influences macrophage functions, macrophages were sorted from ischemic hindlimb tissue by flow cytometry, and their ability to generate growth factors was determined through real-time polymerase chain reaction. In addition, Western blot was utilized to determine protein levels in bone marrow–derived macrophages (BMDMs). As depicted in Figure 3A through 3E, both mRNA and protein levels of TGFβ, fibroblast growth factor (FGF)-2, VEGF, and platelet-derived growth factor subunit B were significantly reduced in AMPKα1−/− macrophages, but not in AMPKα1+ macrophages. In addition, we measured the capability of macrophage to secrete soluble factors in WT and AMPKα1−/− mice and found that there was a lower level of VEGF and TGFβ in AMPKα1−/− BMDM supernatant than in WT BMDM supernatant (Figure 3F and 3G). We also detected levels of VEGF and TGFβ in circulation of mice with FAL. As shown in Figure IIC and IID in the online-only Data Supplement, levels of VEGF and TGFβ in circulation were lower in AMPKα1−/− than in WT mice 3 days after FAL. Overall, these results suggest that AMPKα1 deletion inhibits the generation or secretion of growth factors in macrophages.

Next, we assessed the effects of macrophages isolated from WT and AMPKα1−/− mice on the migration and proliferation of endothelial cells and vascular smooth muscle cells, the 2 main cellular components of arteries. Soluble factors released by both AMPKα1−/− and WT BMDMs increased the migration and proliferation of vascular smooth muscle cells (Figure 4A and 4B). However, the ability of AMPKα1−/− macrophage–conditioned medium to enhance the migration and proliferation of vascular smooth muscle cells was significantly lower than that of WT macrophage–conditioned medium (Figure 4A, 4B, and 4D). Consistent with this, endothelial cells exposed to conditioned medium from AMPKα1−/− macrophages showed less migration than those exposed to WT macrophage–conditioned medium (Figure 4C). To test
a relative contribution of soluble factors secreted by macrophages to SMC and endothelial cell functions, neutralizing antibodies (anti-VEGF and anti-TGFβ) were used to block corresponding factors in WT macrophage supernatant. As expected, the neutralizing antibodies diminished the effect of WT macrophage supernatant on SMC and endothelial cell (Figure 4A through 4C), indicating that macrophage impacts the behavior of SMC and endothelial cell through secretion of growth factors. Collectively, these data show that the ability of macrophages from AMPKα1−/− mice to promote the recruitment and growth of the 2 main cellular components of arteries is reduced compared with that of WT macrophages, and the reduction is attributed to AMPKα1−/− macrophage–secreting less growth factors.

Impairment of Arteriogenesis Is Because of AMPKα1 Deficiency in Macrophages

To more precisely assess the intrinsic role of AMPK in macrophage function, and to demonstrate whether the impairment of arteriogenesis in AMPKα1−/− mice is macrophage dependent, myeloid specific, AMPKα1-deletion mice (called macrophage-specific knockout mice) were generated by breeding Lysozyme Cre and AMPKα1 flox/flox mice and were subjected to femoral artery ligation as was done in the global AMPK knockout mice. Blood flow perfusion was monitored after ligation at different times. As shown in Figure 5A and 5B, hindlimb perfusion recovery was attenuated in AMPKα1 flox; Lys Cre mice, compared with littermate controls. In addition, compared with control mice at day 7 after ligation, collateral modeling in AMPKα1 flox/flox; Lys Cre mice was markedly decreased (Figure 5E and 5F) in parallel with an increase in calf muscle necrosis (Figure 5C and 5D). Taken together, these results suggest that AMPKα1 deletion impairs arteriogenesis by regulating the amount and function of monocytes and macrophages in ischemic tissues.

