

## Intra-arterial AICA-riboside administration induces NO-dependent vasodilation in vivo in human skeletal muscle

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**Bosselaar M, Boon H, van Loon LJ, van den Broek PH, Smits P, Tack CJ.** Intra-arterial AICA-riboside administration induces NO-dependent vasodilation in vivo in human skeletal muscle. *Am J Physiol Endocrinol Metab* 297: E759–E766, 2009. First published July 14, 2009; doi:10.1152/ajpendo.00141.2009.—In animal models, administration of the adenosine analog AICA-riboside has shown beneficial effects on ischemia-reperfusion injury and glucose homeostasis. The vascular and/or metabolic effects of AICA-riboside administration in humans remain to be established. AICA-riboside was infused intra-arterially in four different dosages up to 8 mg·min<sup>-1</sup>·dl<sup>-1</sup> in 24 healthy subjects. Forearm blood flow (FBF) and glucose uptake and plasma glucose, free fatty acid, and AICA-riboside concentrations were assessed. We also combined AICA-riboside infusion (2 mg·min<sup>-1</sup>·dl<sup>-1</sup>) with the intra-arterial administration of the adenosine receptor antagonist caffeine (90 μg·min<sup>-1</sup>·dl<sup>-1</sup>; n = 6) and with the endothelial NO synthase inhibitor L-NMMA (0.4 mg·min<sup>-1</sup>·dl<sup>-1</sup>; n = 6). Additional in vitro experiments were performed to explain our in vivo effects of AICA-riboside in humans. AICA-riboside increased FBF dose dependently from 2.0 ± 0.2 to 13.2 ± 1.9 ml·min<sup>-1</sup>·dl<sup>-1</sup> maximally (P < 0.05 for all dosages). The latter was not reduced by caffeine administration but was significantly attenuated by L-NMMA infusion. Despite high plasma AICA-riboside concentrations, forearm glucose uptake did not change. In vitro experiments showed rapid uptake of AICA-riboside by the equilibrative nucleoside transporter in erythrocytes and subsequent phosphorylation to AICA-ribotide. We conclude that AICA-riboside induces a potent vasodilator response in humans that is mediated by NO. Despite high local plasma concentrations, AICA-riboside does not increase skeletal muscle glucose uptake.

5-aminoimidazole-4-carboxamide; nitric oxide; forearm blood flow; forearm glucose uptake

THE ADENOSINE ANALOG 5-aminoimidazole-4-carboxamide (AICA)-riboside (also known as acadesine) has been shown to improve ischemia-reperfusion injury (7, 17, 21) and glucose homeostasis in various animal models (19). Although the exact molecular mechanisms remain to be elucidated, it seems evident that AICA-riboside-induced activation of AMP-activated protein kinase (AMPK) plays a key role (14). AICA-riboside is taken up by skeletal muscle cells and is subsequently phosphorylated by adenosine kinase to AICA-ribotide (ZMP) (8). ZMP is an intermediate of the de novo purine synthesis and is present in very low concentrations in normal cells. ZMP activates AMPK by mimicking the effects of AMP without disturbing cellular

levels of AMP, ADP, or ATP (8). AMPK activation has been shown to stimulate endothelial nitric oxide (NO) release (15), increase skeletal muscle glucose uptake (14), and inhibit hepatic glucose production (37). Consequently, AMPK represents a promising pharmacological target for the prevention and/or treatment of ischemic heart disease and/or insulin resistance (25).

Both in vitro (14) and in vivo animal studies (19) as well as in vitro human studies (22) have demonstrated that AICA-riboside stimulates glucose transporter 4 translocation to the plasma membrane, resulting in greater insulin sensitivity and increased glucose uptake. Until now, only three recent studies have described the in vivo metabolic effects of AICA-riboside administration in humans (1, 6, 9). In our study, we observed a significant decline in plasma glucose concentrations following intravenous AICA-riboside infusion (6). This might, at least partly, be attributed to increased muscle perfusion leading to increased glucose uptake in skeletal muscle (26, 27), since AICA-riboside has been suggested to have strong vasodilator properties, being an analog to adenosine (17). Adenosine plays an important role in the regulation of vascular tone and is a potent vasodilator (30). So far, the impact of AICA-riboside administration on blood flow in vivo in humans has not been established. In the present study, we investigated the impact of AICA-riboside administration on skeletal muscle forearm blood flow (FBF) and glucose uptake (FGU) in vivo in healthy humans. Additional in vitro experiments were performed to elucidate the mechanisms responsible for the vasodilator properties of AICA-riboside in vivo in humans.

