AMP-Activated Protein Kinase α2 in Neutrophils Regulates Vascular Repair via Hypoxia-Inducible Factor-1α and a Network of Proteins Affecting Metabolism and Apoptosis

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Rationale: The AMP-activated protein kinase (AMPK) is stimulated by hypoxia, and although the AMPKα1 catalytic subunit has been implicated in angiogenesis, little is known about the role played by the AMPKα2 subunit in vascular repair.

Objective: To determine the role of the AMPKα2 subunit in vascular repair.

Methods and Results: Recovery of blood flow after femoral artery ligation was impaired (>80%) in AMPKα2−/− versus wild-type mice, a phenotype reproduced in mice lacking AMPKα2 in myeloid cells (AMPKα2ΔMC). Three days after ligation, neutrophil infiltration into ischemic limbs of AMPKα2ΔMC mice was lower than that in wild-type mice despite being higher after 24 hours. Neutrophil survival in ischemic tissue is required to attract monocytes that contribute to the angiogenic response. Indeed, apoptosis was increased in hypoxic neutrophils from AMPKα2ΔMC mice, fewer monocytes were recruited, and gene array analysis revealed attenuated expression of proangiogenic proteins in ischemic AMPKα2ΔMC hindlimbs. Many angiogenic growth factors are regulated by hypoxia-inducible factor, and hypoxia-inducible factor-1α induction was attenuated in AMPKα2-deficient cells and accompanied by its enhanced hydroxylation. Also, fewer proteins were regulated by hypoxia in neutrophils from AMPKα2ΔMC mice. Mechanistically, isocitrate dehydrogenase expression and the production of α-ketoglutarate, which negatively regulate hypoxia-inducible factor-1α stability, were attenuated in neutrophils from wild-type mice but remained elevated in cells from AMPKα2ΔMC mice.

Conclusions: AMPKα2 regulates α-ketoglutarate generation, hypoxia-inducible factor-1α stability, and neutrophil survival, which in turn determine further monocyte cell recruitment and repair potential. The activation of AMPKα2 in neutrophils is a decisive event in the initiation of vascular repair after ischemia. (Circ Res. 2017;120:99-109. DOI: 10.1161/CIRCRESAHA.116.309937.)

Key Words: diabetes mellitus ■ ischemia ■ metabolomics ■ proteomics ■ vascular remodeling

A MP-activated protein kinase (AMPK) is a nutrient-sensitive kinase that can be regulated by Ca2+-elevating agents that activate upstream kinases and by various cellular stresses including hypoxia.1 In endothelial cells, the AMPK is required for an adequate angiogenic response to hypoxia2 and can induce the generation of vascular endothelial growth factor (VEGF).3 Angiogenesis and vascular repair after ischemia in vivo also rely on the recruitment and activation of circulating cells such as monocytes and neutrophils.4 Little is known about the role of the AMPK in such processes, but the kinase has been implicated in inflammatory signaling in myeloid cells,5 such as leukocyte homing and neutrophil activation,6 and monocyte differentiation.7,8

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The AMPK is a heterotrimeric kinase consisting of a catalytic α subunit and regulatory β and γ subunits. There are 2 different isoforms of the catalytic α subunit, that is, the α1 subunit that is mainly localized to the cytosol and the α2 subunit...

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Reagents and other detailed methods are described in the Online Data Supplement.

**Results**

**Effects of AMPKα2 Deletion on Vascular Repair**

Diabetes mellitus is not only a risk factor for the development of cardiovascular disease but is linked with impaired vascular repair after injury and ischemia.\(^{12,13}\) Given that the AMPKα2 subunit was reported to be a regulator of whole body insulin sensitivity,\(^{14}\) vascular repair after femoral artery ligation was assessed in wild-type and AMPKα2/− mice. The recovery of blood flow in ischemic hindlimbs was markedly attenuated in AMPKα2/− mice compared with their wild-type littermates over 14 days (Figure 1A). The restoration of hindlimb perfusion is dependent on arteriogenesis and angiogenesis, and micro computed tomography analyses revealed a clearly impaired arteriogenesis, defined as the development of remodeled collaterals with a corkscrew morphology, in the AMPKα2/− mice (Figure 1B). The delayed functional recovery was also reflected in the decreased capillary density in the calf muscles 14 days after surgery (Figure 1C).

**AMPKα2 in Endothelial Versus Myeloid Cells**

Arteriogenesis and angiogenesis are determined by endothelial cells and circulating cells.\(^ {4}\) To determine whether the reduced recovery after hindlimb ischemia was dependent on the deletion of AMPKα2 in endothelial cells or other cell types, experiments were repeated in AMPKα2\(^ {2\text{lox/lox}}\) mice crossed with mice expressing the Cre-deleter under the control of the Tie-2 promoter (Tie2−α2 mice) and their wild-type littermates. Although there was delayed recovery of perfusion in the Tie2−α2 versus wild-type mice (Figure 2A), the effect was much less impressive than that in the global knockout animals. Although the Tie-2 promoter is frequently used to target endothelial cells, it is also expressed by a subset of myeloid cells.\(^ {15,16}\) Therefore, endothelial cells were more specifically targeted by crossing AMPKα2\(^ {2\text{lox/lox}}\) mice with mice expressing the Cre-deleter under the control of the VE-cadherin promoter. However, the endothelial cell–specific deletion of the AMPKα2 had no effect on the recovery from hindlimb ischemia (Figure 2B).

As the results indicated that the AMPKα2 in mononuclear cells may explain the observations, the expression of the AMPKα2 subunit was assessed in myeloid cells. AMPKα2 mRNA was clearly expressed in the aorta and in peritoneal macrophages from wild-type mice (Figure 2C). Crossing AMPKα2\(^ {2\text{lox/lox}}\) mice with LysM-Cre mice, to generate animals lacking the AMPKα2 in myeloid cells (AMPKα2\(^ {2\text{lox/lox}}\) mice), resulted in the loss of AMPKα2 mRNA expression in macrophages (Figure 2C) and protein expression in CD11b+ (myeloid lineage) cells (Figure 2D). However, the CD11b− cell population from the same animals did express AMPKα2, confirming the specificity of the LysM-Cre–dependent deletion. The myeloid cell–specific deletion of the AMPKα2 resulted in a decrease in circulating neutrophils under basal conditions that could not be explained by altered hematopoietic stem cell differentiation or mobilization. The deletion of the AMPKα2 in myeloid cells did not alter the composition of hematopoietic stem cell populations or the more committed lineages in bone marrow from untreated mice or after treatment with that can translocate to the nucleus, where it likely plays a more important role in transcription factor activation and gene regulation.\(^ {3}\) Although many studies have reported a role for AMPK activation in angiogenesis in vitro and in vivo, they generally relied on unspecific pharmacological tools that cannot differentiate between the different catalytic subunits. We reported previously that the AMPKα subunit in endothelial cells is a prerequisite for angiogenesis induced by cytokines and VEGF via a mechanism involving transforming growth factor-β–activated kinase 1 and superoxide dismutase 2 expression.\(^ {10}\) The aim of the present study was to assess the role of AMPK, particularly the AMPKα2 subunit, in the regulation of vascular repair in vivo in a model where the outcome is largely dependent on local responses to hypoxia and the mobilization and recruitment of bone marrow–derived cells such as monocytes and neutrophils.

**Methods**

**Animals**

C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany).

Genetically modified mice lacking either the AMPKα1 or the AMPKα2 subunits, their respective wild-type littermates, and floxed AMPKα2 mice were kindly provided by Benoit Viollet (INSERM, U1016, Paris, France) and bred at the Goethe University Hospital animal facility. Floxed AMPKα2 mice were crossed with animals expressing the Cre-deleter under the control of the Tie-2 promoter (B6.Cg-Tg(Tek-cre)12Flv/J; Jackson Laboratories, Bar Harbor, ME) to generate mice lacking the α2 subunit in endothelial cells and some myeloid cells (Tie2−α2), with VE-cadherin-Cre mice (B6-Tg(Cdh5-cre); Polygene, Switzerland) to generate animals lacking the α2 subunit specifically in endothelial cells (AMPKα2\(^ {2\text{lox/lox}}\)), or with LysM-Cre mice (B6.129P2-Lyz2tm1(cre)If/J; Jackson Laboratories) to generate mice lacking the α2 subunit specifically in myeloid cells (AMPKα2\(^ {2\text{lox/lox}}\)).