Decreased Expression of Growth Factors in Macrophages Under Hypoxia Conditions In Vivo and In Vitro

To further demonstrate that AMPKα1 deletion influences growth factor generation in macrophages in vivo, we performed immunofluorescence staining of TGFβ or FGF2 and macrophage markers in ischemic adductor tissues to observe vascular growth factors in macrophages in situ. As shown in Figure 6A through 6D, TGFβ and FGF2 levels were reduced in macrophages from AMPKα1−/− mice when compared with those in WT mice. We also analyzed VEGF, TGFβ, FGF2, and bone morphogenetic protein 4 (BMP4) levels in ischemic adductor tissue by Western blot. As depicted in Figure VA and VB in the online-only Data Supplement, proangiogenic factors were reduced in ischemic settings in AMPKα1−/− mice in comparison with WT mice. In contrast, the level of BMP4, a TGFβ signal pathway, was comparable between the 2-genotype mice.
To mimic ischemia and hypoxia condition in vivo, BMDMs were exposed to 1% oxygen for 3 hours. In agreement with in vivo findings, Western blot showed that hypoxia activated both AMPK and nuclear factor κB (NF-κB) pathways (Figure 6E) and increased TGF-β, FGF2, and FGF21 levels (Figure 6G). However, under hypoxia condition, increase of TGF-β, FGF2, and FGF21 in AMPKα1−/− macrophages was much less when compared with increases in WT macrophages (Figure 6G and 6H), suggesting that AMPKα1 deletion mitigates expression of vascular growth factors in macrophage response to hypoxia. In addition, hypoxia-inducible factor (HIF)-1α levels was significantly reduced (P<0.05) in macrophages from AMPKα1−/− mice under both normoxia and hypoxia conditions (Figure 6E), indicating that AMPK may be involved in HIF1α stability in macrophages.

NF-κB Regulates Growth Factor Expression in Macrophages

We reasoned that the mechanism by which AMPKα1 deletion impairs growth factor expression in macrophages might involve NF-κB because this signaling molecule is not only a central regulator of the stress response but also a transcription factor for many genes. To test whether NF-κB regulates TGFβ1 and FGF2 gene expression, human embryonic kidney 293T (HEK293T) cells were cotransfected with plasmids containing a TGFβ1 promoter reporter and NF-κB p65. The luciferase activity assay showed that NF-κB p65 significantly increased TGFβ1 promoter activity (Figure 7A), indicating that NF-κB may function as a transcription factor for TGFβ1.

AMPKα1 Deletion Leads to Reduction of NF-κB Transcription Factor Activity in Macrophages

To test whether AMPKα1 deletion leads to a reduction of NF-κB activity in macrophages, the nuclear fraction was isolated from BMDMs and subjected to Western blot, to determine the level of phosphorylated NF-κB (on residue serine 536). A distinct reduction in phospho-NF-κB P65 serine 536 levels was observed in AMPKα1−/− BMDMs, compared with WT (Figure 7B). Subsequently, we utilized an electrophoretic mobility shift assay to assess the DNA-binding affinity of NF-κB in AMPKα1−/− macrophages. As shown in Figure 7C, the DNA-binding affinity of NF-κB was modestly reduced in BMDMs from AMPKα1−/− mice when compared with those from WT mice. To explore NF-κB transcription factor activity in AMPKα1−/− macrophages under ischemic conditions in vivo, we analyzed NF-κB activity in macrophages under hypoxic conditions in vitro to mimic ischemic condition in vivo. We found that NF-κB activity is noticeably lower in AMPKα1−/− macrophages than in WT macrophages under hypoxic conditions (Figure 7D). Taken together, these results suggest that AMPKα1 deletion impairs NF-κB activation in the macrophage response to hypoxic stress.
AMPK Directly Phosphorylates Inhibitor of NF-κB Kinase α Threonine 23