### METHODS

**Study population.** A total of 32 healthy subjects (14 males, 18 females; 4 subjects participated twice) were selected to participate (age 21.9 ± 2.2 yr, BMI 21.5 ± 1.7 kg/m<sup>2</sup>; means ± SD). The investigation conformed to the principles outlined in the Declaration of Helsinki. The local ethics committee approved the study, and all subjects gave their written, informed consent.

**Protocol and experimental procedure.** AICA-riboside was infused into the brachial artery [perfused forearm model (38); also see below] for 110 min in four doses (1, 2, 4, or 8 mg·min<sup>-1</sup>·dl<sup>-1</sup> forearm tissue), each dose in a separate group of six volunteers. Hereafter, normal saline was infused intra-arterially for an additional 70 min. The exact dose calculations of AICA-riboside are provided below.

The experiments started at 8:15 AM after an overnight fast in a quiet, temperature controlled room (23–24°C). The subjects abstained from caffeine and alcohol for ≥24 h prior to the experiments (29). A catheter (Angiocath, 20-gauge, 48 mm; Becton Dickinson, Sandy, UT) was inserted into the brachial artery of the nondominant arm (experimental arm) for intra-arterial infusion of AICA-riboside and

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for obtaining arterial blood samples. The brachial artery catheter was connected with an arterial pressure-monitoring line to a Hewlett Packard 78353 B monitor (Hewlett Packard, Böblingen, Germany) for continuous blood pressure monitoring. In both arms, a 20-gauge catheter was inserted retrogradely into a deep forearm vein for blood sampling, enabling measurement of forearm arteriovenous glucose differences ( $\Delta\text{Gluc}_{A-V}$ ) in both arms. Thirty minutes after complete instrumentation, baseline data (FBF,  $\Delta\text{Gluc}_{A-V}$ , plasma insulin level, uric acid level, and hematocrit) were collected. During administration of the highest dose of AICA-riboside ( $8 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ), plasma free fatty acid (FFA), free glycerol, lactate, and triglyceride concentrations were also measured ( $n = 5$ ). We used the venous occlusion plethysmography (34) in our experiments to measure FBF (by a Hokanson EC-4 Plethysmograph) in both arms simultaneously and to measure the local vasodilator response to the administration of AICA-riboside into the brachial artery in one of the two arms. This technique is a well-validated method for these measurements (3, 33). Intrabrachial administration of vasoactive drugs results in a high local concentration in the forearm vascular bed but prevents significant systemic spillover of the drugs and subsequent systemic confounding effects or effects on the contralateral arm. Venous outflow from the forearm is prevented by the placement of a cuff with repetitive inflation around the upper arm using an inclusion pressure of  $\pm 40 \text{ mmHg}$  (E-20 Rapid Cuff Inflator; Hokanson). The arms are slightly elevated (10 cm) above heart level. One minute before the start of the measurements, wrist cuffs are inflated to 100 mmHg above systolic blood pressure to exclude hand and skin flow to enter the deep venous system at the wrist, because the blood flow in hands is predominantly through skin. The rate of swelling of the forearm, measured with mercury-in-silastic strain gauges, is used to assess FBF. We measured FBF in both arms, but we infused AICA-riboside only in the experimental arm. In the contralateral arm, no AICA-riboside was infused, and therefore, we can use this as the "placebo arm." By using this study model, no other placebo experiments are needed because the healthy subjects are their own controls.

To further characterize the vasodilator effects of AICA-riboside, we combined intra-arterial AICA-riboside administration ( $2 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ) with intra-arterial infusion of the adenosine receptor antagonist caffeine ( $90 \text{ } \mu\text{g} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ;  $n = 6$ ) or the endothelial NO synthase inhibitor  $N^G$ -monomethyl-L-arginine (L-NMMA;  $4 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ;  $n = 6$ ). In the caffeine experiment, we used the same experimental procedure as described above, with the exception that, after 90 min of AICA-riboside infusion, caffeine was concomitantly infused for 15 min to inhibit adenosine-induced vasodilation. We have recently demonstrated that this intra-arterial dose of caffeine is effective in reducing adenosine-induced vasodilation (28, 29). AICA-riboside in a dose of  $2 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$  showed approximately the same increase in FBF as adenosine in this study (28). In the experiments with L-NMMA (Clinalpha, Laufelfingen, Switzerland), we started first with 15 min of L-NMMA to completely inhibit NO synthase. Hereafter, we infused AICA-riboside for 110 min. Every 15 min we combined AICA-riboside administration with L-NMMA ( $t = 15\text{--}30, 45\text{--}60$ , and  $75\text{--}90 \text{ min}$ ). We recently reported that this dose of L-NMMA ( $4 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ) maximally inhibits endothelial NO synthase in the human forearm (35).