All animals were housed in conditions that conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23). Both the University Animal Care Committee and the Federal Authority for Animal Research at the Regierungspräsidium Darmstadt (Hessen, Germany) approved the study protocol (#F28/25). For the isolation of organs, mice were euthanized using 4% isoflurane in air and subsequent exsanguination or decapitation.

**Hindlimb Ischemia**

Arteriogenic and angiogenic capacity was investigated in a murine model of hindlimb ischemia using 6- to 8-week-old wild-type and transgenic animals as described.\(^ {11}\)

**Statistical Analysis**

Data are expressed as mean±SEM. Statistical evaluation was performed using Student’s t test for unpaired data, 1-way ANOVA followed by a Bonferroni t test, or ANOVA for repeated measures where appropriate. Values of P<0.05 were considered statistically significant.

<table>
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<tr>
<th>Nonstandard Abbreviations and Acronyms</th>
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granulocyte colony-stimulating factor (Online Figure IA). Neither the proliferation nor the differentiation of mobilized progenitors was altered 5 days after granulocyte colony-stimulating factor administration as assessed by using a colony-forming unit assay (Online Figure IB). These findings fit with the reported lack of effect of AMPK deletion on normal hematoepoietic stem cell function.17

AMPKα2ΔMC mice reproduced the phenotype of the AMPKα2−/− mice in that only minimal recovery of hindlimb blood flow was observed over 14 days after the induction of ischemia (Figure 2E). Fitting with the delayed recovery of blood flow, arteriogenesis was less pronounced in the AMPKα2ΔMC mice compared with their wild-type littermates. The latter animals developed remodeled collaterals that largely failed to form in the AMPKα2ΔMC mice (Figure 2F). Angiogenesis, assessed as the number of capillaries (CD31+ cells) in the semimembranosus muscle (Figure 2G) and gastrocnemius muscle (Figure 2H), was also markedly decreased in ischemic hindlimbs from AMPKα2ΔMC mice. The AMPKα1 subunit is more highly expressed than the AMPKα2 isoform in myeloid cells. Despite this, the deletion of the AMPKα2 subunit attenuated neutrophil AMPK phosphorylation on the activating Thr172 site (the phospho-specific antibody used cannot differentiate between the AMPKα1 and α2 isoforms) under basal conditions and in cells exposed to hypoxia (Online Figure IIA). To confirm the importance of the AMPKα2 isoform in vascular repair, we also generated a myeloid cell–specific AMPKα1-deficient mouse. Although vascular repair after ischemia was attenuated in mice lacking the α1 subunit in myeloid cells, the effect was much less pronounced than that seen in AMPKα2ΔMC mice (compare Online Figure IIB and Figure 2E).

Altered Recruitment of Myeloid Cells to Ischemic Hindlimbs in AMPKα2ΔMC Mice

As the rapid recruitment of myeloid cells to the ischemic tissue has been implicated in arteriogenesis and angiogenesis, the recruitment of the different cell populations was studied 3 days after the induction of ischemia.

Using hematoxylin–eosin staining, distinctly fewer infiltrating cells were detected in ischemic muscles from AMPKα2ΔMC mice compared with their wild-type littermates (Figure 3A). In ischemic muscles from wild-type animals, leukocytes (CD45+ cells), neutrophils (Gr1+ cells), and macrophages (F4/80+ cells) were abundant but were significantly lower in the AMPKα2ΔMC group (Figure 3B). Infiltrating cells have a significant impact on the local environment in the ischemic skeletal muscle, and analysis of mRNA expression of a wide range of angiogenic markers and inflammatory chemokines and cytokines in the ischemic gastrocnemius muscle revealed pronounced differences (Figure 3C; Online Figure III). For example, levels of transforming growth factor (TGF)β, VEGFA and VEGFB, and the VEGF receptor 1 (VEGFR1) and VEGFR2 were lower in ischemic muscles from AMPKα2ΔMC mice compared with their wild-type littermates. On the contrary, the expression of several inflammatory mediators and chemokines were upregulated in ischemic muscles from AMPKα2ΔMC mice including tumor necrosis factor-α, interleukin (IL)-1β, and chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemoattractant factor 1 (MCP-1) and its receptor C-C chemokine receptor type 2 (CCR2). The latter also coincided with endothelial cell activation as indicated by the up-regulation of intercellular adhesion molecule-1, which was confirmed by immunohistochemistry (Figure 3D). This altered gene expression pattern was consistently observed in the gastrocnemius and in the semimembranosus muscles (Online Figure III).

To determine whether myeloid cell recruitment to the ischemic muscle or myeloid cell survival in the hypoxic environment was altered by the deletion of the AMPKα2 subunit, experiments were repeated 1 day after surgery. Within 24 hours of ischemia, leukocyte infiltration was similar in the ischemic hindlimbs from wild-type and AMPKα2ΔMC mice (Figure 4A). However, a significantly higher number of neutrophils were recruited to the gastrocnemius muscle of AMPKα2ΔMC mice than their wild-type littermates.
At this early time point, no macrophages were consistently detected in the ischemic muscles. In vitro migration assays, neutrophils from AMPKα2ΔMC mice more effectively migrated through an endothelial cell monolayer (Figure 4C) even though directed migration toward a cytokine, the murine IL-8 homologue CXCL1, did not differ between the 2 strains (data not shown). Confocal microscopy confirmed the elevated neutrophil infiltration into ischemic muscles from AMPKα2ΔMC mice 24 hours after surgery (Figure 4D). In these samples, intra- and extravascular neutrophils were easier to detect in whole mounts from AMPKα2ΔMC mice versus their wild-type littermates, indicating an improved transendothelial migration also in vivo (Online Figure IV). Moreover, although circulating numbers of neutrophils were lower under basal conditions, they were comparable in wild-type and AMPKα2ΔMC littermates 24 hours after the induction of ischemia, indicating a lack of a defect in mobilization (Online Figure V). Taken together, these data point out that, compared with responses in wild-type littermates, the response to ischemia in hindlimbs from AMPKα2ΔMC mice is linked to an increase in early neutrophil infiltration, the upregulation of inflammatory cytokines, and adhesion molecule expression followed by a later (72 hours) decline in neutrophil numbers.

AMPKα2 Regulates Neutrophil Survival Under Hypoxia

Circulating neutrophils are short-lived cells, but their life span can be prolonged under hypoxic conditions. The decreased recovery of neutrophils from ischemic hindlimbs 3 days after ligation hinted at an increased apoptosis of AMPKα2ΔMC neutrophils. To determine which AMPKα2-regulated proteins were altered by hypoxia, bone marrow–derived neutrophils were isolated from wild-type and

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AMPKα2ΔMC littermates and maintained under normoxic or hypoxic conditions for 16 hours. Thereafter, altered protein expression was determined by mass spectrometry. Online Table I summarizes all of the proteins identified, number of identified peptides, accession numbers, and sequence coverage of each sample. The quantification of the results is summarized in Online Table II.

Fitting with the in vivo observations, neutrophils from AMPKα2ΔMC mice expressed lower levels of the antiapoptotic proteins: translocase of inner mitochondrial membrane 50 and apoptosis inhibitor 5 (Figure 5A and 5B). These changes were accompanied by an increase in caspase-3 cleavage and Bax expression (Figure 5C) and an increase in caspase activity in neutrophils lacking AMPKα2 (Figure 5D). Thus, the expression of the AMPKα2 in neutrophils is required for protection against apoptosis in hypoxic conditions.

Figure 3. Myeloid cell infiltration into the ischemic hindlimb 72 h post surgery. A, Representative hematoxylin–eosin images and quantification of infiltrated cells into gastrocnemius muscle. Bar=100 μm. The graph summarizes data from 5 animals per group. B, CD45+, Gr1+, and F4/80+ cells isolated from the gastrocnemius muscle of the ligated and nonligated hindlimbs from wild-type (WT) and AMPKα2ΔMC (ΔMC) 3 d after ligation (n=5 per group). C, Chemokine and cytokine gene expression analysis in ischemic hindlimbs. Green bars show significantly (P<0.05) downregulated genes, and red bars indicate significantly upregulated genes in AMPKα2ΔMC compared with WT mice (n=4 animals in each group). D, Intercellular adhesion molecule (ICAM)-1 and CD31 immunostaining in the gastrocnemius muscle. Bar=20 μm. *P<0.05, **P<0.01, ***P<0.001. AMPK indicates AMP-activated protein kinase.

