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# AMP-Activated Protein Kinase $\alpha 2$ in Neutrophils Regulates Vascular Repair via Hypoxia-Inducible Factor-1 $\alpha$ 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 AMPK $\alpha 1$  catalytic subunit has been implicated in angiogenesis, little is known about the role played by the AMPK $\alpha 2$  subunit in vascular repair.

**Objective:** To determine the role of the AMPK $\alpha 2$  subunit in vascular repair.

**Methods and Results:** Recovery of blood flow after femoral artery ligation was impaired (>80%) in AMPK $\alpha 2^{-/-}$  versus wild-type mice, a phenotype reproduced in mice lacking AMPK $\alpha 2$  in myeloid cells (AMPK $\alpha 2^{\Delta MC}$ ). Three days after ligation, neutrophil infiltration into ischemic limbs of AMPK $\alpha 2^{\Delta MC}$  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 AMPK $\alpha 2^{\Delta MC}$  mice, fewer monocytes were recruited, and gene array analysis revealed attenuated expression of proangiogenic proteins in ischemic AMPK $\alpha 2^{\Delta MC}$  hindlimbs. Many angiogenic growth factors are regulated by hypoxia-inducible factor, and hypoxia-inducible factor-1 $\alpha$  induction was attenuated in AMPK $\alpha 2$ -deficient cells and accompanied by its enhanced hydroxylation. Also, fewer proteins were regulated by hypoxia in neutrophils from AMPK $\alpha 2^{\Delta MC}$  mice. Mechanistically, isocitrate dehydrogenase expression and the production of  $\alpha$ -ketoglutarate, which negatively regulate hypoxia-inducible factor-1 $\alpha$  stability, were attenuated in neutrophils from wild-type mice but remained elevated in cells from AMPK $\alpha 2^{\Delta MC}$  mice.

**Conclusions:** AMPK $\alpha 2$  regulates  $\alpha$ -ketoglutarate generation, hypoxia-inducible factor-1 $\alpha$  stability, and neutrophil survival, which in turn determine further myeloid cell recruitment and repair potential. The activation of AMPK $\alpha 2$  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

AMP-activated protein kinase (AMPK) is a nutrient-sensitive kinase that can be regulated by  $Ca^{2+}$ -elevating agents that activate upstream kinases and by various cellular stresses including hypoxia.<sup>1</sup> In endothelial cells, the AMPK is required for an adequate angiogenic response to hypoxia<sup>2</sup> and can induce the generation of vascular endothelial growth factor (VEGF).<sup>3</sup> Angiogenesis and vascular repair after ischemia in vivo also rely on the recruitment and activation of circulating cells such as monocytes and neutrophils.<sup>4</sup> Little is known about the role of the AMPK in such processes, but the kinase

has been implicated in inflammatory signaling in myeloid cells,<sup>5</sup> such as leukocyte homing and neutrophil activation,<sup>6</sup> and monocyte differentiation.<sup>7-9</sup>

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The AMPK is a heterotrimeric kinase consisting of a catalytic  $\alpha$  subunit and regulatory  $\beta$  and  $\gamma$  subunits. There are 2 different isoforms of the catalytic  $\alpha$  subunit, that is, the  $\alpha 1$  subunit that is mainly localized to the cytosol and the  $\alpha 2$  subunit

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**Nonstandard Abbreviations and Acronyms**

<b>AMPK</b>	AMP-activated protein kinase
<b>HIF</b>	hypoxia-inducible factor
<b>IDH</b>	isocitrate dehydrogenase
<b>IL</b>	interleukin
<b>PHD</b>	prolyl hydroxylase domain
<b>VEGF</b>	vascular endothelial growth factor

that can translocate to the nucleus, where it likely plays a more important role in transcription factor activation and gene regulation.<sup>1</sup> 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  $\alpha$  subunits. We reported previously that the AMPK $\alpha$ 1 subunit in endothelial cells is a prerequisite for angiogenesis induced by cytokines and VEGF via a mechanism involving transforming growth factor- $\beta$ -activated kinase 1 and superoxide dismutase 2 expression.<sup>10</sup> The aim of the present study was to assess the role of AMPK, particularly the AMPK $\alpha$ 2 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 AMPK $\alpha$ 1 or the AMPK $\alpha$ 2 subunits, their respective wild-type littermates, and floxed AMPK $\alpha$ 2 mice were kindly provided by Benoit Viollet (INSERM, U1016, Paris, France) and bred at the Goethe University Hospital animal facility. Floxed AMPK $\alpha$ 2 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  $\alpha$ 2 subunit in endothelial cells and some myeloid cells (Tie2- $\alpha$ 2), with VE-cadherin-Cre mice (B6-Tg(Cdh5-cre)/J; Polygene, Switzerland) to generate animals mice lacking the  $\alpha$ 2 subunit specifically in endothelial cells (AMPK $\alpha$ 2<sup>ΔEC</sup>), or with LysM-Cre mice (B6.129P2-Lyz2tm1(cre)Ifo/J; Jackson Laboratories) to generate mice lacking the  $\alpha$ 2 subunit specifically in myeloid cells (AMPK $\alpha$ 2<sup>ΔMC</sup>).

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.<sup>11</sup>

**Statistical Analysis**

Data are expressed as mean $\pm$ SEM. Statistical evaluation was performed using Student *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.

Reagents and other detailed methods are described in the [Online Data Supplement](#).

**Results****Effects of AMPK $\alpha$ 2 Deletion on Vascular Repair**

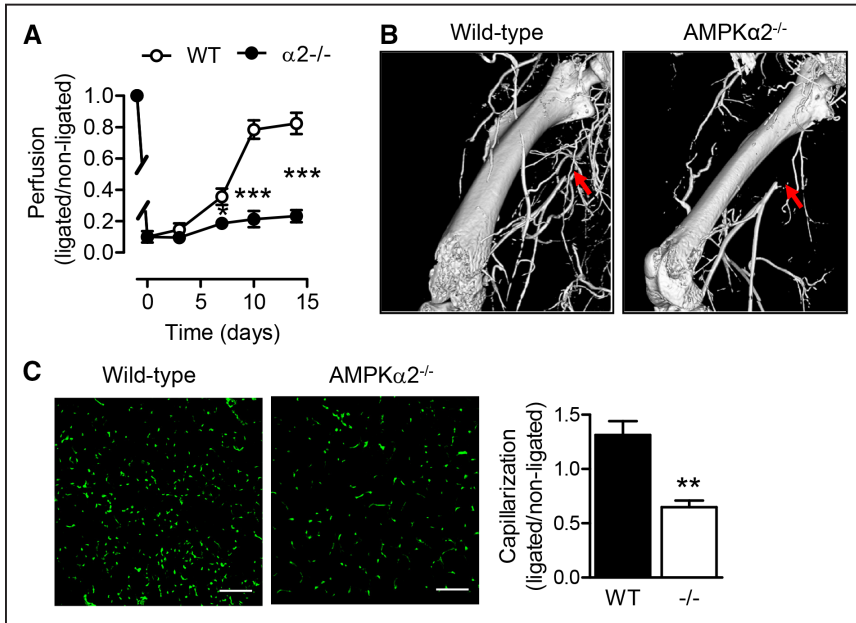
Diabetes mellitus is not only a risk factor for the development of cardiovascular disease but is linked with impaired vascular repair after injury and ischemia.<sup>12,13</sup> Given that the AMPK $\alpha$ 2 subunit was reported to be a regulator of whole body insulin sensitivity,<sup>14</sup> vascular repair after femoral artery ligation was assessed in wild-type and AMPK $\alpha$ 2<sup>-/-</sup> mice. The recovery of blood flow in ischemic hindlimbs was markedly attenuated in AMPK $\alpha$ 2<sup>-/-</sup> 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 AMPK $\alpha$ 2<sup>-/-</sup> 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).