We next tested how AMPKα1 regulates NF-κB activation in macrophages, and whether phosphorylation of inhibitor kappa B kinase alpha (IKKα) at threonine 23 affects NF-κB activity and function. AMPK is a Thr/Ser protein kinase that can phosphorylate various kinds of substrates, including acetyl-CoA carboxylase, endothelial nitric oxide synthase, Unc-51 like autophagy activating kinase 1, tuberous sclerosis complex 2, and mammalian target of rapamycin to regulate all aspects of cellular function, including autophagy, cell polarity, cell growth, and cell proliferation. Based on the consensus site of the substrate recognized by AMPK\(^\alpha\) (Figure 8A), we speculated that AMPK might directly phosphorylate IKK. To test this, we first measured total threonine/serine phosphorylation of IKK in BMDMs. As depicted in Figure 8B, AMPKα1 may increase IKKα threonine phosphorylation, but not that of serine or IKKβ. Next, we determined whether AMPKα1
directly phosphorylates IKKα, and which threonine residues in the IKKα protein are phosphorylated, by measuring AMPK kinase activity in vitro. As shown in Figure 8C, recombinant AMPKα1/β1/γ1 may significantly increase the phosphorylation of recombinant IKKα protein at threonine 23, suggesting that AMPK may directly phosphorylate IKKα at Thr23.

To further confirm Thr23 of IKKα as the phosphorylation target of AMPKα1, we generated a site-directed mutant construct of IKKα (threonine to alanine, T23A). HEK293 cells were transfected with IKKα or the IKKα T23A mutant. After the transfection, they were treated with A769662, a selective activation for AMPK. As expected, A769662 promoted AMPK phosphorylation at Thr172 in cells transfected with IKKα or the IKKα T23A mutant (Figure 8D). IKKα phosphorylation at Thr23 was markedly increased in cells transfected with WT IKKα, but not in those transfected IKKα mutants (Figure 8D), confirming that threonine 23 is the target of AMPKα threonine phosphorylation.

To further test whether AMPKα1 directly phosphorylates IKKα threonine 23 in cells in vivo, we measured the levels of IKKα Thr 23 phosphorylation in WT and AMPKα1−/− peritoneal macrophages and BMDMs. We observed significantly decreased levels of Thr 23 phosphorylation in AMPKα1−/− compared with WT macrophages (Figure 6E and 8E) under both normoxic and hypoxic conditions.

**Discussion**

In the present study, we have demonstrated that AMPKα1 plays an essential role in the arteriogenesis of collateral remodeling. We found that the AMPKα1 knockout mouse displayed a manifest decrease in the restoration of blood flow after femoral artery ligation. Histological evaluation revealed defects in arteriogenesis as a result of impaired collateral remodeling. An AMPK α1 flox/flox; Lyz-Cre mouse, in which AMPK α1 was deleted in macrophages, exhibited similar phenotypes in terms of blood flow recovery and remodeling of collateral arterioles, suggesting macrophage AMPK as a regulator for arteriogenesis. We further found less accumulation of AMPKα1−/− macrophages in the arteriogenesis setting and lower levels of growth factor production in AMPKα1−/− macrophages. In addition, we demonstrated that AMPK directly phosphorylates IKKα and subsequently activates NF-κB, which functions as a transcription factor for TGFβ.

The most important finding in this study was that macrophage AMPK plays an essential role in arteriogenesis through its regulation of growth factor generation. We did observe a decrease in macrophage accumulation and macrophage growth factor generation in AMPKα1−/− mice and further generated macrophage-specific AMPKα1−/− mice to demonstrate that the impairment of arteriogenesis can be attributed to the loss of macrophage AMPK. The data from both global knock-out and AMPK α1 flox/flox; Lyz-Cre mouse models suggest that macrophage AMPK is important in growth factor generation and arteriogenesis.

Another important finding is how AMPK regulates the production of growth factors. We revealed that AMPK regulates NF-κB activation through the phosphorylation of threonine 23 on IKKα (IkB kinase), which in turn phosphorylates IkBα in macrophages under either hypoxic or normoxic conditions. Next, we validated that NF-κB as a transcription factor can bind to the TGFβ promoter to enhance its expression (Figure 7). On deletion of AMPKα1 in macrophages, the ability of macrophages to generate growth factors was markedly reduced. This link between AMPK, NF-κB, and growth factor levels, to our knowledge, has not yet been reported. Indeed, monocytes/macrophages have long been thought to be crucial to arteriogenesis, in part, because of their production of VEGF and FGF2. Apart from VEGF and FGF2, we found that there were significantly decreased levels of TGFβ, FGF21, and platelet-derived growth factor subunit B in AMPKα1-deficient macrophages compared with WT macrophages, not only under hypoxic but also under normoxic conditions.