AMPKα2 Regulates Hypoxia-Inducible Factor-1α Hydroxylation and Stabilization and Hypoxia-Inducible Factor-1α–Dependent Responses in AMPKα2-Deficient Neutrophils

The most prominent difference in the proteome of neutrophils from the 2 strains was that the neutrophils lacking AMPKα2 demonstrated an impaired response to hypoxia (Figure 6A; Online Figure VI). This finding fits with reports that myeloid cell function is strongly dependent on and mediated by hypoxia-inducible factor (HIF)-1α, which is crucial for prolonging neutrophil survival under hypoxic conditions.19

HIF-1α protein levels can be modified by several kinases,20 and previous studies in a prostate cancer cell line indicated that global AMPK inhibition may attenuate hypoxia-induced responses such as HIF-1α target gene expression.21 Therefore, we determined whether or not AMPK can affect HIF-1α stability in HEK293 cells, which express both the AMPKα1 and α2...
therefore, we determined the ability of AMPK after exposure of cells to hypoxia (1% O2, 6 and 16 hours) or levels were not detectable, but the protein was clearly induced hydroxylase domain (PHD)–containing enzymes, which hy-
mediated phosphorylation of HIF-1α; however, we were unable to confirm the AMPK-
ction factor-1, was decreased in lipopolysaccharide-stimulated neu-
 striking the e

cubation of cells exposed to hypoxia (1% O2, 6 and 16 hours) or cobalt chloride (150 μmol/L, 6 hours; Figure 6B). Although the downregulation of the AMPKα subunit had little effect on the induction of HIF-1α by either stimulus, HIF-1α levels were con-
sistently lower in HEK293 cells treated with small interfering RNA directed against the AMPKα subunit. The serine phos-
phorylation of HIF-1α has been linked with its destabilization22,23; therefore, we determined the ability of AMPKα2 to phosphoryl-
late a recombinant HIF-1α peptide (corresponding to amino acids 1–735) in vitro. We found that AMPKα2 was able to elicit the serine but not the threonine phosphorylation of HIF-1α (Online Figure VII); however, we were unable to confirm the AMPK-
mediated phosphorylation of HIF-1α in isolated neutrophils.

HIF-1α protein levels are barely detectable in normoxic conditions largely because of the high activity of the prolyl hydroxylase domain (PHD)–containing enzymes, which hydroxylate HIF-1α (on Pro564) thus promoting its subsequent proteasome-mediated degradation.24 In response to hypoxia, PHD enzymes and the degradation of HIF-1α are inhibited. Interestingly, the downregulation of AMPKα2 enhanced the hydroxylation of HIF-1α on proline 564 in cells exposed to hypoxia, indicating that the effect of AMPKα2 on HIF-1α may be indirectly regulated via PHD activity. Indeed, treatment with the PHD inhibitor dimethylglyoxal-glycine restored HIF-
α levels in cells treated with AMPKα2 siRNA (Figure 6C). This implies that AMPKα2 regulates HIF-1α stabilization under hypoxia by interfering with its hydroxylation and thereby inhibiting its proteasomal degradation. Consistent with the observed effects on HIF-1α destabilization, the expression of the hypoxia-regulated genes, VEGF and stromal cell–derived factor-1, was decreased in lipopolysaccharide-stimulated neutrophils from AMPKα2ΔMC mice (Figure 7A). In the same cells, the expression of the proinflammatory markers, tumor necro-
sis factor-α and IL-1β, was increased (Figure 7B) and fit well with the altered cytokine and growth factor expression profile detected in the ischemic muscles (Figure 3C). Neutrophil recrui-
tment to ischemic tissues involves several secreted factors including cathelicidin-related antimicrobial peptide (LL-37 in humans) and CD147 (basigin), both of which were increased in hypoxic neutrophils from wild-type mice but not in those lacking the α2 subunit (Figure 7C and 7D).

**AMPKα2 Regulates HIF-1α Hydroxylation by Modulating α-Ketoglutarate Levels**

Because HIF-1α also regulates the cellular metabolic state, we analyzed metabolic parameters in isolated neutrophils. Under normoxic conditions, the deletion of AMPKα2 in neutrophils was associated with increased oxygen consumption, both in the absence of stimulation and when maximally stimulated with FCCP (Figure 7F; Online Figure VIII A through VIII E). The latter fits well with an increased phorbol ester–induced generation of reactive oxygen species (Figure 7G). There was no difference in glycolysis in neutrophils from the 2 strains under the conditions studied (Online Figure VIII F). Hypoxia is a potent activator of AMPK, and because the Seahorse analyses could not be performed under hypoxic conditions, mass spectrometry was used to assess metabolic alterations in hypoxic neutrophils. Consistent with the extracellular acidification data, there were no detectable differences in glucose or lactate levels between the 2 strains of neutrophils maintained in normoxic or hypoxic conditions (Online Figure VIII G through VIII J).

Proteomic analysis revealed a significant, hypoxia-induced decrease in 2 isoforms of the isocitrate dehydrogenase (IDH), that is, cytosolic IDH1 and mitochondrial IDH3b (Figure 8A and 8B). In the same cells, metabolomic analyses revealed
that although the IDH product α-ketoglutarate decreased in wild-type neutrophils exposed to hypoxia, it failed to do so in hypoxic neutrophils from AMPKα2ΔMC mice (Figure 8C). α-Ketoglutarate is required as a cofactor for PHD enzymes and the subsequent hydroxylation of HIF-1α. The hydroxyla-
duced decrease in α-ketoglutarate production prevents the hydroxylation and degradation of HIF thus facilitating the induction of HIF-1α-regulated genes. Certainly, incubating human leukemic monocyte THP-1 cell line with octyl-α-
ketoglututarate, a cell-permeable ester of α-ketoglutarate, was sufficient to abrogate HIF-1α expression in cells exposed to hypoxia (Figure 8D).

Discussion

The results of the present study indicate that the AMPKα2 sub-
unit is important in determining neutrophil survival and function in ischemic tissue. The mechanisms involved are related to the stabilization of HIF-1α protein by preventing its hydroxylation under hypoxia and the expression of a network of proteins—many of which affect mitochondrial function and cell survival. In the absence of these mechanisms, neutrophil survival is attenuated, and the recruitment of proangiogenic monocytes to ischemic areas is reduced, thus impairing the processes of arteriogenesis and angiogenesis (Online Figure IX).

Limb ischemia as a consequence of vascular injury initiates a series of events that involve an inflammatory phase, character-
ized by cell infiltration and the initiation of angiogenesis, followed by a resolution phase. The processes involved are com-
plex, but many pharmaceutical tools, such as metformin, have been reported to improve vascular repair, at least in wild-type mice.25 One key signaling event that is altered in diabetes mellitus and targeted by metformin is the activation of the AMPK.26 To determine the importance of the AMPK in vascular repair, the recovery of blood flow after ischemia was assessed in wild-
type and AMPKα-deficient mice. The focus of the studies was the AMPKα2 subunit, given its reported link to diabetes melli-
tus24 and its activation by hypoxia.25 We found that the recovery of blood flow was significantly impaired in ischemic hindlimbs from animals completely deficient in the AMPKα2 subunit. Interestingly, targeting AMPKα2 in endothelial cells did not im-
pair vascular repair, whereas its deletion in myeloid cells repro-
duced the effects observed in the AMPKα2 mice. Certainly, the AMPKα2 isoform was clearly expressed in CD11b+ cells, and in animals lacking AMPKα2 in the myeloid lineage (AMPKα2ΔMC mice), arteriogenesis and angiogenesis in the ischemic hindlimb were substantially reduced. The myeloid cell–specific deletion of the AMPKα1 subunit also delayed the recovery of blood flow after femoral artery ligation, but the defect was much less pro-
nounced than that in the AMPKα2ΔMC mice. Although in our study the role of AMPKα2 deletion in neutrophils was more pronounced, it is possible that the AMPKα1 isoform plays a signifi-
cant role in the regulation of monocyte function as described in an article published during the revision of this article.27

Consistent with a previous report that the deletion of the AMPK had no effect on normal hematopoietic stem cell differ-
entiation or function,17 the myeloid cell–specific deletion of the AMPKα2 was without effect on hematopoietic stem cell differ-
entiation. Interestingly, circulating levels of neutrophils were attenuated by the deletion of the AMPKα2 under basal condi-
tions but were comparable in wild-type and AMPKα2ΔMC mice 24 hours after femoral artery ligation indicating normal neutrophil generation and mobilization.