**AMPK $\alpha$ 2 in Endothelial Versus Myeloid Cells**

Arteriogenesis and angiogenesis are determined by endothelial cells and circulating cells.<sup>4</sup> To determine whether the reduced recovery after hindlimb ischemia was dependent on the deletion of AMPK $\alpha$ 2 in endothelial cells or other cell types, experiments were repeated in AMPK $\alpha$ 2<sup>fllox/fllox</sup> mice crossed with mice expressing the Cre-deleter under the control of the Tie-2 promoter (Tie2- $\alpha$ 2 mice) and their wild-type littermates. Although there was delayed recovery of perfusion in the Tie2- $\alpha$ 2 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.<sup>15,16</sup> Therefore, endothelial cells were more specifically targeted by crossing AMPK $\alpha$ 2<sup>fllox/fllox</sup> mice with mice expressing the Cre-deleter under the control of the VE-cadherin promoter. However, the endothelial cell-specific deletion of the AMPK $\alpha$ 2 had no effect on the recovery from hindlimb ischemia (Figure 2B).

As the results indicated that the AMPK $\alpha$ 2 in mononuclear cells may explain the observations, the expression of the AMPK $\alpha$ 2 subunit was assessed in myeloid cells. AMPK $\alpha$ 2 mRNA was clearly expressed in the aorta and in peritoneal macrophages from wild-type mice (Figure 2C). Crossing AMPK $\alpha$ 2<sup>fllox/fllox</sup> mice with LysM-Cre mice, to generate animals lacking the AMPK $\alpha$ 2 in myeloid cells (AMPK $\alpha$ 2<sup>ΔMC</sup> mice), resulted in the loss of AMPK $\alpha$ 2 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 AMPK $\alpha$ 2, confirming the specificity of the LysM-Cre-dependent deletion.

The myeloid cell-specific deletion of AMPK $\alpha$ 2 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 AMPK $\alpha$ 2 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



**Figure 1. Recovery of blood flow and revascularization in ischemic hindlimbs after femoral artery ligation in diabetic and AMPK $\alpha$ 2<sup>-/-</sup> mice.** **A**, Reperfusion after femoral artery ligation as measured by laser Doppler analysis in wild-type (WT) and AMPK $\alpha$ 2<sup>-/-</sup> littermates (n=9 per group). **B**, Micro computed tomography angiography of ligated limbs from WT and AMPK $\alpha$ 2<sup>-/-</sup> mice 7 d post surgery. Arrows indicate site of ligation. Similar results were obtained in an additional 2 animals in each group. **C**, CD31+ capillaries in transverse sections of ischemic gastrocnemius muscles from WT and AMPK $\alpha$ 2<sup>-/-</sup> mice (bar=100  $\mu$ m; n=4 per group). \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs control group. AMPK indicates AMP-activated protein kinase.

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 hematopoietic stem cell function.<sup>17</sup>

AMPK $\alpha$ 2<sup>AMC</sup> mice reproduced the phenotype of the AMPK $\alpha$ 2<sup>-/-</sup> 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 AMPK $\alpha$ 2<sup>AMC</sup> mice compared with their wild-type littermates. The latter animals developed remodeled collaterals that largely failed to form in the AMPK $\alpha$ 2<sup>AMC</sup> 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 AMPK $\alpha$ 2<sup>AMC</sup> mice. The AMPK $\alpha$ 1 subunit is more highly expressed than the AMPK $\alpha$ 2 isoform in myeloid cells. Despite this, the deletion of the AMPK $\alpha$ 2 subunit attenuated neutrophil AMPK phosphorylation on the activating Thr172 site (the phospho-specific antibody used cannot differentiate between the AMPK $\alpha$ 1 and  $\alpha$ 2 isoforms) under basal conditions and in cells exposed to hypoxia (Online Figure IIA). To confirm the importance of the AMPK $\alpha$ 2 isoform in vascular repair, we also generated a myeloid cell-specific AMPK $\alpha$ 1-deficient mouse. Although vascular repair after ischemia was attenuated in mice lacking the  $\alpha$ 1 subunit in myeloid cells, the effect was much less pronounced than that seen in AMPK $\alpha$ 2<sup>AMC</sup> mice (compare Online Figure IIB and Figure 2E).

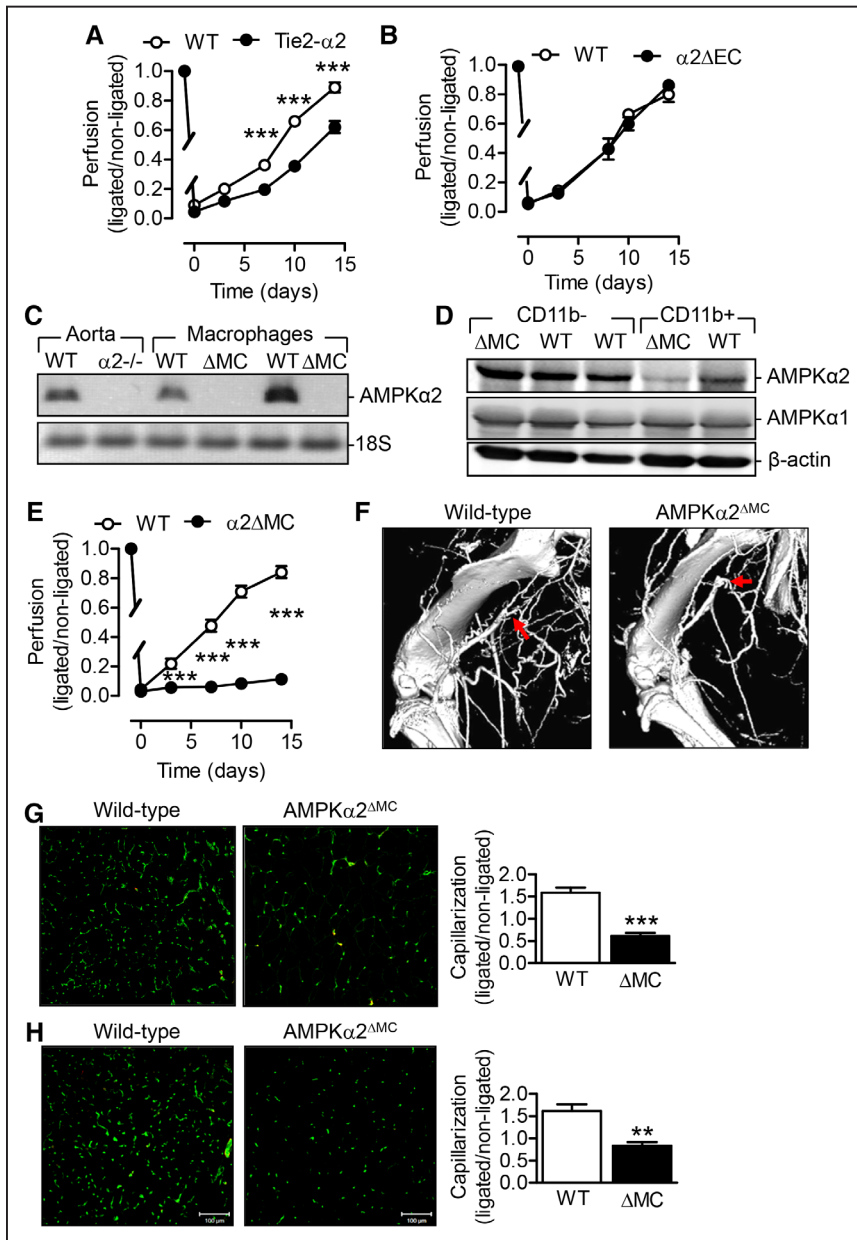
### Altered Recruitment of Myeloid Cells to Ischemic Hindlimbs in AMPK $\alpha$ 2<sup>AMC</sup> Mice