Although studies have reported that TGFβ expression is increased in growing collaterals and that local application of...
exogenous TGFβ might foster arteriogenesis,30–32 the generation and regulation of TGFβ is elusive. Our findings show that macrophages express high levels of TGFβ and that AMPK affects its expression via the regulation of NF-κB. However, emerging studies indicate that AMPK signaling can suppress the inflammatory responses induced by the NF-κB system.33,34 NF-κB subunits are not direct phosphorylation substrates of AMPK; rather, the inhibition of NF-κB signaling is mediated through its downstream targets, eg, SIRT1 (NAD+-dependent histone deacetylase 1), PGC-1α (peroxisome proliferator-activated receptor-γ coactivator-1α), p53, and FoxO1, in different cell types.35

Previous studies,36–38 mainly focused on endothelial NF-κB and found that NF-κB was a key regulator of adult and developmental arteriogenesis and collateral formation. Shear stress7 and inflammation31 have long been considered key drivers for arteriogenesis in adult tissues. During arterial occlusion, increased shear stress causes NF-κB activation in the endothelium. This activation of the endothelial NF-κB cascade can augment the expression of adhesion molecules to recruit blood-derived monocytes/macrophages. Although endothelial NF-κB is squarely at the center of both developmental and adult arteriogenesis,36,39 we focused on the mechanism underlying the activation of recruited monocytes/macrophages during adult arteriogenesis. We found that AMPKα1 deletion results in decreased NF-κB activity, which subsequently leads to a lack of macrophage-secreting growth factors, thereby affecting the interaction between macrophages and SMCs/endothelial cells. In this regard, macrophage NF-κB also plays an important role in arteriogenesis. However, AMPK might play additional roles in other cell types (such as endothelial cells) in response to fluid shear stress, hypoxia, and ischemic condition during arteriogenesis and angiogenesis. Both in vivo and in vitro studies40–42 indicate that AMPK might be activated by hypoxia, ischemia, and fluid shear stress in endothelial cells, with the subsequent interplay between AMPK, SIRT1, and endothelial nitric oxide synthase performing an atheroprotective function. Findings in this study demonstrate that hypoxia activates macrophage AMPK and sequentially increases generation of growth factors that are beneficial for arteriogenesis and angiogenesis (Figure 6). Moreover, we found that small-molecule AMPK activator, A769662, enhances NF-κB signal, and increases TGFβ level in macrophage (Figure 8E), indicating
that activation of macrophage AMPK is helpful for neovascularization. However, the clinical application of macrophage-specific AMPKα1-selective pharmacological activator is unclear because this molecule may promote proinflammatory and atherogenic signaling mechanisms in other organs.

Consistent with recent studies, which have suggested that HIF levels are controlled by transcription factor NF-κB and that NF-κB can stabilize HIF1α levels both under hypoxic and normoxic conditions, our findings demonstrated that the HIF1α levels in AMPKα1 macrophages are significantly lower than those in WT macrophages under both normoxia and hypoxia, indicating that AMPK might regulate VEGF levels indirectly through the NF-κB/HIF1α pathway.

In summary, we found that macrophage AMPKα1 plays an important role in the regulation of adult arteriogenesis and the formation of collateral circulation via-NF-κB signaling and through the control of monocyte-macrophage production of TGF-β, platelet-derived growth factor subunit B, FGF2, and VEGF.

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Disclosures
None.

