Activation of AMP-Activated Protein Kinase by 5-Aminoimidazole-4-Carboxamide-1-β-D-Ribofuranoside in the Muscle Microcirculation Increases Nitric Oxide Synthesis and Microvascular Perfusion

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Objective—To investigate the effects of activation of the AMP-activated protein kinase (AMPK) on muscle perfusion and to elucidate the mechanisms involved.

Methods and Results—In a combined approach, we studied the vasoactive actions of AMPK activator by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) on rat cremaster muscle resistance arteries and on microvascular perfusion in the rat hindlimb in vivo. In isolated resistance arteries, AICAR increased Thr172 phosphorylation of AMPK in arteriolar endothelium, which was predominantly located in microvascular endothelium. AICAR induced vasodilation (19±4% at 2 mmol/L, P<0.01), which was abolished by endothelium removal, inhibition of NO synthase (with N-nitro-L-arginine), or AMPK (with compound C). Smooth muscle sensitivity to NO, determined by studying the effects of the NO donor S-nitroso-N-acetylpenicillamine (SNAP), was not affected by AICAR except at the highest dose. AICAR increased endothelial nitric oxide synthase activity, as indicated by Ser1177 phosphorylation. In vivo, infusion of AICAR markedly increased muscle microvascular blood volume (≈60%, P<0.05), as was evidenced by contrast-enhanced ultrasound, without effects on blood pressure, femoral blood flow, or hind leg glucose uptake.

Conclusion—Activation of AMPK by AICAR activates endothelial nitric oxide synthase in arteriolar endothelium by increasing its Ser1177 phosphorylation, which leads to vasodilation of resistance arteries and recruitment of microvascular perfusion in muscle. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: AICAR ■ AMPK ■ microcirculation ■ muscle ■ hemodynamics

The AMP-activated protein kinase (AMPK) is a serine/threonine kinase that has a ubiquitous tissue distribution. Its activity increases in response to cellular stressors such as hypoxia, oxidant stress, and hyperosmolarity. Muscle contraction also leads to the activation of AMPK, which in turn can increase glucose uptake, independently of insulin, both in vivo and in vitro. In addition, AMPK has been proposed to mediate the insulin-sensitizing effects of exercise, to be responsible for the beneficial effects on blood pressure, and to reduce atherogenic risk factors associated with the insulin resistance syndrome, but the mechanisms involved have not been fully elucidated.

AMPK is activated by hormones that act via Gs receptors, adiponectin, leptin, pharmacological agents such as metformin and thiazolidinediones, and oxidants such as ONOO− and H2O2. AMPK is expressed in both endothelial cells and smooth muscle, but the isoform expression of the AMPKα units differs between different arteries. Endothelial AMPK may have many important physiological functions, including regulation of endothelial nitric oxide synthase (eNOS) activation, regulation of inflammation, angiogenesis, and maintenance of perfusion. In endothelial cells, AMPK is phosphorylated and activated by 2 AMPK kinase pathways, LKB1 and CAMKKβ. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), a drug that is metabolized inside cells into 5-aminoimidazole-4-carboxamide-riboside, an analogue of AMP, also activates AMPK in endothelial cells. AMPK has been shown to regulate endothelial function. In conduit arteries, AMPK enhances endothelium-dependent vasodilatation, increases eNOS phosphorylation at Ser1177, decreases cholesterol synthesis, and increases fatty acid oxidation. Interestingly, AMPK associates with eNOS in the microcirculation of the heart, suggesting a role for AMPK in the regulation of NO.
activity in microvessels. In the microcirculation, a functional role of AMPK has not been demonstrated.

Direct activation of AMPK by AICAR enhances insulin-mediated glucose uptake in muscle and ameliorates insulin resistance. The activation of the AMPK pathway is thus an attractive therapeutic target for cardiovascular diseases associated with obesity, type 2 diabetes, stroke, and atherosclerosis. There is evidence that suggests that the insulin-independent effects of AICAR in vivo and the sensitizing effects of exercise and AMPK on insulin-mediated glucose uptake in muscle may be mediated in part by the muscle microcirculation. First, insulin-mediated glucose uptake in muscle is determined by myocellular glucose uptake, as well as glucose delivery, ie, enhancement of nutritive muscle blood flow. Second, exercise-induced enhancement of insulin sensitivity is independent of known insulin signaling in myocytes. Third, the enhancement of insulin-mediated muscle blood flow by exercise has been shown to correlate strongly to its enhanced metabolic effects. In agreement with a role for the microcirculation, genetic deletion of the AMPKα2 subunit in mice causes insulin resistance but does not alter insulin-stimulated glucose uptake in the isolated muscles that obtain their nutrients by diffusion independently from the vasculature. Therefore, a role of AMPK in regulating microvascular perfusion in muscle may be a mechanism relevant to insulin resistance.