Although neutrophils are early infiltrating cells that control critical steps during angiogenesis, collateral growth, and per-
fusion recovery, they have a short half-life within the ischemic tissue.24,25 Comparing the numbers of neutrophils that infiltrat-
ed ischemic skeletal muscle 1 and 3 days after femoral artery ligation revealed that despite increased neutrophil infiltration in ischemic AMPKα2ΔMC hindlimbs after 24 hours, the situation was reversed after 72 hours. These observations indicated that neutrophil survival was attenuated in the absence of the
AMPKα2 subunit. Certainly, AMPK activation has previously been linked with neutrophil survival, and in this study, apoptosis was increased in AMPKα2-deficient neutrophils. Some of the proteins significantly altered in hypoxic AMPKα2ΔMC versus wild-type neutrophils were linked to apoptosis, in particular translocase of inner mitochondrial membrane 50, a component of the mitochondrial translocator, and apoptosis inhibitor 5, a negative regulator of E2F1-dependent cell death. Little is known about the role played by either protein in neutrophils, but the loss of translocase of inner mitochondrial membrane 50 has also been reported to increase apoptosis in breast cancer cells.

Part of the arteriogenic/angiogenic response can be attributed to the liberation of neutrophil-derived VEGF, matrix...
metalloproteinases, and other proangiogenic growth factors, but these cells also release factors such as cathelicidins that promote the recruitment of monocytes, which then amplify the proangiogenic factors in hindlimbs from AMPK mice. How can the increase in neutrophil infiltration at 24 hours be accounted for? This may be related a combination of enhanced neutrophil migratory activity and crosstalk between the neutrophils and the vasculature. Certainly, the expression of adhesion molecules such as intercellular adhesion molecule-1 was increased in hindlimbs from AMPK mice, hinting that endothelial cell activation was enhanced. This is a neutrophil-dependent effect as endothelial cells from wild-type and AMPK mice responded to IL-1β and lipopolysaccharide stimulation with similar increases in adhesion molecule expression (R. Abdel Malik et al, unpublished data, 2016).

The inhibition of neutrophil apoptosis in a hypoxic environment is dependent on the activation of HIF-1α and interactions between HIF-1α and AMPK have been reported. Indeed, increased AMPKα expression and activity are paralleled by phosphorylation. We found that the downregulation of the AMPKα2 but not the AMPKα1 subunit attenuated the increase in HIF-1α levels induced by either hypoxia or cobalt chloride. In agreement with our in vitro observations, the expression of the HIF-1α-regulated factors, stromal cell–derived factor-1 and VEGF, was attenuated in neutrophils from AMPKα2 mice. AMPK has also been proposed to regulate HIF-1α protein stability by phosphorylation. However, even though we could confirm these findings in an in vitro assay, we were unable to detect an AMPKα2-dependent phosphorylation of HIF-1α in intact cells. Instead, it was possible to demonstrate that the deletion of the AMPKα2 subunit enhanced the hydroxylation of HIF-1α on Pro564 in cells exposed to hypoxia. The latter response is indicative of increased PHD activity and proteasomal degradation. Indeed, treating AMPKα2-deficient neutrophils with a PHD inhibitor prevented the increased hydroxylation and rescued HIF-1α induction.

Unlike the AMPKα1 subunit, the AMPKα2 can translocate to the nucleus and affect gene and protein expression by several mechanisms. Mass spectrometry was used to ascertain which neutrophil proteins could be affected by AMPKα2 deletion and revealed that although wild-type neutrophils exposed to hypoxia increased expression of the granule protein cathelicidin-related antimicrobial peptide (LL-37 in humans) and CD147 (basigin or extracellular matrix metalloproteinase inducer), effects were blunted in AMPKα2−/− neutrophils. The latter proteins were of interest as both are regulated by HIF-1α and both are involved in neutrophil recruitment, invasion, and survival in ischemic tissues. Of interest for vascular repair is that CD147 is reportedly required for the increased synthesis of VEGF and matrix metalloproteinase-9, thus, the lower levels of matrix metalloproteinase-9 in the AMPKα2−/− neutrophils may be related to their failure to increase CD147 expression.

Prolyl hydroxylases require oxygen and the electron donor α-ketoglutarate to execute the hydroxylation and inactivation of HIF-1α. Neutrophil adaption to hypoxia and the stabilization of HIF-1α, therefore, require accompanying changes in metabolism and a decrease in α-ketoglutarate production. Isocitrate dehydrogenases catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate, and 2 IDH isoforms, that is, IDH1 and IDH3b, were differentially regulated by hypoxia in neutrophils from AMPKα2−/− mice. Specifically, the decrease in IDH expression detected in wild-type neutrophils exposed to hypoxia was blunted in AMPKα2−/− neutrophils. The latter proteins were of interest as both are regulated by HIF-1α and both are involved in neutrophil recruitment, invasion, and survival in ischemic tissues. Thus, the lower levels of matrix metalloproteinase-9 in the AMPKα2−/− neutrophils may be related to their failure to increase CD147 expression.
hypoxia failed to occur in AMPKα2-deficient neutrophils. The changes in IDH expression were reflected in α-ketoglutarate levels, which were decreased by hypoxia in wild-type neutrophils but remained elevated in neutrophils from AMPKα2-/- mice. It is interesting to note that higher IDH1 levels have been linked with increased HIF-1α degradation and the sensitization of melanoma cells to hypoxia-induced apoptosis, effects that parallel the fate of AMPKα2-deficient neutrophils. Moreover, inhibition of IDH by oxalocalic acid or IDH knockdown was reported to elevate HIF-1α levels. It, therefore, seems that the links between AMPKα2 and HIF-1α are complex and may be regulated at more levels than the direct phosphorylation of the transcription factor.

Taken together, these observations indicate that the activation of AMPKα2 in neutrophils is a decisive event in the initiation of vascular repair after ischemia. It is tempting to speculate that dysregulation of AMPK signaling, such as is the case in diabetes mellitus, may attenuate vascular repair by a combination of effects on HIF-1α stability mediated by alterations in phosphorylation and myeloid cell metabolism.

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Disclosures

None.

References

Neutrophil AMPKa2 Controls Vascular Repair

Novelty and Significance

Limb ischemia, as a consequence of vascular injury, initiates a series of events that involve an inflammatory phase, characterized by cell infiltration and the initiation of angiogenesis, followed by a resolution phase. Neutrophils are early infiltrating cells that liberate several key factors that promote the subsequent recruitment of monocytes, which then amplify the arteriogenic and angiogenic processes. This study shows that the activation of AMPKa2 in neutrophils is required for arteriogenesis and angiogenesis in the ischemic hindlimb. In its absence, neutrophils fail to survive to recruit monocytes to the ischemic region, and as a consequence, the ischemic area is maintained in an inflammatory rather than reparative resolution of inflammation.

What Is Known?
- Myeloid cells are rapidly recruited to ischemic tissue and are required for the initiation of reparative vascular repair.
- Neutrophils are the first cell population to infiltrate ischemic tissue and secrete factors that recruit a second wave of monocytes.
- Neutrophils are short lived, but their half-life is prolonged in ischemic tissue by increased expression of the hypoxia-inducible factor (HIF)-α.
- HIF-1α protein levels are barely detectable in normoxic conditions because of the high activity of the prolyl hydroxylase domain-containing enzymes that hydroxylate HIF-1α and promote its degradation.

What New Information Does This Article Contribute?
- Global deletion of the AMP-activated protein kinase (AMPKa2) subunit or its specific deletion in myeloid cells abrogate arteriogenesis and angiogenesis and prevents the recovery of hindlimb blood flow after femoral artery ligation.
- Activation of the AMPKa2 subunit in neutrophils is essential for their survival in the ischemic hindlimb and subsequent monocyte recruitment.
- In the absence of AMPKa2, the changes in neutrophil protein expression elicited by hypoxia are attenuated, a phenomenon attributed to the failure to stabilize HIF-1α protein expression.
- In hypoxic conditions, AMPKa2 activation leads to a decrease in the expression of 2 isoforms of the isocitrate dehydrogenase and the generation of α-ketoglutarate, which is required as a cofactor for prolyl hydroxylase domain enzymes and the subsequent HIF-1α hydroxylation.
AMP-Activated Protein Kinase α2 in Neutrophils Regulates Vascular Repair via Hypoxia-Inducible Factor-1 α and a Network of Proteins Affecting Metabolism and Apoptosis

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Materials

RPMI 1640 and fetal calf serum (FCS) were from Gibco (Invitrogen, Karlsruhe, Germany), G-CSF was from Amgen (Gauting, Germany), MethoCult M3434 from Stem Cell Technologies (Köln, Germany) and collagenase II from Worthington (Troisdorf, Germany). All other materials were obtained from Sigma (Munich, Germany) or Applichem (Darmstadt, Germany).