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**Highlights**

- Occlusive vascular diseases remain the most important causes of morbidity and death in developed countries and cause >50% of mortalities before the age of 75 years.
- Therapies that promote the compensatory growth of blood vessels could prove superior to palliative surgical intervention in managing occlusive vascular diseases.
- However, therapies that improve collateral growth are not available because the underlying mechanisms that control the complex process of arteriolar remodeling remain undefined.
- To define suitable targets for pharmacological therapies, a description of key molecules orchestrating the activity of macrophages, vascular smooth muscle cells, and endothelial cells is necessary.
- Our study suggests that AMPK signaling plays a key role in collateral remodeling and arteriogenesis.
- Genetic ablation of AMPKα1 in mice impairs collateral growth by inhibiting both monocyte to macrophage terminal differentiation and the secretion of arteriogenic factors by macrophages in the area surrounding an arterial occlusion.
- From a clinical point of view, enhancing the activation of AMPK favors collateral remodeling and arteriogenesis and thus may be an alternative therapeutic strategy for ischemic disorders.
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AMP-activated protein kinase (AMPK) α1 in macrophages promotes collateral remodeling and arteriogenesis in mice *in vivo*

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Materials and Methods

Materials

Antibodies for western blotting directed against AMPK, phospho-Thr172 AMPK, TGFβ1, IKKα, and IκBα were purchased from Cell Signaling (Danvers, MA, USA). Antibodies against human/mouse HIF1α were obtained from R&D Systems (Minneapolis, MN, USA), APC-Cy7-Streptavidin and rat anti-mouse CD31 were procured from BD Pharmingen (San Jose, CA, USA). Fluorescein isothiocyanate (FITC)-labeled CD45 antibody, peridinin chlorophyll protein complex (PerCP)- and Cy5.5-labeled CD11b antibody, allophycocyanin (APC)-labeled F4/80 antibody, phycoerythrin (PE)-labeled Ly6C antibody, biotin-labeled CD90.2, B220, CD49b, NK1.1, and Ly6G antibodies were provided by Biolegend (San Diego, CA, USA). Anti-monocyte/macrophage antibody (Moma2) was ordered from AbD Serotec (Kidlington, UK), anti-mouse F4/80 antibody for immunostaining was from eBioscience (San Diego, CA, USA), and anti-phospho-IKKα (T23) antibody was from Santa Cruz (Dallas, TX, USA). The dual-luciferase
reporter assay kit was ordered from Promega (Madison, WI, USA). Anti-mouse FGF2, FITC-anti-mouse smooth muscle α-actin, and all other substances were obtained from Sigma (St. Louis, MO, USA).

Mice

AMPKα1−/−, AMPKα2−/− mice backcrossed to the C57BL/6 background for at least eight generations were used for these experiments, as described previously1. Both AMPKα1 flox/flox and LysM Cre knock-in mice are C57BL/6-congenic mice, and were purchased from Jackson labs. Experimental wild-type and AMPKα −/−, AMPKα1 flox/flox, and Cre mice were used at ages of 10 to 12 weeks. All experimental procedures have been reviewed and approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center.

Ischemic Hind Limb Model: Surgery and Laser Doppler Imaging

The surgical procedure was performed on left side of the hind limb as previously described2. Briefly, the left femoral artery was exposed aseptically through a 2-mm incision, isolated from vein and nerve, and ligated just distal to the origin of the arteria profunda femoris. Relative blood flow to the foot was measured under standardized conditions by Laser Doppler imaging (LDI) as described3. Measurements of plantar perfusion were performed before, immediately after surgery, and on post-surgery days 3, 7, 14, 21, and 28. The left-to-right (L/R) ratio (ligated versus nonligated leg) was calculated for each animal (n=10–15 for each group), in order to compare various time points.
Evaluation of Foot Movement Score

To evaluate the functional recovery of the ligated limbs, we used a scoring system based on active foot movement. Each individual mouse was placed in a cage and evaluated as follows: score 4, unrestricted movement; score 3, use of complete foot and spreading of toes; score 2, active foot use; score 1, use of leg only; 0, dragging of foot. Scores were ascribed directly prior to anesthetizing mice for LDI, starting 7 days after surgery in a randomized and blinded fashion.