In the present study, we tested the hypotheses that (1) activation of AMPK with AICAR has direct vasodilatory actions on muscle resistance arteries ex vivo and in vivo and (2) these effects are achieved by increasing NO activity.

### Materials and Methods

#### Ex Vivo Effects of AICAR on Muscle Resistance Arteries

Segments of muscle resistance arteries were studied in the pressure myograph as described. To assess vasoactive effects of AMPK activation, segments were treated with AICAR (0.2 or 2 mmol/L) alone or in combination with the AMPK inhibitor compound C (40 μmol/L). To determine the role of arteriolar endothelium in vasoactive effects of AICAR, endothelium was removed by air bubble treatment as described. To assess whether NO mediates the vasoactive effects of AICAR, segments were treated with either the nitric oxide synthase inhibitor N-nitro-L-arginine (L-NA; 0.1 mmol/L) or a combination of AICAR and L-NA.

#### Western Blotting and 3D-Fluorescence Microscopy

The effects of AICAR on Thr172 phosphorylation of AMPKα were assessed by Western blotting and 3D deconvolution microscopy of the vessel wall as described in the Supplemental Data (available online at http://atvb.ahajournals.org) and by Eringga et al. Briefly, segments of cremaster arterioles were stimulated with AICAR (2 mmol/L) in the pressure myograph, fixed with 4% paraformaldehyde, permeabilized with 0.1% triton, and stained for Thr172 phosphorylated AMPKα (pAMPKα) as described. Specificity of staining was determined in each experiment by staining vessel segments of the same arterioles without the primary antibody. All Western blotting and microscopy experiments were carried out in a paired fashion: pAMPKα or phosphorylated eNOS was determined in segments from the same arteriole that were treated with solvent, AICAR, or AICAR in combination with compound C. Ser1177 phosphorylation of eNOS was determined as a surrogate measure of eNOS activation. To confirm that AMPK mediates effects of AICAR on eNOS phosphorylation, effects of AICAR were determined in the absence and presence of compound C.

### In Vivo Effects of AICAR and Exercise on Muscle Perfusion

Microvascular perfusion of the adductor magnus and semimembranosus muscles of the rat hindlimb was measured by contrast-enhanced ultrasound, as described in the online Supplemental Data. Microvascular perfusion was subsequently measured under control conditions, during intravenous infusion of AICAR, and during muscle contraction.

Details regarding animal housing, vessel myography, contrast-enhanced ultrasound assessment of muscle perfusion, and statistics are given in the online Supplemental Data.

### Results

#### AICAR Dilates Muscle Resistance Arteries Ex Vivo by Activating AMPK in Endothelium and Increasing Ser1177 Phosphorylation of eNOS

Passive intraluminal diameters of arterial segments averaged 176±6 μm when pressurized to 65 mm Hg. During the equilibration period, all vessel segments developed spontaneous tone, reducing the diameter by 94±7 μm (54±3%) to 82±7 μm. When stimulated with the endothelium-dependent vasodilator acetylcholine (0.1 μmol/L), vessels dilated by 43±11%.