Animals

Genetically modified mice lacking either the AMPKα1 or the AMPKα2 subunits, their respective wild-type littermates and floxed AMPKα1 and α2 mice were kindly provided by Benoit Viollet (INSERM, U1016, Paris, France) and bred at the Goethe University Hospital animal facility. Floxed AMPKα2 mice were crossed with animals expressing the Cre-deleter under the control of the Tie-2 promoter (B6.Cg-Tg(Tek-cre)12Flv/J; Jackson Laboratories, Bar Harbor, USA) to generate mice lacking the α2 subunit in endothelial cells and some myeloid cells (Tie2-α2) or with VE-cadherin-Cre mice (B6-Tg(Cdh5-cre)/J; Polygene, Switzerland) to generate animals lacking the α2 subunit specifically in endothelial cells (AMPKα2ΔEC). Floxed AMPKα1 and α2 mice were bred with LysM-Cre mice (B6.129P2-Lyz2tm1(cre)Ifo/J; Jackson Laboratories) to generate mice lacking the AMPKα1 or α2 subunit specifically in myeloid cells.

All animals were housed in conditions that conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication No. 85-23). Both the University Animal Care Committee and the Federal Authority for Animal Research at the Regierungspräsidium Darmstadt (Hessen, Germany) approved the study protocol (#F28/25). For the isolation of organs, mice were sacrificed using 4% isoflurane in air and subsequent exsanguination or decapitation.

Blood Count

Blood was harvested (cardiac puncture) with a syringe containing 10% (vol/vol) EDTA buffer (10 mg/mL) to prevent clotting. Blood profiles were analyzed using a Vetscan HM5 (Abaxis, Griesheim, Germany) or by flow cytometric analysis on a BD FACSVersus flow cytometer (BD, Heidelberg, Germany). CD45 was used for total leukocyte number, myeloid cells were defined as CD45+ and CD11b+, neutrophils as CD45+ Ly6G+ cells, T-cells as CD45+ CD3+ and B-cells as CD45+ CD19+ cells. For the identification of leukocyte-platelet or neutrophil-platelet aggregates CD45 CD61 or Ly6G CD61 double positive cells were gated.

Stem and Progenitor Cell Populations

Mice were administered G-CSF (subcutaneous injection, 250 μg/kg/day, 4 days) and stem and progenitor cell populations in bone marrow were analyzed by flow cytometry (BD BIOS Aria or FACS Cantoll (BD Bioscience). Lineage staining: cKit (CD117; 2B8) BV421 (Biolegend, Munich, Germany), CD11b (M1/70) PE (Biolegend), Ly6G (1A8) A647 (eBioscience, Frankfurt, Germany), B220 (CD45R; RA3-6B2) BV510 (Biolegend), CD3 (145-2C11) PE-Cy7 (eBioscience), Ter119 (TER-119) APC-eF780 (eBioscience), CD71 (R7217) FITC (Biolegend), Fc block (STEMCELL Technologies). Stem and Progenitor staining: CD3 (145-2C11), CD19 (1D3), CD41 (MwReg30), Ter119 (TER-119), B220 (RA3-6B2), CD11b (M1/70), Gr-1 (RB6-8C5) (all biotinylated, eBioscience); Streptavidin APC-eF780 (eBioscience), Sca-1 (D7) PB or PerCP-Cy5.5 (eBioscience), CD117 (2B8) PE-Cy7 (eBioscience), CD150 (TC15-12F12.2) PerCP-Cy5.5 or PE (eBioscience), CD48 (HM48.1) FITC (Biolegend), CD34 (RAM34) eF660 (eBioscience).

Colony Forming Unit Assay

After 4 days of G-CSF treatment 100µl of peripheral blood was obtained from the tail vein, transferred into tubes (buffered with 10% (vol/vol) EDTA (10 mg/mL) and erythrocytes were
lysed. The resulting cell suspension was carefully mixed with 3 mL of pre-warmed methylcellulose media (M3434, STEMCELL Technologies) supplemented with growth factors and plated on 35 mm dishes. After 6 days colonies formed were counted with an inverted microscope.

**Hindlimb Ischemia**

Arteriogenic and angiogenic capacity was investigated in a murine model of hindlimb ischemia using 6 to 8 week old wild-type and transgenic animals as described. Briefly, the deep femoral artery was ligated using an electric coagulator (ERBOTOM ICC50, ERBE). Afterwards the superficial femoral artery and vein as well as the epigastric arteries were completely excised. The overlying skin was closed with 3 surgical staples. Relative blood flow was determined by laser Doppler imaging (Laser Doppler Perfusion Imager System, Wilmington, Germany) at regular intervals for up to 28 days post-ligation. The perfusion of the ischemic and non-ischemic limb was calculated on the basis of colored histogram pixels. To minimize variables including ambient light and temperature and to maintain a constant body temperature, mice were exposed to infrared light for 10 minutes before laser Doppler scans. Perfusion was expressed as the ratio of the ischemic to the non-ischemic hindlimb.

**µ-CT Analyses**

Mice were sacrificed and intravascular perfused through the aorta using a roller pump with 20 mL of a heparinized NaCl solution supplemented with sodium nitroprusside at a rate of 5 mL/minute. The perfusion was immediately followed by perfusion with a solution of 1% paraformaldehyde in 0.9% NaCl for prefixation of the vasculature. Mice were then perfused (5 mL in 2 minutes) with a contrast agent consisting to 20% of a BaSO4 solution (Micropaque, Guerbet, Villepint, France) and 80% polyurethane (Dispercoll, Bayer, Leverkusen, Germany). Fixation was achieved with 50% formic acid for 5 minutes. Mice were stored overnight in a 4% PFA solution (4°C) and were subsequently scanned using a micro-CT (Skyscan 1176, Bruker micro-CT, Kontich, Belgium) at 50kV X-ray with a 0.5 mm aluminum filter, 9 μm isotopic resolution and 7 projection images per 0.3° rotation step. Data were reconstructed with the NRecon/ InstaRecon CBR Server software (Skyscan, Kontich, Belgium/ InstaRecon, Champaign, Illinois, USA). Image analysis and segmentation were performed with the Imalytics Preclinical Software (Gremse-IT, Aachen, Germany).

**Immunohistochemistry**

Semimembranosus and gastrocnemius muscles were embedded in TissueTek OCT Compound (Sakura, Staufen, Germany) and immediately frozen in dry ice. Transverse cryosections (10 µm) were cut at 3 different sites of the muscle (upper, middle and lower). Sections were dried at 37°C for 10 minutes and fixed in a solution of 4% PFA in PBS at room temperature for 15 minutes. After washing in PBS, samples were incubated with blocking buffer (PBS containing 3% BSA and 0.03% Triton X-100) at room temperature for 45 minutes, followed by incubation with anti-CD31 antibody, overnight at 4°C. After extensive washing and exposure to the secondary anti-rat antibody and anti-smooth muscle actin-Cy3 (1 hour at room temperature) sections washed and covered with fluorescent mounting medium (Dako) and analyzed using a confocal microscope (Zeiss, LSM 780). For each section, 5 non-overlapping pictures were taken and evaluated. For the visualization of infiltrating cells and ICAM expression, hindlimb muscles (M. semimembranosus and M. gastrocnemius) were placed in zinc fixative solution for 24 hours, dehydrated and cleared in ethanol and xylol and embedded in paraffin. Sections were cut in 10µm slices using a microtome and stretched by floating in 40°C warm water bath. Slices were dried on a heating plate at 37°C, deparaffinized and rehydrated in a series of xylol (5 minutes), 100 % ethanol (3 minutes), 96 % ethanol (3 minutes), 80 % ethanol (3 minutes), 70 % ethanol (3 minutes). After washing in PBS samples were incubated with blocking buffer (PBS containing 10% horse serum, 1% BSA and 0.3% Triton X-100) at room temperature for 2 hours before
being exposed to primary antibody (4°C, overnight). After extensive washing and exposure to
the appropriate secondary antibody (1 hour at room temperature) samples were mounted in
fluorescent mounting medium (Dako) and analyzed with a confocal microscope (Zeiss, LSM
780).