Immunofluorescence

Crystal sections of adductor or calf tissue were fixed in acetone (15 minutes). Subsequently, the slides were washed three times with phosphate-buffered saline (PBS), then permeabilized with PBS containing 0.1% (v/v) Triton X-100, and blocked with PBS containing 2% (v/v) donkey serum and 5% (w/v) bovine serum albumin, before incubation with the appropriate primary antibodies. After thorough washing with PBS, the slides were incubated with the appropriate secondary antibody conjugated to Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647. The sections were mounted in prolonged mounting medium from life technologies (Carlsbad, CA, USA) and visualized on a Leica TCS SP2 confocal microscope. Image analysis was performed using Leica software.

Immunohistochemistry

For the identification of collateral arteries, 6-μm thick sections of adductor muscle were fixed in acetone (15 minutes) and incubated with 3% H2O2 for 30 minutes to block endogenous peroxidase activity. After washing with PBS, sections were incubated for
30 minutes in 1% bovine serum albumin in PBS, then stained for 12 hours at 4°C with a rat monoclonal antibody against murine smooth muscle actin-alpha. Immunoperoxidase signals were visualized by incubation with diaminobenzidine. Nuclei were counterstained with hematoxylin.

**Western Blot Analysis**

Protein extracts were prepared by lysing cells in radioimmunoprecipitation assay buffer (150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid [EDTA], and 50 mM Tris-HCl, pH 8.0) supplemented with complete EDTA-free protease inhibitor cocktail tablets (Santa Cruz) and phosphatase inhibitor cocktails (Sigma-Aldrich, USA). Cleared lysates were separated on 4–20% Bis-Tris gels (Invitrogen, USA) followed by transfer onto polyvinylidene difluoride membranes. After blocking in 5% skim milk, blots were probed using the following primary antibodies: anti-mouse TGFβ, FGF2, VEGF, AMPKα1, phospho-AMPKα (T172), and IκBα S32, all of which were purchased from Cell Signaling; and anti-mouse IKKα T23, which was purchased from Santa Cruz Biotechnology.

**Flow Cytometry**

Three days after femoral artery ligation, AMPKα1−/− and WT mice were anesthetized and perfused by cardiac puncture with Dulbecco’s PBS containing 2 mM EDTA. Adductor or calf tissues were cut from ischemic hind limbs, and digested with 3 mg/ml collagenase type I in PBS containing 20 mM HEPES at 37°C for 40 minutes. The digested muscles were gently forced through a 70-μm strainer to obtain a single-cell suspension. Cells were incubated with Fc block for 10 min at 4°C, then with biotin-labeled CD90.2, B220,
CD49b, NK1.1, and Ly6G antibodies for 20 min at 4°C, wash twice, then with FITC-CD45, APC-Cy7-streptavidin, APC-F4/80, and PerCP-Cy5.5-CD11b antibodies for 20 min at 4°C and analyzed by flow cytometry. Macrophages were identified as CD45+, lineage\(^lo\), CD11b\(^+\), F4/80\(^hi\)/Ly6C\(^lo\) cells, and monocytes were identified as CD45\(^+\), CD11b\(^+\), lineage\(^lo\), F4/80\(^lo\)/Ly6C\(^hi\) cells.

**Whole-mount Visualization of Arteries by Pigment Particle Perfusion**

For gross imaging of collateral arteries in whole-mount limb muscles, we referred to a specific pigment particle perfusion protocol\(^3\). Briefly, mice with femoral artery ligation for 1 week were sacrificed by cervical dislocation. Each animal was immediately placed in the perfusion tray, the limbs were fixed, and the chest was then opened to remove the ventral vertebrosternal. Twenty to 40 ml of vasodilation solution were infused at 10–15 ml/min for pigment staining over 2–3 min. Then, 20–40 ml of pigment solution was infused at a rate of 10–15 ml/min. The mouse was transferred to a clean tray and the fur was made wet with 70% ethanol. The muscles were dissected, and the thigh muscles washed for 1 min in distilled water. Tissues were immediately transferred into perfusion-fixation solution and incubated at 4°C overnight. The thigh muscles were dehydrated through immersion in a series of alcohol solutions (70%, 85%, and 96% ethanol) for 2 h each, followed by immersion in isopropanol for 2 h. The tissue samples were then transferred into transparency solution, and shaken gently at 20 rpm at room temperature until the muscle tissue became transparent. The tissue pieces were then photographed under an inverted microscope.