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 AMPK $\alpha$ 2<sup>AMC</sup> 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 AMPK $\alpha$ 2<sup>AMC</sup> 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) $\beta$ , VEGFA and VEGFB, and the VEGF receptor 1 (VEGFR1) and VEGFR2 were lower in ischemic muscles from AMPK $\alpha$ 2<sup>AMC</sup> mice compared with their wild-type littermates. On the contrary, the expression of several inflammatory mediators and chemokines were upregulated in ischemic muscles from AMPK $\alpha$ 2<sup>AMC</sup> mice including tumor necrosis factor- $\alpha$ , interleukin (IL)-1 $\beta$ , 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 upregulation 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 AMPK $\alpha$ 2 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 AMPK $\alpha$ 2<sup>AMC</sup> mice (Figure 4A). However, a significantly higher number of neutrophils were recruited to the gastrocnemius muscle of AMPK $\alpha$ 2<sup>AMC</sup> mice than their wild-type littermates



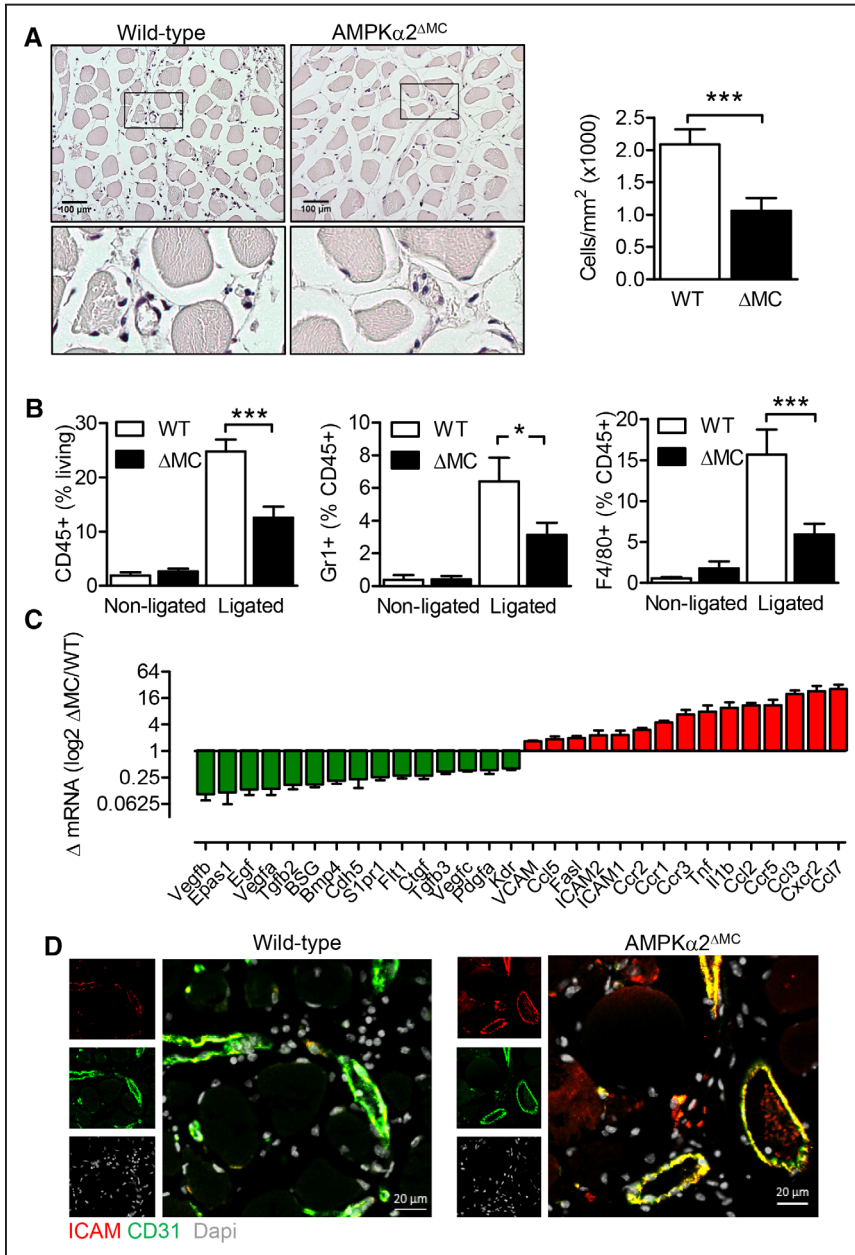
**Figure 2. Comparison of the consequences of endothelial cell and myeloid cell AMP-activated protein kinase (AMPK)  $\alpha$ 2 deletion on vascular repair.** **A** and **B**, Recovery of blood flow after femoral artery ligation in hindlimbs from **(A)** wild-type (WT), Tie2- $\alpha$ 2 littermates (n=8 per group), and **(B)** WT and AMPK $\alpha$ 2 $\Delta$ EC littermates (n=4 per group). **C**, AMPK $\alpha$ 2 mRNA expression in aorta and macrophages isolated from WT, AMPK $\alpha$ 2 $^{-/-}$  or AMPK $\alpha$ 2 $\Delta$ MC mice. Similar results were obtained in 2 additional experiments. **D**, AMPK $\alpha$ 2 and AMPK $\alpha$ 1 protein expression in CD11b $^{-}$  and CD11b $^{+}$  cells isolated from spleens from WT and AMPK $\alpha$ 2 $\Delta$ MC littermates. Similar results were obtained in 2 additional experiments. **E**, Recovery of blood flow after femoral artery ligation in hindlimbs from WT and AMPK $\alpha$ 2 $\Delta$ MC mice (n=9 per group). **F**, Micro computed tomography angiography of ligated limbs from WT and AMPK $\alpha$ 2 $\Delta$ MC mice, 7 d post surgery. Arrows indicate site of ligation. **G** and **H**, Anti-CD31 immuno-stained capillaries (green) in the semimembranosus (**G**) and gastrocnemius (**H**) muscles of ligated limbs from WT and AMPK $\alpha$ 2 $\Delta$ MC mice 14 d after surgery. The quantification was performed relative to the nonligated limb (n=5 per group). \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs WT.