Antibodies: Anti-CD144 (1:300, SC6458) was from Santa Cruz (Heidelberg, Germany), ICAM
(1:100; Santa Cruz, sc-1511) anti-Ly6G (1:100 rat) and anti-CD31 (1:200, rat) were from BD
Pharmixing and anti-alpha-smooth muscle actin-Cy3 (1/1000) was from Sigma.

Whole Mount Immunofluorescence

One day after femoral artery ligation mice were sacrificed using 4% isoflurane in air and animals
were perfused with 5 mL NaCl (0.9%) and 5 mL formalin (4%). Semimembranosus and
gastrocnemius muscles were taken and fixed for 24 hours in 4% formalin. Tissue was then
permeabilized for 1 hour with 1% SDS in PBS, extensively washed in PBS, then incubated with
glycine (100 mmol/L, 3 times for 15 minutes). After blocking for 3 hours (0.1% BSA, 0.2% Triton
X-100, 0.05% Tween 20, 10% goat serum in PBS, 37°C) the tissue was incubated for 40 hours
in primary Ly6G antibody (rat anti mouse BD 1:100, 37°C). After washing in PBS secondary
antibodies (1% BSA in PBS) were applied together with smooth muscle actin (CY3 Sigma,
1:1000) and DAPI (20 µg/ml) and incubated for a further 16 hours (37°C). After washing 4 times
with PBS the tissue was transferred into CUBIC2 solution.3 Pictures were taken as Z-stack with
a confocal microscope (Zeiss, LSM 780) with a 40x objective (EC-Plan-Neofluar 40x1.3 Oil DIC
M27).

RNA Isolation and Quantitative Real Time PCR (RT-qPCR)

Total RNA was extracted using TRIzol (Sigma-Aldrich). For the generation of
complementary DNA (cDNA) total RNA (1 μg) was reverse transcribed using the SuperScriptIII
(Life Technologies GmbH, Darmstadt, Germany) and random hexamer primers according to the
manufacturer’s protocol. The amount of mRNA was quantified using the cycle threshold (cT)
value using a SYBR green master mix (ABgene, Dreieich, Germany) with intron spanning
primers in a Mx4000 multiplex qPCR system (Stratagene, Heidelberg, Germany). C_T values
obtained were converted into relative amounts on the basis of a standard curve and mRNA
levels were normalized to 18S rRNA. For the quantification of gene expression by RT-qPCR the
following primers were used: 18S rRNA forward (5’-CTTGTGCTGCTCGCTC-3’), 18S rRNA
reverse (5’-CGACTGGACCTGCTTCTGCTGTA-3’), VEGF forward (5’-GAATTGATCCTCTCAGCCAGC-3’),
VEGF reverse (5’-GGCTGGATCGCTTGCTGCTGTA-3’), TNFα forward (5’-GGCGACCTGCTTCTGCTGTA-3’),
TNFα reverse (5’-CCGGCCCTCCAATAAATAC-3’), Interleukin-1β forward (5’-CAGGCCAGCAGATCAGTCCTCA-3’),
Interleukin-1β reverse (5’-AGCTCATATGGTCCGACAGC-3’), MMP9 forward (5’-GAAGGCACCCCGTCTGTT-3’)
reverse (5’-AGACTGACTGCTTGCCCAGGA-3’) and SDF1α primer set from Qiagen (PPM02965E,
Heidelberg, Germany). For the mRNA expression profiling of the angiogenic markers total RNA
was isolated from the M. semimembranosus or M. gastrocnemius of the control and ischemic
limbs 3 days after the femoral artery ligation and qRT-PCR was performed using a custom array
(Qiagen).

Immunoblotting

Cells were lysed in Triton X-100 buffer and detergent-soluble proteins were solubilized in SDS-
PAGE sample buffer, separated by SDS-PAGE and subjected to Western blotting as described.4
Proteins were visualized by enhanced chemiluminescence using a commercially available kit
(Amersham, Freiburg, Germany). The antibodies against AMPKα2 (1:1000 A-20 goat), Bax
(1:1000, sc-493), Api5 (1:1000) and phospho-serine (1:1000, AB1603) were from Santa Cruz
Biotechnology (Heidelberg, Germany). The antibody against AMPKα1 (1:1000, 8056) was from
Eurogentec (Seraing, Belgium), anti-β-actin (1:5000, A5541) was from Sigma, anti-non-muscle
myosin IIA (1:2000, ab24762) from ABCAM (Cambridge, UK), anti-Tim50 (1:500, ab23938, goat) was from ABCAM, anti-CRAMP (1:100, PA-CRPL-100) from Innovagen (Lund, Sweden), anti-IDH1 (1:1000, NBP2-32150) from Novus Biologicals (Abingdon, United Kingdom), anti-IDH3b (1:1000, ab121016) from ABCAM, anti-cleaved caspase-3 (1:800, 9662) from New England Biolabs GmbH (Frankfurt, Germany), anti-HIF-1α (1:800, 610959) from BD Pharmingen (Heidelberg, Germany), anti-hydroxy Pro564-HIF-1α (1:800, NB110-74679) from Novus Biologicals and anti-phospho-threonine (1:1000, AB1607) from Merck Millipore (Darmstadt, Germany).

**Flow Cytometric Analysis of Infiltrated Immune Cells**

One and three days after femoral artery ligation mice were sacrificed and perfused with 15 mL NaCl solution containing 5 U/mL heparin. Intact hindlimb muscles (M. semimembranosus) were removed and minced carefully. Single cell suspension was generated from the entire muscle by subsequent digestion in DMEM/F12 containing 300 U/mL Collagenase Type II at 37°C for 60 minutes. Cell suspension was passed through a 70 µm filter and washed in PBS. For flow cytometric analysis cells were incubated in Fc block solution (CD16/32 1/100 in PBS) to prevent non-specific antibody binding. Staining with corresponding antibodies were performed in PBS containing 2% FCS and 1 mmol/L EDTA and analyzed on a BD FACSVersa flow cytometer. Neutrophils were defined as CD45+, CD11b+ and Gr1+ cells, macrophages were defined as CD45+, CD11b+ and F4/80+ cells.

**Neutrophil Isolation**

Murine bone marrow cells were obtained from femurs and tibias and polymorphonuclear neutrophils (PMNs) were either isolated by using the EasySep™ Mouse Neutrophil Enrichment Kit (Stemcell Technologies, Köln, Germany) according to the manufacturer’s protocol, or by discontinuous Percoll gradient (52%/64%/72%) centrifugation at 1000 g for 30 minutes and 4°C. Neutrophils were harvested from the 64%/72% interface, washed in PBS, and maintained in RPMI 1640 medium supplemented with 10% FCS as indicated in the results section. Neutrophil viability was greater than 95% as assessed by the trypan blue exclusion test and purity was greater than 98% as analyzed by microscopy using Hemacolor staining (Merck, Darmstadt, Germany).

**In vitro Transendothelial Migration**

Mouse lung endothelial cells were grown to a confluent monolayer on a fibronectin coated transwell filter (3µm pore size; BD Pharmingen). Endothelial cells were stimulated with 10 ng/mL tumor necrosis factor α (Peprotech, Hamburg, Germany) for 5 hours. After careful washing neutrophils (5x10⁵ cells) were added on top of the endothelial cell monolayer and allowed to migrate towards a stromal cell-derived factor (SDF1) 1α gradient (100 ng/mL; Peprotech, Hamburg, Germany) for 2 hours. Cells that migrated to the lower chamber were collected and counted by flow cytometry (BD FACSVersa).

**AMPK Downregulation**

HEK293 cells were transiently transfected with either control oligonucleotides or small interfering RNA (siRNA) directed against the AMPKα1 or AMPKα2 subunits (Eurogentec) using Lipofectamin 2000 (Invitrogen, Göttingen, Germany) according to the manufacturer’s protocol.