**The transwell migration assay**
During this assay, smooth muscle cells or endothelial cells (5x10⁴) are placed on the upper layer of a cell permeable membrane and a base medium containing macrophage condition medium is placed below the cell permeable membrane. Following incubation at 37°C, 5% CO₂ environment for 4-6 hours, the cells that have migrated through the membrane are fixed, then stained and counted. The membrane is from Corning Incorporated –Life Sciences (pore size is 8 μm). To test proangiogenic activity of macrophage supernatant, 5ug/ml of anti-VEGF (Clone: 2G11-2A05 from Biolegend) and 50 ug/ml of anti-TGFβ antibody (Clone: 1D11 from R$D systems) were pre-incubated with WT macrophage supernatant for 30 minutes at room temperature respectively, then were used as a trigger to perform transwell migration assay.

Statistical Analysis

All data were presented as means ± standard errors of the mean. Two-group comparisons were performed using the unpaired Student’s t-test. Multiple group comparisons were performed using one-way analysis of variance followed by Bonferroni multiple comparison post-test. P values of less than 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism Version 5 (San Diego, CA).

References


Supplemental Figure I: A: Quantitative analyses of collateral lumen diameter (*p<0.05 versus WT, n=15). B: smooth muscle α-actin+ vascular area per vessel (*p<0.05 versus WT, n=15). C: the ratio of smooth muscle α-actin+ vascular area in whole muscle (*p<0.05 versus WT, n=15).
Supplemental Figure II: A: Immunofluorescence staining of CD31, an endothelial cell marker in cross-sections of crural muscle from WT and KO mice with ligated limb for 3 days, (scale bar: 50 μm). B: Quantitation of capillary density per 139876 μm² ischemic crural muscle. *p<0.05 versus WT, n=4-5. C and D: ELISA measurement of serum VEGF (C) and TGFβ (D) level in mice 3 days after FAL. *p<0.05 versus WT, n=5. E: Flow cytometry analysis of endothelial progenitor cells in circulation of mice suffered from FAL (n=5).
Supplemental Figure III: A: Representative 2, 3, 5-triphenyltetrazolium chloride (TTC) staining for crural muscle from ligated and nonligated limbs in KO and WT mice. B: Quantification of TTC staining was decreased in AMPKα1−/− mice 7 days after femoral artery ligation (*p<0.05 versus WT, n=5). C: Foot movement was determined and scored between 0 and 4, active foot movement was significantly weakened in AMPKα1−/− mice. *p<0.05 versus WT, n=15.
Supplemental Figure IV: A: Quantification of CD45+ cells in adductor (A) and calf tissue (B) from WT and AMPKα1-/- mice 3 days after femoral artery ligation. C and D: Quantification of the ratio of blood-derived mononuclear cells (monocyte/macrophage, M&M) in CD45+ cells in adductor (C) and calf (D) muscle from WT and AMPKα1-/- mice 3 days after femoral artery ligation. E and F: Quantification of the ratio of monocytes in blood-derived mononuclear cells. *p<0.05 versus WT, n=7.
Supplemental Figure V: A: Western blot determination of TGFβ, VEGF, FGF2 and BMP4 level in ischemic adductor tissue. The blot is three of five mice tissue. B: Qualification of western blot image from five mice, *p<0.05 versus WT, n=5. C: Western blot analysis of phospho-eNOS Ser1177, phospho-mTOR Ser2448, Notch I and δ-like ligand 1 (DLL1) and DLL4 in adductor tissue lysates from WT and prkaa1−/− mice 3 days after femoral artery ligation.