In isolated muscle resistance arteries, AICAR induced a dose-dependent vasodilatation. At 0.2 mmol/L, AICAR induced a transient vasodilatation of 6±1% (P<0.05; Figure 1B) that reached a maximum at between 5 and 10 minutes. At 2 mmol/L, AICAR induced a sustained vasodilatation of 19±4% (P<0.01) that started approximately 5 minutes after addition of AICAR and reached a plateau after 15 to 25 minutes (Figure 1A and 1B). Removal of vascular endothelium by air bubble treatment fully inhibited AICAR-induced vasodilatation (Figure 1B). Furthermore, AICAR-induced vasodilatation was abrogated by inhibition of nitric oxide synthase (with L-NA; P<0.01). Finally, the vasodilator effect of AICAR was abolished by inhibition of AMPK (with compound C; Figure 1B), demonstrating a critical role for AMPK activity. AMPK inhibition reduced AICAR-induced vasodilatation to 3±2%. In the absence of AICAR, inhibition of NO synthesis by L-NA caused a small vasostriction (Figure 1B), indicating basal NO synthesis. To assess whether AICAR enhances NO production in endothelium or NO sensitivity of smooth muscle, we studied vasodilatation of endothelium-denuded resistance arteries to the NO donor S-nitroso-N-acetylpenicillamine (SNAP) in the absence and presence of AICAR. AICAR did not enhance SNAP-induced vasodilatation in endothelium-denuded resistance arteries except at the highest concentration (0.1 mmol/L; Figure 1C). In addition, AICAR did not increase vasodilatation in response to acetylcholine (data not shown). This indicates that AICAR enhances endothelial NO synthesis rather than smooth muscle sensitivity to NO.

To elucidate the mechanism underlying AICAR-mediated vasodilatation in muscle resistance arteries in more detail, we first studied the localization of AICAR-induced AMPK activity by Western blotting and 3D deconvolution microscopy of the vessel wall. Figure 2 shows that AICAR activated AMPK in the resistance arteries by significantly increasing the phosphorylation of its catalytic subunit, AMPKα, at Thr172 (P<0.01; Figure 2A). Using 3D microscopy of the arteriolar wall, we found that activated AMPKα was found predominantly in the arteriolar endothelium (Figure 2B). In contrast to AICAR-treated segments, there was a markedly lower phosphorylation of AMPKα in control segments (Figure 2B). Nonspecific staining at identi-
cal exposure times was negligible (Figure 2B), demonstrating that the fluorescence signal was indeed Thr172 pAMPK.

The increased amount of activated AMPKα in endothelium was associated with a significant ($P<0.05$) increase in the phosphorylation of eNOS at Ser1177 in resistance arteries that was fully inhibited by pretreatment with compound C (Figure 2C).

**In Vivo Effects of AICAR**

Injection of 20 mg · kg⁻¹ AICAR IV at the commencement of the experiment followed by constant infusion of AICAR IV at
3.75 mg · min\(^{-1}\) · kg\(^{-1}\) gave rise to a plasma concentration of 266±12 μmol/L and a tissue concentration at the end of the experiment (gastrocnemius muscle) of 98±9 nmol/g wet weight of AICAR and 207±27 nmol/g wet weight of aminoimidazole-4-carboxamide-riboside (Table). Figure 3 shows that AICAR had no effect on blood pressure, femoral blood flow, or hind leg glucose uptake but had a small but significant effect of lowering the heart rate (38 bpm from a resting rate of 380±15 bpm). This effect on heart rate is probably a direct action on the heart, as it has been observed previously with isolated perfused hearts.28

However, despite the failure of AICAR to increase femoral blood flow, there was a marked increase in microvascular perfusion (reflected by an increase in the microvascular volume filled by the microbubbles) in the semimembranosus, adductor magnus, and biceps femoris muscles of the hindlimb. Muscle contraction also increased microvascular perfusion compared with the saline control. Figure 4 compares data for measurement of the microvascular blood volume and microvascular flow rate at commencement (\(t=0\) minutes), after treatment with saline or AICAR (\(t=60\) minutes), and during muscle contraction (\(t=70\) minutes). Saline infusion had no effect on either volume or velocity over the 60 minutes. AICAR increased microvascular blood volume but not microvascular flow rate relative to the value at \(t=0\) minutes. Contraction increased the microvascular blood volume in both saline-treated and AICAR-treated animals (Figure 4). Muscle contraction significantly \((P<0.05)\) increased microvascular flow rate in both saline- and AICAR-treated animals, and this effect was significantly greater in AICAR-treated compared with saline-treated animals.