(Figure 4B). At this early time point, no macrophages were consistently detected in the ischemic muscles. In *in vitro* migration assays, neutrophils from AMPK $\alpha$ 2 $\Delta$ MC 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 AMPK $\alpha$ 2 $\Delta$ MC mice 24 hours after surgery (Figure 4D). In these samples, intra- and extravascular neutrophils were easier to detect in whole mounts from AMPK $\alpha$ 2 $\Delta$ MC 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 AMPK $\alpha$ 2 $\Delta$ MC 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 AMPK $\alpha$ 2 $\Delta$ MC 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.

### AMPK $\alpha$ 2 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 AMPK $\alpha$ 2 $\Delta$ MC neutrophils. To determine which AMPK $\alpha$ 2-regulated proteins were altered by hypoxia, bone marrow-derived neutrophils were isolated from wild-type and



**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  $\mu$ m. 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 AMPK $\alpha$ 2<sup>ΔMC</sup> ( $\Delta$ MC) 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 AMPK $\alpha$ 2<sup>ΔMC</sup> compared with WT mice (n=4 animals in each group). **D**, Interleukin adhesion molecule (ICAM)-1 and CD31 immunostaining in the gastrocnemius muscle. Bar=20  $\mu$ m. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ . AMPK indicates AMP-activated protein kinase.

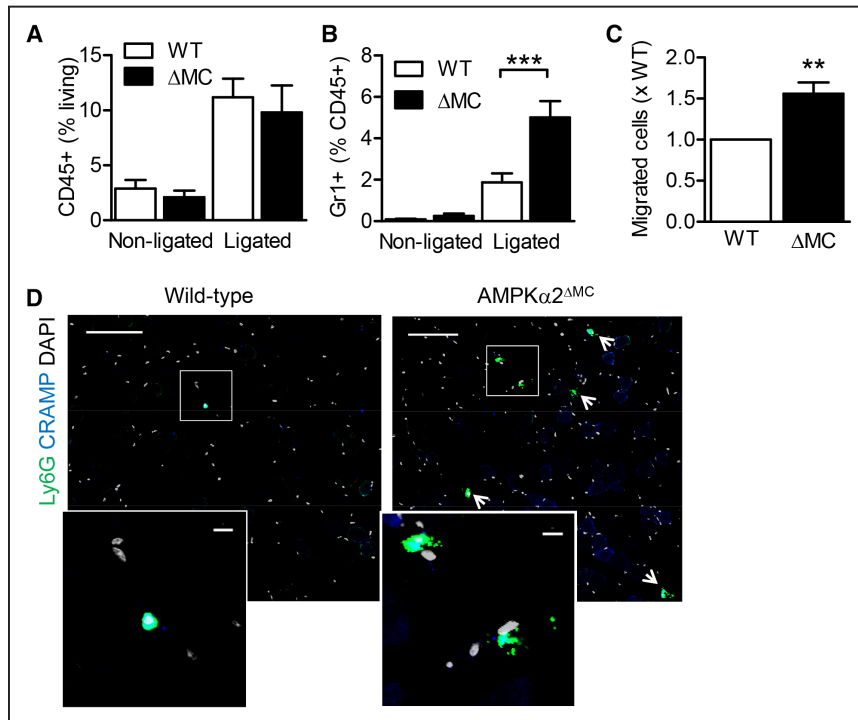
AMPK $\alpha$ 2<sup>ΔMC</sup> littermates and maintained under normoxic or hypoxic conditions for 16 hours. Thereafter, altered protein expression was determined by mass spectrometry. Online Table I summarizes all of the proteins identified, number of identified peptides, accession numbers, and sequence coverage of each sample. The quantification of the results is summarized in Online Table II.

Fitting with the *in vivo* observations, neutrophils from AMPK $\alpha$ 2<sup>ΔMC</sup> 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 AMPK $\alpha$ 2 (Figure 5D). Thus, the expression of the AMPK $\alpha$ 2 in neutrophils is required for protection against apoptosis in hypoxic conditions.

### AMPK $\alpha$ 2 Regulates Hypoxia-Inducible Factor-1 $\alpha$ Hydroxylation and Stabilization and Hypoxia-Inducible Factor-1 $\alpha$ -Dependent Responses in AMPK $\alpha$ 2-Deficient Neutrophils

The most prominent difference in the proteome of neutrophils from the 2 strains was that the neutrophils lacking AMPK $\alpha$ 2 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)-1 $\alpha$ ,<sup>18</sup> which is crucial for prolonging neutrophil survival under hypoxic conditions.<sup>19</sup>

HIF-1 $\alpha$  protein levels can be modified by several kinases,<sup>20</sup> and previous studies in a prostate cancer cell line indicated that global AMPK inhibition may attenuate hypoxia-induced responses such as HIF-1 $\alpha$  target gene expression.<sup>21</sup> Therefore, we determined whether or not AMPK can affect HIF-1 $\alpha$  stability in HEK293 cells, which express both the AMPK $\alpha$ 1 and  $\alpha$ 2



**Figure 4. Myeloid cell infiltration into the ischemic hindlimb 24 h post surgery.** **A** and **B**, CD45+ and Gr1+ cells isolated from the gastrocnemius muscle of the ligated and nonligated hindlimbs from wild-type (WT) and AMPK $\alpha$ 2 $\Delta$ MC ( $\Delta$ MC) mice 24 h after ligation (n=6 per group). **C**, In vitro stromal cell–derived factor-1–induced transendothelial migration of bone marrow–derived neutrophils from WT and AMPK $\alpha$ 2 $\Delta$ MC mice (n=4–6 per group). **D**, Ly6G+ and CRAMP+ neutrophils in ischemic semimembranosus muscles from WT and  $\Delta$ MC mice 1 d post surgery. The images are representative of data obtained in an additional 3 animals per group. **Upper**: bar=200  $\mu$ m; **lower**: bar=10  $\mu$ m. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs WT. AMPK indicates AMP-activated protein kinase.

subunits. In cells maintained under normoxic conditions, HIF-1 $\alpha$  levels were not detectable, but the protein was clearly induced after exposure of cells to hypoxia (1% O<sub>2</sub>, 6 and 16 hours) or cobalt chloride (150  $\mu$ mol/L, 6 hours; Figure 6B). Although the downregulation of the AMPK $\alpha$ 1 subunit had little effect on the induction of HIF-1 $\alpha$  by either stimulus, HIF-1 $\alpha$  levels were consistently lower in HEK293 cells treated with small interfering RNA directed against the AMPK $\alpha$ 2 subunit. The serine phosphorylation of HIF-1 $\alpha$  has been linked with its destabilization<sup>22,23</sup>; therefore, we determined the ability of AMPK $\alpha$ 2 to phosphorylate a recombinant HIF-1 $\alpha$  peptide (corresponding to amino acids 1–735) in vitro. We found that AMPK $\alpha$ 2 was able to elicit the serine but not the threonine phosphorylation of HIF-1 $\alpha$  (Online Figure VII); however, we were unable to confirm the AMPK-mediated phosphorylation of HIF-1 $\alpha$  in isolated neutrophils.