**AICA-riboside dose calculation.** AICA-riboside has been administered intravenously to healthy subjects (12, 13) and to patients with cardiac disease (10, 24) in studies regarding ischemic protection during major surgery. All dosages were well tolerated. Studies suggested that dosages up to 8 g over 30 min do not impose any health risk (13). Assuming a baseline FBF of  $2 \text{ ml} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$  (and a plasma flow of  $\sim 1 \text{ ml} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ), an infusion rate of  $1 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$  would result in a local plasma AICA-riboside concentration of 1 mg/ml, equalling 3.87 mmol/l (molecular weight 258.24 g). In vitro, AICA-riboside appeared to be pharmacologically active in this range (22). AICA-riboside (Toronto Research Chemi-

cals, Toronto, ON, Canada) was prepared for human use by our Department of Clinical Pharmacy and was dissolved in normal saline.

**Measurement of AICA-riboside and ZMP concentrations.** In the AICA-riboside experiments ( $8 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ), we measured plasma AICA-riboside concentrations and AICA-riboside and ZMP concentrations in erythrocytes in venous blood samples taken from the experimental as well as from the control arm (at  $t = 0, 5, 30, 60, 90$ , and 180 min). The erythrocyte data were used as a model for the intracellular handling of AICA-riboside in the vascular wall. One milliliter of blood was drawn in lithium-heparin tubes and stored directly on ice. To avoid further uptake or metabolism of AICA-riboside after sampling, the tubes were first filled with dipyridamole (Boehringer Ingelheim, Alkmaar, The Netherlands) (used concentrations were based on our own measurements; see RESULTS and Fig. 6) and A-13497, with final blood concentrations of 25  $\mu\text{M}$  and 225 nM, respectively. Dipyridamole inhibited the endogenous equilibrative nucleoside transporter (ENT) and was found to block AICA-riboside uptake in erythrocytes (vide infra). A-13497 blocks adenosine kinase, an enzyme that converts AICA-riboside into ZMP. Erythrocytes were washed twice in normal saline and lysed with 10 volumes of aqua pure (cold). Lysed erythrocytes and plasma were stored at  $-80^\circ\text{C}$  until final analysis.

**In vitro characterization of AICA-riboside uptake in cells.** In additional in vitro experiments, we further characterized the AICA-riboside uptake by erythrocytes. Assuming that AICA-riboside is taken up by the ENT, we measured intracellular AICA-riboside and ZMP concentrations under varying concentrations of the ENT inhibitor dipyridamole. We incubated at  $37^\circ\text{C}$  washed erythrocytes (50  $\mu\text{l}$  of 20% erythrocytes added to MOPS buffer) with dipyridamole (concentration range 0–50  $\mu\text{M}$ ) 5 min before adding 3 mM AICA-riboside. After 5 min, 250  $\mu\text{M}$  dipyridamole was added to block the uptake of AICA-riboside completely and immediately stored on ice.

**Laboratory measurements and analysis.** Plasma glucose concentrations were measured by the glucose oxidation method (Beckman Glucose Analyzer II; Beckman, Fullerton, CA). Plasma insulin was assessed in duplicate against IS 83/500 by an in-house radioimmunoassay using an anti-human insulin antiserum raised in guinea pig and radioiodinated human insulin as a tracer. Bound-free separation was carried out by addition of sheep anti-guinea pig antiserum and precipitation by means of polyethylene glycol. Between- and within-run coefficients of variation were 4.6 and 5.8%, respectively, at a level of 33 mU/l.

At the highest AICA-riboside dose, blood was collected for determination of plasma FFA, glycerol, triglyceride, and lactate concentrations by regular assays. Plasma AICA-riboside concentrations and AICA-riboside and ZMP levels in the erythrocytes were determined by high-performance liquid chromatography (HPLC), with ultraviolet detection set at 260 nm. The used column was Hypersil BDS C18 5 $\mu$  200  $\times$  4.6 mm (Thermo Quest). The mobile phase consisted of methanol, 10 mmol/l tetrabutylammonium hydrogen sulphate, and 5 mmol/l  $\text{K}_2\text{HPO}_4$ , pH 8.2 (20:80, vol/vol). For the in vitro experiments, the AICA-riboside concentration in the supernatant was determined with HPLC by use of a Polaris column. The mobile phase consisted of methanol, 50 mmol/l  $\text{NH}_4\text{H}_2\text{PO}_4$ , and 5 mmol/l hexanesulfonic acids, pH 3.0 (2:98, vol/vol). Plasma caffeine concentrations were determined by use of reversed-phase HPLC, with ultraviolet detection set at 273 nm.