**Discussion**

The present study shows for the first time that (1) AMPK activation by AICAR directly causes vasodilation of skeletal muscle resistance arteries by enhancing NO production in endothelium and (2) acute AICAR-induced stimulation of AMPK in vivo enhances microvascular perfusion in skeletal muscle. This is the first report of such an effect, which suggests a role for AMPK in the vasodilation responsible for increased microvascular perfusion, the purpose of which is to increase available capillary surface area for increased nutrient and hormone delivery to skeletal muscle myocytes. We found that AICAR activated AMPK in endothelium and, to a minor extent, in smooth muscle cells of muscle resistance arteries and relaxes these arteries by increasing NO synthesis. Thus, AICAR-induced AMPK activation and endothelial NO production is the most likely mechanism underlying AICAR-stimulated microvascular perfusion in muscle in vivo.

Our results strongly suggest that AICAR's vasodilator effects in muscle resistance arteries are achieved through

### Table. Plasma and Muscle Contents of AICAR and ZMP From AICAR-Treated Animals

<table>
<thead>
<tr>
<th></th>
<th>AICAR (nmol/g Wet Weight)</th>
<th>ZMP (nmol/g Wet Weight)</th>
<th>AICAR (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrocnemius group</td>
<td>98±9</td>
<td>207±27</td>
<td>266±12</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td>266±12</td>
</tr>
</tbody>
</table>

Values are means±SE from groups \((n=4\) for the muscle and \(n=10\) for the plasma\) determined at \(t=70\) minutes.

Figure 3. Effect of AICAR on mean arterial blood pressure, heart rate, femoral arterial blood flow, and hind leg glucose uptake. Mean±SE values are shown for saline-treated (open symbols) \((n=6)\) and AICAR-treated (filled symbols) \((n=10)\) rats. When not visible, the error bars are within the symbol. *Significantly different \((P<0.05)\) from saline values.

Figure 4. Microvascular volume and flow rate. Measurements of microvascular volume (A value) (A) and microvascular flow rate (A×β value) (B) for basal levels at 0 minutes (white bars), after 60 minutes of treatment with either saline (cross-hatched bars) or AICAR (black bars), and during muscle contraction at 70 minutes (hatched bars). Values are means±SE for \(n=6\) in each group. *Significantly different \((P<0.05)\) from 0 minutes, basal; #70 minutes, contraction, significantly different \((P<0.05)\) from 0- and 60-minute values.
increased NO production in endothelium. Although AICAR enhanced smooth muscle relaxation in response to the NO donor SNAP to a minor extent, this occurred only at a SNAP/NO concentration of 0.1 mmol/L, which induced ≈50% vasodilatation (Figure 1C). As AICAR-induced vasodilation is mediated by NO and averaged 19±4% (Figure 1B), sensitization of smooth muscle to NO by AICAR cannot explain this effect. Our results differ from those of Goirand et al., who found that AICAR-induced vasodilatation of the mouse aorta was independent from endothelium. This difference indicates that the mechanisms causing AICAR-induced vasodilatation vary between different vascular beds.

In macrovascular endothelial cells, it has been reported that activation of AMPK by AICAR increases phosphorylation of eNOS at Ser1177 and Ser635, resulting in increased NO production. In agreement with these data, we found that AICAR enhances Ser1177 phosphorylation of eNOS (Figure 2C), which we propose to be the mechanism for vasodilatation in the isolated resistance arteries. However, our results do not exclude other mechanisms contributing to eNOS activation, such as phosphorylation of eNOS at Ser635.

When considering the mechanisms involved in AICAR-induced vasodilatation, the specificity of AICAR and compound C have to be considered. First, AICAR is an analogue of adenosine that in some studies has been reported to have physiological actions in the heart via adenosine receptor stimulation. However, adenosine-induced vasodilatation in our model develops within 1 minute (E. Eringa, unpublished results) and is endothelium independent. Furthermore, the adenosine receptor blocker 8-phenyltheophylline did not inhibit AICAR-induced vasodilatation in isolated resistance arteries (data not shown). Therefore, our results do not support a mechanism for AICAR-induced vasodilatation involving adenosine receptors. In contrast to dilatation of muscle resistance arteries, the decrease in heart rate induced by AICAR may be due to interaction with adenosine receptors in pacemaker cells. Second, it is theoretically possible that AICAR and compound C regulate vascular tone through an AMPK-independent mechanism. However, as there is no known mechanism that is both activated by AICAR and inhibited by compound C, there is no evidence to support an AMPK-independent mechanism. Third, AICAR could have increased Ser1177 phosphorylation of eNOS by activating Akt. We found that AICAR does not activate Akt in cremaster resistance arteries (unpublished data), ruling out this possibility. Taken together, all available data suggest that AICAR’s vasodilator effects in the muscle microcirculation are mediated by AMPK.