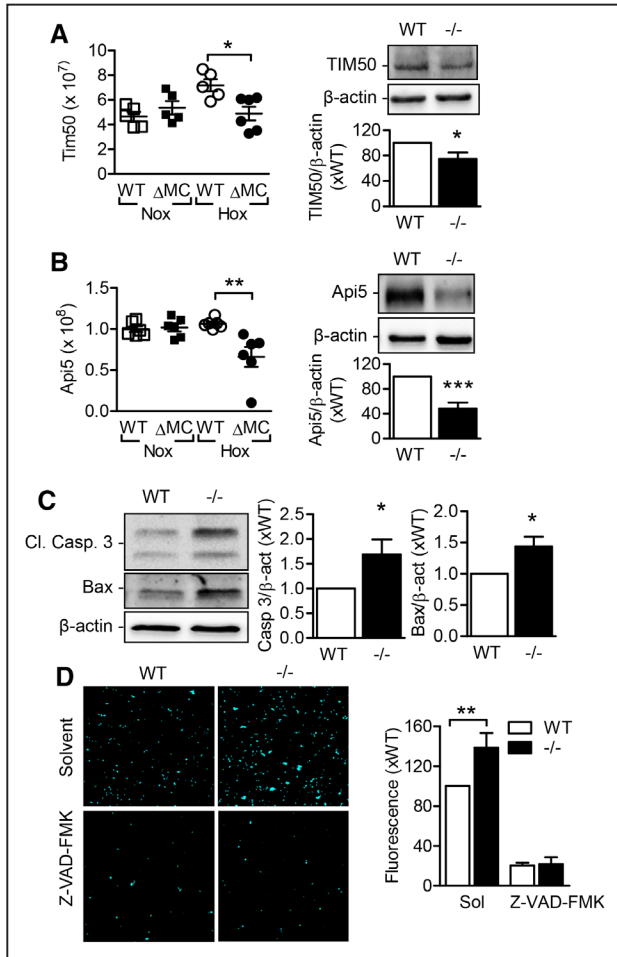
HIF-1 $\alpha$  protein levels are barely detectable in normoxic conditions largely because of the high activity of the prolyl hydroxylase domain (PHD)–containing enzymes, which hydroxylate HIF-1 $\alpha$  (on Pro564) thus promoting its subsequent proteasome-mediated degradation.<sup>24</sup> In response to hypoxia, PHD enzymes and the degradation of HIF-1 $\alpha$  are inhibited. Interestingly, the downregulation of AMPK $\alpha$ 2 enhanced the hydroxylation of HIF-1 $\alpha$  on proline 564 in cells exposed to hypoxia, indicating that the effect of AMPK $\alpha$ 2 on HIF-1 $\alpha$  may be indirectly regulated via PHD activity. Indeed, treatment with the PHD inhibitor dimethylxallylglycine restored HIF-1 $\alpha$  levels in cells treated with AMPK $\alpha$ 2 siRNA (Figure 6C). This implies that AMPK $\alpha$ 2 regulates HIF-1 $\alpha$  stabilization under hypoxia by interfering with its hydroxylation and thereby inhibiting its proteasomal degradation. Consistent with the observed effects on HIF-1 $\alpha$  destabilization, the expression of the hypoxia-regulated genes, VEGF and stromal cell–derived factor-1, was decreased in lipopolysaccharide-stimulated neutrophils from AMPK $\alpha$ 2 $\Delta$ MC mice (Figure 7A). In the same cells,

the expression of the proinflammatory markers, tumor necrosis factor- $\alpha$  and IL-1 $\beta$ , was increased (Figure 7B) and fit well with the altered cytokine and growth factor expression profile detected in the ischemic muscles (Figure 3C). Neutrophil recruitment 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  $\alpha$ 2 subunit (Figure 7C and 7D).

#### AMPK $\alpha$ 2 Regulates HIF-1 $\alpha$ Hydroxylation by Modulating $\alpha$ -Ketoglutarate Levels

Because HIF-1 $\alpha$  also regulates the cellular metabolic state, we analyzed metabolic parameters in isolated neutrophils. Under normoxic conditions, the deletion of AMPK $\alpha$ 2 in neutrophils was associated with increased oxygen consumption, both in the absence of stimulation and when maximally stimulated with FCCP (Figure 7F; Online Figure VIIIA through VIIIE). 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 VIIIF). 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 VIIIG through VIIIJ).

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



**Figure 5. Effect of AMP-activated protein kinase (AMPK)  $\alpha$ 2 deletion on neutrophil apoptosis.** Neutrophils from wild-type (WT) and AMPK $\alpha$ 2 <sup>$\Delta$ MC</sup> ( $\Delta$ MC) littermates were incubated under normoxic (Nox) or hypoxic (Hox) conditions for 16 h (n=6 per group). **A**, Translocase of inner mitochondrial membrane 50 (TIM50) label-free quantification (LFQ) intensity (left) and Western blot of neutrophils maintained under hypoxic conditions (right). **B**, Api5 LFQ intensity (left) and Western blot of neutrophils maintained under hypoxic conditions (right). **C**, Expression of cleaved caspase-3 (Cl. Casp. 3) and Bax in bone marrow–derived neutrophils from wild-type (WT) and AMPK $\alpha$ 2 <sup>$\Delta$ MC</sup> ( $\Delta$ MC) littermates (n=8 per group for Cl. Casp. 3 and n=5 per group for Bax expression). **D**, Caspase-3/7 activity measured as DEVD (7-amino-4-trifluoromethyl coumarin) fluorescence after cleavage from Asp-Glu-Val-Asp in neutrophils from WT and  $\Delta$ MC littermates (n=5 per group). Cells were incubated with solvent or the caspase inhibitor (Z-VAD-FMK, 10  $\mu$ mol/L; n=5 per group). \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

that although the IDH product  $\alpha$ -ketoglutarate decreased in wild-type neutrophils exposed to hypoxia, it failed to do so in hypoxic neutrophils from AMPK $\alpha$ 2 <sup>$\Delta$ MC</sup> mice (Figure 8C).  $\alpha$ -Ketoglutarate is required as a cofactor for PHD enzymes and the subsequent hydroxylation of HIF-1 $\alpha$ . The hypoxia-induced decrease in  $\alpha$ -ketoglutarate production prevents the hydroxylation and degradation of HIF thus facilitating the induction of HIF-1 $\alpha$ -regulated genes. Certainly, incubating human leukemic monocyte THP-1 cell line with octyl- $\alpha$ -ketoglutarate, a cell-permeable ester of  $\alpha$ -ketoglutarate, was sufficient to abrogate HIF-1 $\alpha$  expression in cells exposed to hypoxia (Figure 8D).