**In vitro Phosphorylation Assay of HIF-1α**

Human recombinant HIF-1α (100 ng per sample; ABCAM) was incubated with human recombinant AMPKα2 (150 µg per sample; Calbiochem, Darmstadt, Germany) in the presence of ATP for 30 minutes at 37°C. Samples without HIF-1α or AMPKα2 addition were used as controls. The kinase reaction was stopped and phosphorylation was assessed by SDS-PAGE.
Oxidative Phosphorylation and Glycolytic Flux Analyses

Neutrophils were seeded at 5x10^5 cells per well on Seahorse XF96 polystyrene tissue culture plates (Seahorse Bioscience, Boston, USA) in RPMI 1640 medium with 10% FCS. After three hours medium was changed to unbuffered DMEM assay medium for 1 hour before measuring in an XFe 96 extracellular flux analyzer (Seahorse Bioscience). Inhibitors and activators were used at the following concentrations: oligomycin (1 µmol/L), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; 5 µmol/L), rotenone (5 µmol/L), antimycin A (5 µmol/L), glucose (10 mmol/L) and 2-deoxy-D-glucose (100 mmol/L). Each measurement was averaged from triplicate values.

Assay of Reactive Oxygen Species production

Neutrophils (1x10^6 cells/mL) were incubated with 2′,7′-dichlorofluorescein diacetate (Life Technologies, Darmstadt, Germany) and with or without phorbol 12-myristate 13-acetate (PMA, 100 ng/mL) for 20 minutes at 37°C. To stop the reaction cells were washed with ice-cold PBS and stored on ice until flow cytometric analysis. The production of reactive oxygen species was quantified by assessing the mean fluorescence intensity (excitation λ=492-495nm, emission λ =517-527nm).

Caspase-3/7 Activity Assay

Neutrophils (1.5x10^5 cells per well) were seeded onto a fibronectin coated 96 well plate in RPMI 1640 containing 10% FCS and caspase 3/7 reagent (5 µmol/L; Essen Bioscience, Michigan, USA). The pan caspase inhibitor Z-VAD-FMK (10 µmol/L; Enzo Life Sciences, Lörbach, Germany) was added to some wells as a control. Fluorescence was assessed using an automated imaging system (IncuCyte system 2010A; Essen Bioscience). Green fluorescence and phase contrast pictures were analyzed using ImageJ (version 1.47 National Institutes of Health). Each value was averaged from triplicates.

THP-1 cell culture

Monocytic THP-1 cells were differentiated to macrophages using PMA (10 nmol/L) in RPMI medium supplemented with heat-inactivated fetal calf serum (10%), sodium pyruvate (1 mmol/L), penicillin (100 U/mL) and streptomycin (100 μg/mL). After 5 days, medium was changed to PMA-free medium and the differentiated cells were then incubated with or without octyl-α-ketoglutarate (Cayman Europe, Hamburg, Germany) under normoxic or hypoxic (1% O_2) conditions for 4 hours.

Proteomics: Sample Preparation

Pellets of isolated neutrophils were solubilized in 10% SDS, 150 mmol/L NaCl, 100 mmol/L Tris/HCl pH 7.6, 100 mmol/L DTT. Samples were sonicated for 5 seconds and heated at 95°C for 5 minutes to facilitate protein solubilization. Samples were then incubated at 56°C for 30 min and centrifuged to remove insoluble material. Total protein (100 µg per sample) was diluted by adding 200 µL, 8 mol/L urea, 50 mmol/L Tris/HCl, pH 8.5 and loaded onto spin filters with a 30 kDa cut off (Microcon, Merck/Millipore, Darmstadt, Germany), and prepared as described. Proteins were digested overnight with trypsin (sequencing grade, Promega, Mannheim, Germany) and eluted peptides were acidified by trifluoroacetic acid to a final concentration of 0.1 % and fractionated on multi-stop-and-go tips (StageTips) containing three strong cation exchange (SCX) disks and a stack of three C18-disks on top. SCX fractionation by StageTips was performed in four steps as described. The first and second fractions were combined and all three fractions of each sample were eluted in wells of microtiter plates. Peptides were dried and resolved in 1% acetonitrile and 0.1 % formic acid.
Proteomics: Liquid Chromatography/Mass Spectrometry (LC/MS)

LC/MS was performed using a Q Exactive Plus (Thermo Scientific, Dreieich, Germany) equipped with an ultra-high performance LC unit (Dionex Ultimate 3000, Thermo Scientific) and a Nanospray Flex Ion-Source (Thermo Scientific). Peptides were loaded on a C18 reversed-phase precolumn followed by separation on a 2.4 μm reprosil C18 resin (Dr. Maisch GmbH, Ammerbuch, Germany) in-house packed picotip emitter tip (diameter 100 μm, 15 cm long from New Objectives, Woburn, USA) using a gradient from mobile phase A (4% acetonitrile, 0.1% formic acid) to 44 % mobile phase B (80% acetonitrile, 0.1% formic acid) for 105 minutes with a flow rate 500 nL/minute. Runs were finished by column washout with 99% mobile phase B for 5 minutes and re-equilibration in 1% mobile phase B.

MS data were recorded by data dependent acquisition using the Top10 method to select the most abundant precursor ions in positive mode for higher-energy collisional dissociation fragmentation. The lock mass option was enabled to ensure high mass accuracy during many following runs. The full MS scan range was 300 to 2000 m/z with a resolution of 70000, and an automatic gain control value of 3x10⁶ total ion counts with a maximal ion injection time of 240 ms. Only higher charged ions (2+) were selected for MS/MS scans with a resolution of 17500, an isolation window of 2 m/z and an automatic gain control value set to 10⁵ ions with a maximal ion injection time of 150 ms. Selected ions were excluded in a time frame of 30s following fragmentation event. Fullscan data were acquired in profile and fragments in centroid mode by Xcalibur software. The LC Unit was controlled by Chromeleon Xpress software (Thermo Scientific, Dreieich, Germany). The performance of both units LC and MS was integrated by DCMSLink.

Proteomics: MS Data Analysis

Xcalibur raw files were analyzed using the Max Quant (1.5.2.8; MPI of Biochemistry, Martinsried, Germany, http://141.61.102.17/maxquant_doku/doku.php?id=start) proteomics software. The enzyme specificity was set to trypsin, missed cleavages were limited to 2. Acetylation of N-terminus (+42.01) and oxidation of methionine (+15.99) were selected for variable modification, carbamidomethylation (+57.02) on cysteines was set as fixed modification. The mouse reference proteome set from the UniProt Knowledgebase (download June 2015, 76086 entries) was used to identify peptides and proteins and the false discovery rate was set to 5%. Label free quantification values were obtained from at least one identified peptide. Online Table I summarizes all protein identification, number of identified peptides, accession numbers and sequence coverage of each sample. For further analysis data were uploaded into Perseus software software (1.5.2.6, http://www.perseus-framework.org/; MPI of Biochemistry, Martinsried, Germany). Identifications from reverse decoy database and known contaminants were excluded. For quantification, proteins were quality filtered according to a minimum of three valid values per group. For statistical comparison ANOVA and subsequent post hoc t-tests were used. Quantification results are summarized in Online Table II.

Metabolomics: Sample Preparation

Isolated neutrophils (2.5×10⁶ cells) stimulated as described, were recovered by centrifugation, washed with PBS and solubilized in ice-cold methanol/water (85/15, v/v). One 3 mm stainless steel bead was added to each tube and samples were vortexed for at least one minute. Samples were centrifuged (10000g, 4°C, 10 minutes), and the supernatants were immediately transferred to a fresh reaction tube and snap frozen in liquid nitrogen. Before further processing isotope labeled internal standards were added. Samples were evaporated in a vacuum concentrator plus (Eppendorf, Hamburg, Germany) at 45°C, resolved in 50 μl water and subsequently transferred to the LC-MS system.
Metabolomics: LC/MS

LC was performed on an Agilent 1290 Infinity pump system (Agilent, Waldbronn, Germany). Separation was performed on a Waters Acquity HSS T3 column (150 mm × 2.1 mm, 1.8 µm) at 40°C. Separation started with a 1.5 minute isocratic step using water/acetonitrile (98/2, v/v) and 0.15% formic acid followed by a 3 minute linear increase to 100% acetonitrile plus 0.15% formic acid at a flow rate of 400 µl/minute. Separation was followed by a cleaning and equilibration step, making 10 minutes total LC run time. Mass spectrometry was performed using a QTrap 5500 mass spectrometer (Sciex, Darmstadt, Germany) with electro spray ionization at 400°C with 4500 V in positive and -4500 V negative modes. MS parameters were set to CUR 30 psi, GS1 45 psi, and GS2 25 psi for both ionization modes. Data acquisition and instrument control were managed through the software Analyst 1.6.2. Peak integration, data processing, and analyte quantification were performed using MultiQuant 3.0 (Sciex, Darmstadt, Germany). Area under the peak was used as the quantitative measurement. The specific MRM transition for every compound was normalized to appropriated isotope labeled internal standards.