The stimuli that activate AMPK in microvascular endothelium and smooth muscle leading to increased NO activity under physiological conditions are still to be resolved from the many candidates (adiponectin, leptin, circulating hormones, oxidants etc). In macrovascular endothelial cells, AMPK has been shown to be involved in shear stress–mediated activation of eNOS. However, it is important to note that the AICAR-mediated increase in microvascular perfusion in vivo occurred in the absence of any increase in total femoral flow. Therefore, a direct effect of AICAR to activate AMPK is likely and could not have occurred indirectly by shear stress through increased flow.

Although steady-state infusion of AICAR has led to greater microvascular perfusion of muscle in vivo and a direct vasodilator effect on resistance vessels in vitro has been demonstrated, we cannot entirely rule out an indirect vascular effect of AICAR. One such possibility relates to the stimulation of nitric oxide synthase in skeletal muscle, with a spillover of NO to vasodilate nearby resistance vessels controlling microvascular perfusion.

It is important to note that in the present study, AICAR increased microvascular perfusion without increasing muscle glucose uptake. Therefore, we conclude that in the absence of an increase in glucose extraction, greater microvascular perfusion alone is not sufficient for increasing muscle glucose uptake. At doses higher than the one used in our study, AICAR has been reported to increase GLUT-4 translocation in isolated muscle and to increase muscle glucose uptake. Our data are in agreement with a recent study by Boon et al., in which humans were administered AICAR to raise their plasma concentrations to ≈160 μmol/L, without a resulting increase in whole body glucose disposal. Taken together, these data would suggest that the action on microvascular perfusion is more sensitive than glucose uptake to AICAR.

The effects of AICAR on the microcirculation are likely to be relevant to regulation of insulin-stimulated glucose uptake, as AICAR? and muscle microvascular perfusion have been shown to regulate insulin sensitivity. In rats, increased microvascular perfusion, induced by small amounts of muscle contraction, enhances insulin sensitivity even without enhancing basal glucose uptake or total limb blood flow. Increased microvascular perfusion has been proposed to facilitate insulin delivery and uptake and thus contribute to insulin action in muscle. These and our data suggest that AMPK activation in vascular endothelium contributes to regulation of insulin sensitivity.

In the present study, AICAR increased microvascular perfusion in vivo, but unlike muscle contraction, it did so without increasing femoral blood flow or microvascular flow rate. This suggests that AICAR redistributes blood from the nonnutritive route to the nutritive route in muscle. Low levels of exercise have also been reported to increase microvascular blood volume with no increase in microvascular flow rate where there is only a minimal increase in limb blood flow. Thus, selective vasodilation within the nutritive route would appear to be sufficient to allow flow to be redistributed. This redistribution is still apparent when total muscle blood flow is increased with contraction. In the present study, the intensity of contraction increased total muscle blood flow in both saline- and AICAR-treated animals, and because of the redistribution, the microvascular flow rate was significantly greater for the AICAR-treated rats than for saline-treated ones (Figure 4B). As a final note, our data do not prove or disprove the possibility that AMPK mediates exercise-induced microvascular recruitment, as AMPK was not inhibited during exercise. This question remains to be resolved in future studies.

In summary, low-dose AICAR stimulates microvascular perfusion in skeletal muscle in vivo at a dosage that does not increase glucose uptake. We have found that AICAR-mediated vasodilatation in muscle resistance arteries results from AMPK-mediated NO production through increased Ser1177 phosphorylation of eNOS. Our findings highlight a key link between...
AMPK, vascular function, and metabolism, as greater microvascular perfusion potentially enhances hormone and nutrient delivery to muscle myocytes. A vascular action may be a vital factor for AICAR's potential as a therapeutic agent for treating muscle insulin resistance and the associated cardiovascular disease.

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Disclosures

None.

References

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