**Calculations and statistical analysis.** FBF registrations of the last 2 min were averaged to a single value. FGU was calculated from  $\Delta\text{Gluc}_{A-V}$  and FBF, assuming that whole blood glucose =  $[1 - (0.3 \times \text{hematocrit})] \times \text{plasma blood glucose}$  (11) and  $\text{FGU} = \Delta\text{Gluc}_{A-V} \times [1 - (0.3 \times \text{hematocrit})] \times \text{FBF}$ .

All data are expressed as means  $\pm$  SE unless indicated otherwise. To detect changes in plasma metabolite concentrations and hemodynamic values over time, a repeated-measures ANOVA was applied. A Scheffé post hoc test was applied in cases of a significant  $F$  ratio to

detect specific differences. A  $P$  value of  $<0.05$  was considered significant.

## RESULTS

**Hemodynamic effects of AICA-riboside.** AICA-riboside induced a time- and dose-dependent vasodilation in the experimental arm only. From baseline to the end of the infusion (110 min), AICA-riboside increased FBF for each of the four doses (all  $P < 0.05$ ; Fig. 1, top). After discontinuation of AICA-riboside, FBF declined and returned toward baseline values. FBF in the contralateral arm did not increase during AICA-riboside infusion at any of the four dosages (Fig. 1, middle).

Fifteen minutes of L-NMMA infusion induced vasoconstriction ( $1.6 \pm 0.2$  to  $1.2 \pm 0.2$   $\text{ml} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ,  $P < 0.05$ ). During concomitant AICA-riboside infusion ( $2 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ), L-NMMA attenuated the vasodilator response to AICA-riboside significantly compared with the experiments with AICA-riboside ( $2 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ) only (Fig. 2). Concomitant infusion of caffeine did not alter the vasodilator response to AICA-riboside. In this series, AICA-riboside ( $2 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ) infusion

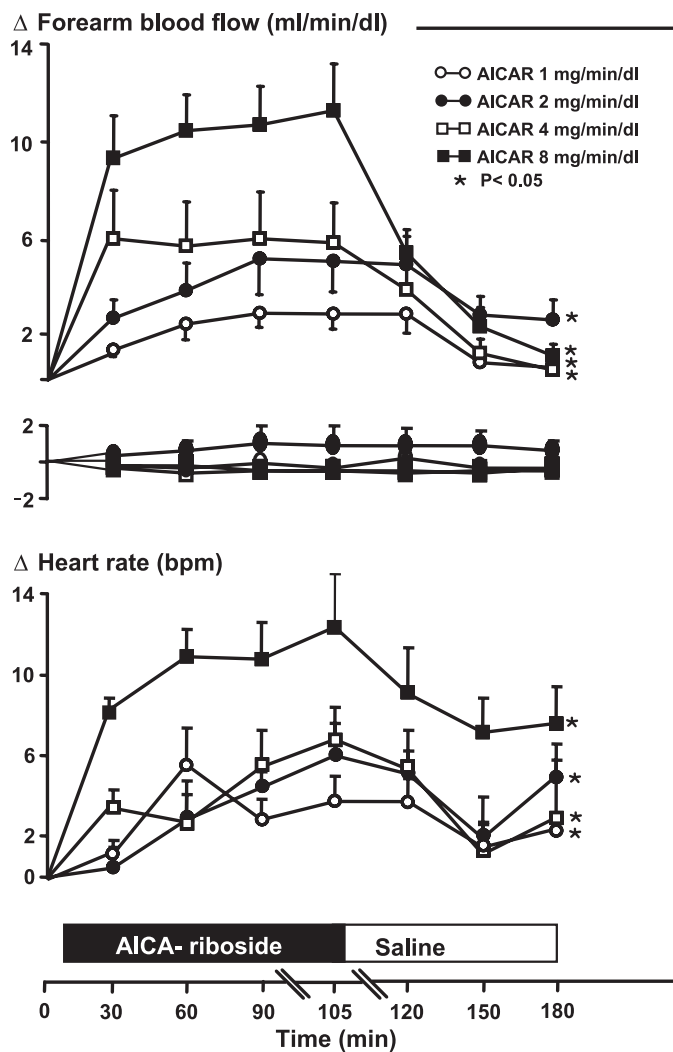


Fig. 1. Mean ( $\pm$  SE) absolute change in forearm blood flow of the experimental arm (top) and control arm (middle) and heart rate (bottom) during the 4 dosages of 5-aminoimidazole-4-carboxamide (AICA) riboside (AICAR). \*Significant changes over time ( $P < 0.05$ ).