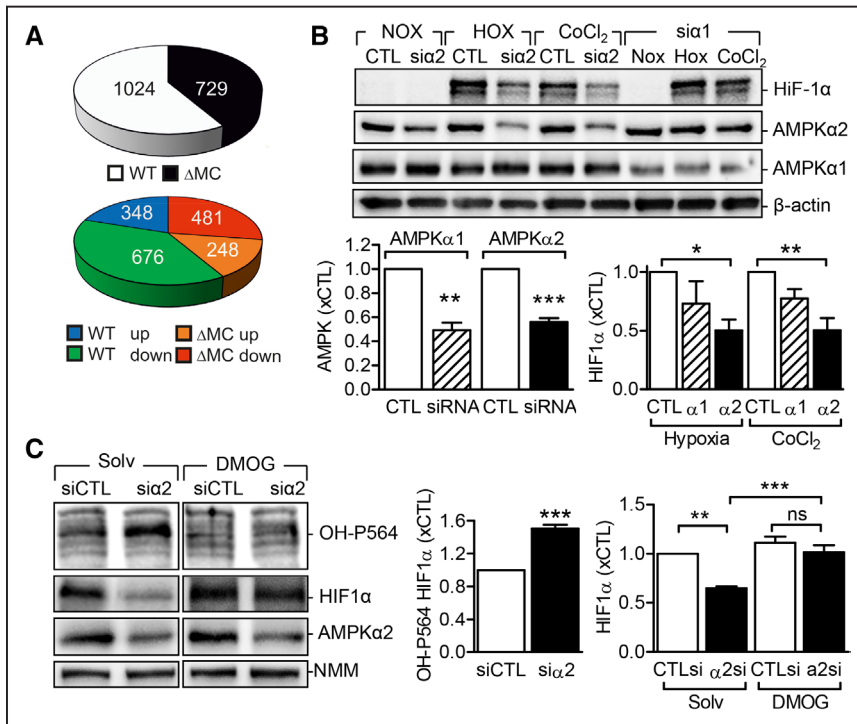
## Discussion

The results of the present study indicate that the AMPK $\alpha$ 2 subunit is important in determining neutrophil survival and function in ischemic tissue. The mechanisms involved are related to the stabilization of HIF-1 $\alpha$  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, characterized by cell infiltration and the initiation of angiogenesis, followed by a resolution phase. The processes involved are complex, but many pharmaceutical tools, such as metformin, have been reported to improve vascular repair, at least in wild-type mice.<sup>25</sup> One key signaling event that is altered in diabetes mellitus and targeted by metformin is the activation of the AMPK.<sup>26</sup> To determine the importance of the AMPK in vascular repair, the recovery of blood flow after ischemia was assessed in wild-type and AMPK $\alpha$ -deficient mice. The focus of the studies was the AMPK $\alpha$ 2 subunit, given its reported link to diabetes mellitus<sup>14</sup> and its activation by hypoxia.<sup>21</sup> We found that the recovery of blood flow was significantly impaired in ischemic hindlimbs from animals completely deficient in the AMPK $\alpha$ 2 subunit. Interestingly, targeting AMPK $\alpha$ 2 in endothelial cells did not impair vascular repair, whereas its deletion in myeloid cells reproduced the effects observed in the AMPK $\alpha$ 2<sup>-/-</sup> mice. Certainly, the AMPK $\alpha$ 2 isoform was clearly expressed in CD11b+ cells, and in animals lacking AMPK $\alpha$ 2 in the myeloid lineage (AMPK $\alpha$ 2 <sup>$\Delta$ MC</sup> mice), arteriogenesis and angiogenesis in the ischemic hindlimb were substantially reduced. The myeloid cell–specific deletion of the AMPK $\alpha$ 1 subunit also delayed the recovery of blood flow after femoral artery ligation, but the defect was much less pronounced than that in the AMPK $\alpha$ 2 <sup>$\Delta$ MC</sup> mice. Although in our study the role of AMPK $\alpha$ 2 deletion in neutrophils was more pronounced, it is possible that the AMPK $\alpha$ 1 isoform plays a significant role in the regulation of monocyte function as described in an article published during the revision of this article.<sup>27</sup>

Consistent with a previous report that the deletion of the AMPK had no effect on normal hematopoietic stem cell differentiation or function,<sup>17</sup> the myeloid cell–specific deletion of the AMPK $\alpha$ 2 was without effect on hematopoietic stem cell differentiation. Interestingly, circulating levels of neutrophils were attenuated by the deletion of the AMPK $\alpha$ 2 under basal conditions but were comparable in wild-type and AMPK $\alpha$ 2 <sup>$\Delta$ MC</sup> 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 perfusion recovery, they have a short half-life within the ischemic tissue.<sup>28,29</sup> Comparing the numbers of neutrophils that infiltrated ischemic skeletal muscle 1 and 3 days after femoral artery ligation revealed that despite increased neutrophil infiltration in ischemic AMPK $\alpha$ 2 <sup>$\Delta$ MC</sup> hindlimbs after 24 hours, the situation was reversed after 72 hours. These observations indicated that neutrophil survival was attenuated in the absence of the