Statistical analysis

Data are expressed as mean ± SEM. Statistical evaluation was performed using Student’s t test for unpaired data, one-way ANOVA followed by a Bonferroni t test or ANOVA for repeated measures where appropriate. Values of P<0.05 were considered statistically significant.

References


Online Figure I. Hematopoietic stem cell differentiation and mobilization. (A-G) Wild-type (WT) and AMPKα2ΔMC (ΔMC) mice were administered G-CSF (250µg/kg/day, 5 days) and stem and progenitor cell populations, and differentiated cells in bone marrow as % of the cKit+Sca1+Lin- (%KSL), cKit+Sca1-Lin- (%KL) or living cell population (% living) were analyzed by flow cytometry. (A) Hematopoietic stem cells, (B) long-term hematopoietic stem cells, (C) multipotent progenitors, (D) granulocyte-macrophage progenitors, (E) megakaryocyte-erythrocyte progenitors, (F) lineage negative (Lin-) population, and (G) differentiated cell populations. (H) Proliferation and differentiation ability of mobilized progenitors as analyzed by using the colony-forming unit assay 5 days after G-CSF treatment. The graphs summarize data from 3-6 different animals per group (3 animals in control group; 6 animals in G-CSF treated group).
Online Figure II. Consequence of myeloid cell-specific deletion of AMPKα1 on vascular repair after ischemia and AMPK phosphorylation in response to hypoxia. (A) Neutrophils isolated from wild-type (WT) and AMPKα2ΔMC mice (ΔMC) were incubated under normoxic (Nox) or hypoxic (Hox) conditions for 16 hours and AMPK phosphorylation (on Thr172) determined by Western blotting (n=3 different animals per group). Non muscle myosin (NMM) was used as an overall loading control. The grey line indicates that non adjacent bands from the same Western blot are shown. (B) Wild-type (WT) and AMPKα1ΔMC (α1ΔMC) mice were subjected to femoral artery ligation and the recovery of blood flow in the ligated limbs was monitored over 14 days by laser Doppler imaging and quantified relative to the non-ligated limb (n=7 different animals per group). **P<0.01, ***P<0.001 versus WT.
Online Figure III. Altered gene expression profile in ischemic hindlimbs 72 hours post-surgery. Chemokine and cytokine gene expression analysis in ischemic hindlimbs. Green bars show > 1.5 fold downregulated genes and red bars indicate significantly > 1.5 fold upregulated genes in AMPK\(\alpha_2^{\Delta MC}\) compared to wild-type mice (n=4 animals in each group).
Online Figure IV. Neutrophil infiltration in the ischemic hindlimb 24 hours after ligation. (A) Overview of infiltrated neutrophils in the murine M. gastrocnemius showing smooth muscle actin (red), Ly6G (green) and DAPI (grey) superimposed and inverted with a confocal laser scanning system (LSM 780); bar = 20 µm. (B) Intra- and extra-vascular neutrophils in the ischemic M. gastrocnemius; VE-cadherin (blue), smooth muscle actin (red), Ly6G (green) and DAPI (white). Shown is a plane of a Z-stack (lower left), the dotted lines indicate the planes of the x and y axes; bar = 20 µm. Similar results were obtained in 4 different animals per genotype.
Online Figure V. Time course of changes in circulating monocyte and neutrophil numbers after femoral artery ligation. (A-C) Time course of changes in circulating white blood cells (WBC), monocytes (MON) and neutrophils (NEU) in blood from wild-type (WT) and AMPKα2ΔMC littermates without ischemia (day 0) or 1, 3 or 14 days after femoral artery ligation. Blood was harvested by cardiac puncture and analyzed using a cell counter; n=6-14 animals per group. (D-I) Composition of circulating leukocytes 24 hours after femoral artery ligation in AMPKα2ΔMC mice versus wild-type littermates; n = 5 animals per group. (J&K) Circulating leukocyte-platelet (CD45+CD61) or neutrophil-platelet (Ly6G+CD61) aggregates as determined by flow cytometry 24 hours after femoral artery ligation; n = 5 animals per group. *P<0.05, **P<0.01 (ANOVA and Newman–Keuls Multiple Comparison Test).
Online Figure VI. Differently expressed proteins in neutrophils from wild-type (WT) and AMPKα2ΔMC (ΔMC) under conditions of normoxia (NOX) and hypoxia (HOX). Volcano plots showing p-values (-log10) versus protein ratio (log2) of (A) HOX/NOX in ΔMC, (B) HOX/NOX in WT, (C) ΔMC/WT in NOX, (D) ΔMC/WT in HOX. Significantly altered proteins are colored. Color code for upregulated proteins: ΔMC NOX (red); ΔMC HOX (orange); WT NOX (green); WT HOX (blue). (E) Two-way Venn diagram summaries of unique and overlapping proteins differentially expressed in ΔMC and WT cells under normoxia and hypoxia. Comparison of upregulated (upper panel) and downregulated (lower panel) proteins in ΔMC and WT neutrophils cultured under hypoxia.
Online Figure VII. Link between AMPKα2 and HIF-1α. In vitro phosphorylation of a HIF-1α peptide by recombinant human AMPKα2 protein. Phosphorylation was assessed by Western blotting with anti-serine or anti-threonine antibodies. Similar results were obtained in 2 additional experiments.
Online Figure VIII. Effect of AMPKα2 deletion on metabolism in neutrophils. Oxygen consumption rate (OCR) was assessed in bone marrow-derived neutrophils from wild-type (WT) and AMPKα2ΔMC (ΔMC) mice. Quantification of mitochondrial respiration function parameters from Fig 5C: (A) basal respiration (B) non-mitochondrial respiration (C) maximal respiration. (D) spare respiratory capacity, and (E) proton leak. (F) Extracellular acidification rate (ECAR) profile showing glycolytic function in bone marrow derived neutrophils from WT and AMPKα2ΔMC littermates. Vertical lines indicate the addition of glucose (Gluc; 10 mmol/L), oligomycin (Oligo; 1 μmol/L), and 2-deoxy-D glucose (2DG; 100 mmol/L). (G-I) Levels of hexose i.e. fructose + glucose (G), glucose-6-phosphate (G-6-P; H) and lactate (I) in neutrophils from wild-type (WT) and AMPKα2ΔMC (ΔMC) mice maintained under normoxic (Nox) or hypoxic (Hox) conditions for 16 hours. Graphs summarize data from 8-9 (A-F) or 5-6 (G-J) different animals per group; *P<0.05, **P<0.01 versus wild-type, ***P<0.001 versus normoxia.
Online Figure IX. Scheme showing the consequences of ischemia-induced AMPKα2 activation in neutrophils. (A) Normoxia (AMPKα2 inactive). Isocitrate dehydrogenases (IDH) catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) by utilizing NAD(P)+ as electron acceptor. α-KG is required as a cofactor for prolyl hydroxylases (PHD) and is thereby decarboxylated to succinate and CO₂. Active PHDs hydroxylate hypoxia-inducible factor-1α (HIF-1α) at proline residue 402 and 564. The hydroxylated proline residues are recognized and captured by the von-Hippel-Lindau protein (pVHL), leading to HIF-1α ubiquitylation and subsequent proteasomal degradation. (B) Hypoxia (AMPKα2 active). Phosphorylated and activated AMPKα2 leads to the inhibition of IDH and to a decrease in cellular α-KG levels which results in the inactivation of PHDs. Consequently, HIF-1α is not hydroxylated and can escape pVHL-mediated ubiquitylation and proteasomal degradation. Stabilized HIF-1α then translocates to the nucleus where, together with HIF-1β, it forms an active HIF complex that induces the expression of angiogenic cytokine-α chemokines, and genes that support survival and decrease mitochondrial respiration. Less mitochondrial respiration reduces ROS formation and the the availability of NAD(P)+. Reduced NAD(P)+ availability concomitanly limits the activity of IDH to keep levels of α-KG and PHD activity low.