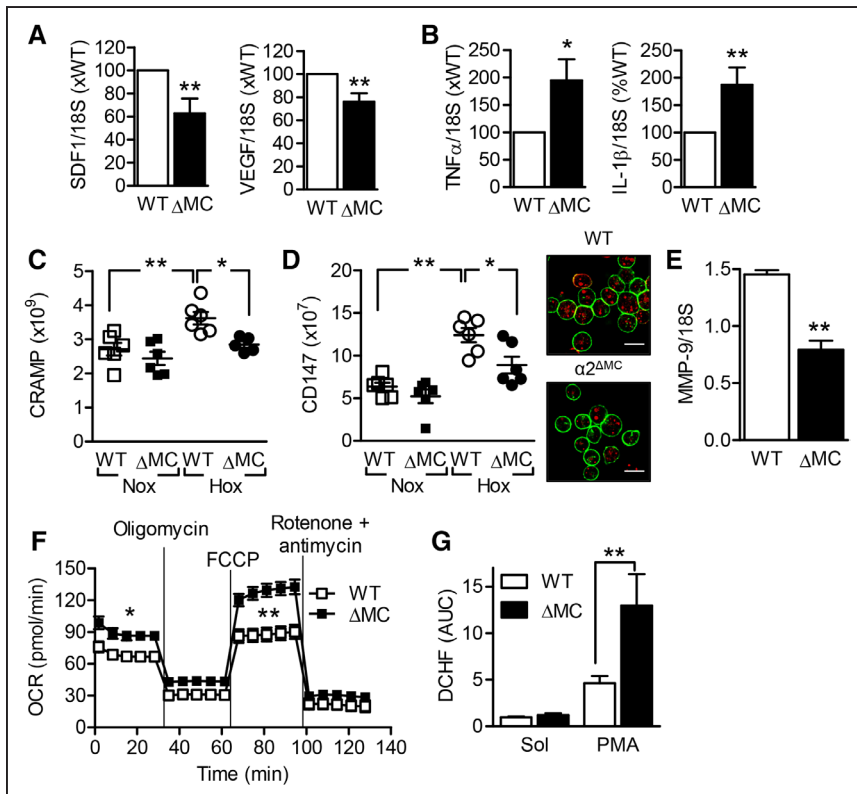


**Figure 6. Link between AMPK-activated protein kinase (AMPK) α2 and hypoxia-inducible factor-1α (HIF-1α).** **A**, Pie charts depicting significantly regulated proteins in neutrophils from wild-type (WT) and AMPKα2<sup>ΔMC</sup> (ΔMC) mice after 16-h exposure to hypoxia. **Upper**: total number of significantly regulated proteins. **Lower**: number of significantly up- and downregulated proteins. **B**, HIF-1α levels in HEK293 cells transfected with a control siRNA (CTL) or siRNA directed against AMPKα1 (siα1) or AMPKα2 (siα2) and then either maintained under normoxic conditions, exposed to hypoxia (1% O<sub>2</sub>, 6 h) or stimulated with CoCl<sub>2</sub> (150 μmol/L, 6 h). Graphs summarize data from 5 independent experiments. **C**, HEK293 cells transfected with a control siRNA (siCTL) or siRNA-directed against AMPKα2 (siα2) were exposed to hypoxia in the presence of solvent (Solv) or dimethylthioallylglycine (DMOG; 1 mmol/L) for 16 h. HIF-1α levels and its hydroxylation on proline 564 (OH-P564) were analyzed by Western blotting (n=9–12 per group). The break in the blots indicates that nonadjacent lanes in the same Western blot are presented. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

AMPKα2 subunit. Certainly, AMPK activation has previously been linked with neutrophil survival,<sup>30</sup> and in this study, apoptosis was increased in AMPKα2-deficient neutrophils. Some of the proteins significantly altered in hypoxic AMPKα2<sup>ΔMC</sup> versus wild-type neutrophils were linked to apoptosis, in particular translocase of inner mitochondrial membrane 50, a component of the mitochondrial translocator,<sup>31</sup> and

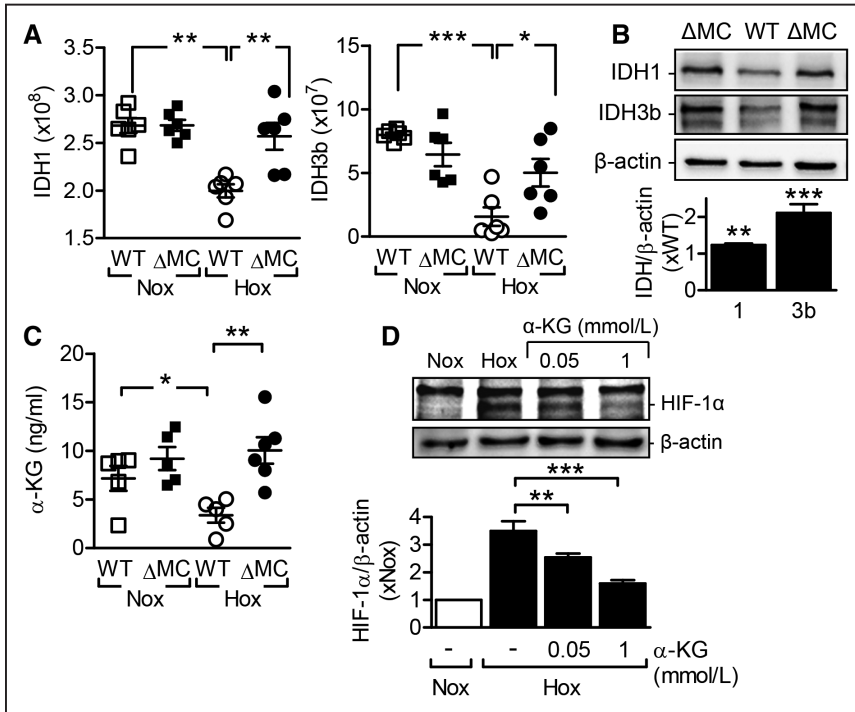
apoptosis inhibitor 5, a negative regulator of E2F1-dependent cell death.<sup>32</sup> Little is known about the role played by either protein in neutrophils, but the loss of translocase of inner mitochondrial membrane 50 has also been reported to increase apoptosis in breast cancer cells.<sup>33</sup>

Part of the arteriogenic/angiogenic response can be attributed to the liberation of neutrophil-derived VEGF, matrix



**Figure 7. Hypoxic response in neutrophils and mitochondrial metabolism.** **A** and **B**, Bone marrow neutrophils were isolated from wild-type (WT) and AMPKα2<sup>ΔMC</sup> (ΔMC) littermates, and the expression of the angiogenic (VEGF and stromal cell-derived factor-1 [SDF-1]) and inflammatory markers (tumor necrosis factor [TNF]-α and interleukin [IL]-1β) were analyzed. The graphs summarize data from 8 to 9 individual animals in each group from 3 independent experiments. **C** and **D**, Neutrophils from WT and ΔMC littermates were incubated under normoxic (Nox) or hypoxic (Hox) conditions for 16 h (n=6 per group). **C**, cathelicidin-related antimicrobial peptide (CRAMP) protein expression (label-free quantification [LFQ] intensity). **D**, CD147 protein levels (LFQ intensity) and immunohistochemistry, CD147 (red), and phalloidin (green). Bar=10 μm. **E**, Matrix metalloproteinase (MMP)-9 mRNA expression (n=4 per group). **F**, Oxygen consumption rate (OCR) profile as an index of mitochondrial respiration in bone marrow-derived neutrophils from WT and ΔMC mice treated with solvent (Sol) or PMA (phorbol 12-myristate 13-acetate; 100 ng/mL) for 20 min (n=7–8 per group). \*P<0.05, \*\*P<0.01, \*\*\*P<0.001. AMPK indicates AMP-activated protein kinase.





**Figure 8. AMP-activated protein kinase (AMPK) regulates levels of isocitrate dehydrogenases and  $\alpha$ -ketoglutarate.**

Neutrophils from wild-type (WT) and AMPK $\alpha$ 2 $\Delta$ MC ( $\Delta$ MC) littermates were incubated under normoxic (Nox) or hypoxic (Hox) conditions for 16 h (n=6 per group). **A**, Isocitrate dehydrogenase (IDH) 1 and IDH3b expression (label-free quantification intensity). **B**, Western blot confirmation of altered IDH1 and IDH3b expression. **C**,  $\alpha$ -Ketoglutarate ( $\alpha$ -KG) levels in neutrophils from WT and  $\Delta$ MC littermates incubated under Nox or Hox conditions for 16 h (n=5–6 per group). **D**, HIF-1 $\alpha$  levels in THP-1 cells incubated in Hox conditions for 6 h with indicated concentrations of octyl- $\alpha$ -KG, a cell-permeable ester of  $\alpha$ -KG (n=6 independent experiments). \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001.

metalloproteinases, and other proangiogenic growth factors,<sup>34</sup> but these cells also release factors such as cathelicidins that promote the recruitment of monocytes,<sup>35,36</sup> which then amplify the angiogenic and arteriogenic processes. Thus, the observed decrease in monocyte recruitment to the ischemic tissue 72 hours after ligation is likely to be linked to neutrophil loss. The same mechanism likely underlies the decreased expression of proangiogenic factors in hindlimbs from AMPK $\alpha$ 2 $\Delta$ MC 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 $\alpha$ 2 $\Delta$ MC mice, hinting that endothelial cell activation was enhanced. This is a neutrophil-dependent effect as endothelial cells from wild-type and AMPK $\alpha$ 2 $\Delta$ MC mice responded to IL-1 $\beta$  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-1 $\alpha$ ,<sup>19</sup> and interactions between HIF-1 $\alpha$  and AMPK have been reported. Indeed, increased AMPK $\alpha$  expression and activity are paralleled by the upregulation of HIF-1 $\alpha$  in cancer cells,<sup>37</sup> and the inhibition of AMPK was found to impair the nuclear accumulation of HIF-1 $\alpha$  under hypoxia or low glucose conditions.<sup>38</sup> We found that the downregulation of the AMPK $\alpha$ 2 but not the AMPK $\alpha$ 1 subunit attenuated the increase in HIF-1 $\alpha$  levels induced by either hypoxia or cobalt chloride. In agreement with our in vitro observations, the expression of the HIF-1 $\alpha$ -regulated factors, stromal cell-derived factor-1 and VEGF, was attenuated in neutrophils from AMPK $\alpha$ 2 $\Delta$ MC mice. AMPK has also been proposed to regulate HIF-1 $\alpha$  protein stability by phosphorylation.<sup>23</sup> However, even though we could confirm these findings in an

in vitro assay, we were unable to detect an AMPK $\alpha$ 2-dependent phosphorylation of HIF-1 $\alpha$  in intact cells. Instead, it was possible to demonstrate that the deletion of the AMPK $\alpha$ 2 subunit enhanced the hydroxylation of HIF-1 $\alpha$  on Pro564 in cells exposed to hypoxia. The latter response is indicative of increased PHD activity and proteasomal degradation. Indeed, treating AMPK $\alpha$ 2-deficient neutrophils with a PHD inhibitor prevented the enhanced hydroxylation and rescued HIF-1 $\alpha$  induction.

Unlike the AMPK $\alpha$ 1 subunit, the AMPK $\alpha$ 2 can translocate to the nucleus and affect gene and protein expression by several mechanisms.<sup>1</sup> Mass spectrometry was used to ascertain which neutrophil proteins could be affected by AMPK $\alpha$ 2 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 AMPK $\alpha$ 2 $\Delta$ MC neutrophils. The latter proteins were of interest as both are regulated by HIF-1 $\alpha$ <sup>39,40</sup> and both are involved in neutrophil recruitment, invasion, and survival in ischemic tissues.<sup>35,41–43</sup> Of interest for vascular repair is that CD147 is reportedly required for the increased synthesis of VEGF and matrix metalloproteinase-9<sup>44,45</sup>; thus, the lower levels of matrix metalloproteinase-9 in the AMPK $\alpha$ 2 $\Delta$ MC neutrophils may be related to their failure to increase CD147 expression.

Prolyl hydroxylases require oxygen and the electron donor  $\alpha$ -ketoglutarate to execute the hydroxylation and inactivation of HIF-1 $\alpha$ .<sup>46</sup> Neutrophil adaption to hypoxia and the stabilization of HIF-1 $\alpha$ , therefore, require accompanying changes in metabolism and a decrease in  $\alpha$ -ketoglutarate production. Isocitrate dehydrogenases catalyze the oxidative decarboxylation of isocitrate to  $\alpha$ -ketoglutarate, and 2 IDH isoforms, that is, IDH1 and IDH3b, were differentially regulated by hypoxia in neutrophils from wild-type and AMPK $\alpha$ 2 $\Delta$ MC mice. Specifically, the decrease in IDH expression detected in wild-type neutrophils exposed to

hypoxia failed to occur in AMPK $\alpha$ 2-deficient neutrophils. The changes in IDH expression were reflected in  $\alpha$ -ketoglutarate levels, which were decreased by hypoxia in wild-type neutrophils but remained elevated in neutrophils from AMPK $\alpha$ 2<sup>MC</sup> mice. It is interesting to note that higher IDH1 levels have been linked with increased HIF-1 $\alpha$  degradation and the sensitization of melanoma cells to hypoxia-induced apoptosis,<sup>47</sup> effects that parallel the fate of AMPK $\alpha$ 2-deficient neutrophils. Moreover, inhibition of IDH by oxalomalic acid or IDH knockdown was reported to elevate HIF-1 $\alpha$  levels.<sup>48</sup> It, therefore, seems that the links between AMPK $\alpha$ 2 and HIF-1 $\alpha$  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 AMPK $\alpha$ 2 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-1 $\alpha$  stability mediated by alterations in phosphorylation and myeloid cell metabolism.

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### Disclosures

None.

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## Novelty and Significance

### What Is Known?

- Myeloid cells are rapidly recruited to ischemic tissue and are required for the initiation of 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)-1 $\alpha$ .
- HIF-1 $\alpha$  protein levels are barely detectable in normoxic conditions because of the high activity of the prolyl hydroxylase domain-containing enzymes that hydroxylate HIF-1 $\alpha$  and promote its degradation.

### What New Information Does This Article Contribute?

- Global deletion of the AMP-activated protein kinase (AMPK $\alpha$ 2) 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 AMPK $\alpha$ 2 subunit in neutrophils is essential for their survival in the ischemic hindlimb and subsequent monocyte recruitment.
- In the absence of AMPK $\alpha$ 2, the changes in neutrophil protein expression elicited by hypoxia are attenuated, a phenomenon attributed to the failure to stabilize HIF-1 $\alpha$  protein expression.
- In hypoxic conditions, AMPK $\alpha$ 2 activation leads to a decrease in the expression of 2 isoforms of the isocitrate dehydrogenase and the generation of  $\alpha$ -ketoglutarate, which is required as a cofactor for prolyl hydroxylase domain enzymes and the subsequent HIF-1 $\alpha$  hydroxylation.

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 AMPK $\alpha$ 2 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 an arteriogenic/angiogenic state. A combination of proteomic and metabolomic analyses show that AMPK $\alpha$ 2 determines the response of neutrophils in an ischemic environment, partly by decreasing isocitrate dehydrogenase expression. The latter step is essential to decrease cellular levels of  $\alpha$ -ketoglutarate and prevent the hydroxylation and degradation of HIF-1 $\alpha$ . 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. Given that AMPK activity is dysregulated in diabetes mellitus, the rapid application of AMPK activators may promote vascular repair in diabetic individuals by a combination of effects on neutrophil metabolism and HIF-1 $\alpha$  stability.