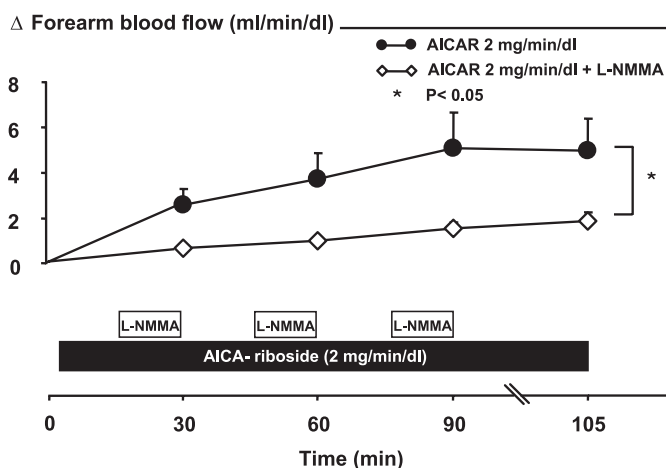


Fig. 2. Mean ( $\pm$  SE) absolute change in forearm blood flow of the experimental arm during AICAR ( $2 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ) only and during concomitant infusion with  $N^G$ -monomethyl-L-arginine (L-NMMA;  $4 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ). L-NMMA significantly attenuated the increase in forearm blood flow compared with AICAR only.

alone ( $t = 0-90$  min) induced a significant vasodilator response from  $2.4 \pm 0.3$  to  $5.7 \pm 0.7$   $\text{ml} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ . After 15 min of concomitant caffeine infusion ( $t = 90-105$  min), FBF amounted to  $5.0 \pm 0.8$   $\text{ml} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$  [not significant (NS)]. Basal plasma caffeine concentrations were  $0.1 \pm 0.1$  mg/l and increased significantly to  $10.0 \pm 1.9$  mg/l in the experimental arm and  $0.3 \pm 0.1$  mg/l in the control arm.

AICA-riboside infusion had no effect on blood pressure (MAP was  $78 \pm 1$  mmHg at baseline vs.  $78 \pm 2$  mmHg at the end of the highest dose of AICA-riboside,  $P = \text{NS}$ ), but heart rate increased significantly following AICA-riboside administration in all four dosages (Fig. 1, bottom).

**Metabolic effects of AICA-riboside.** Arterial (i.e., systemic) plasma glucose levels decreased from  $4.9 \pm 0.1$  to  $4.4 \pm 0.1$  mmol/l (pooled data;  $n = 24$ ,  $P < 0.001$ ) after 110 min of AICA-riboside infusion. This decrease was not strictly dose dependent, although the most pronounced results were obtained at the highest dose ( $P < 0.05$  for 2 and  $8 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ,  $P = 0.06$  for  $4 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ; Fig. 3, top). The decrease in systemic plasma glucose concentrations at the highest dose of AICA-riboside was most pronounced after 60 min. After discontinuation of AICA-riboside administration, arterial plasma glucose levels returned toward baseline values at all dosages (pooled data;  $n = 24$ ,  $P = 0.4$  vs. baseline values). Venous blood glucose levels decreased in parallel with those of the arterial blood in both the experimental and control arms ( $P < 0.05$  for AICA-riboside at the doses of 4 and  $8 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ; Fig. 3, middle and bottom). As a result,  $\Delta\text{Gluc}_{A-V}$  decreased, and as such, intrabrachial AICA-riboside infusion had no effect on the calculated FGU at all dosages. Figure 4 shows  $\Delta\text{Gluc}_{A-V}$  and FGU data for the highest dose of AICA-riboside. During this experiment, FGU did not increase compared with the control arm. Comparable observations were obtained for all lower AICA-riboside dosages.

Plasma insulin concentrations increased significantly after  $t = 90$  and 120 min in the experiments with AICA-riboside at a dosage of  $4 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ . During the highest dose of AICA-riboside, insulin levels decreased significantly from  $37 \pm 7$  at baseline to a nadir of  $26 \pm 5$  pmol/l at  $t = 180$  min

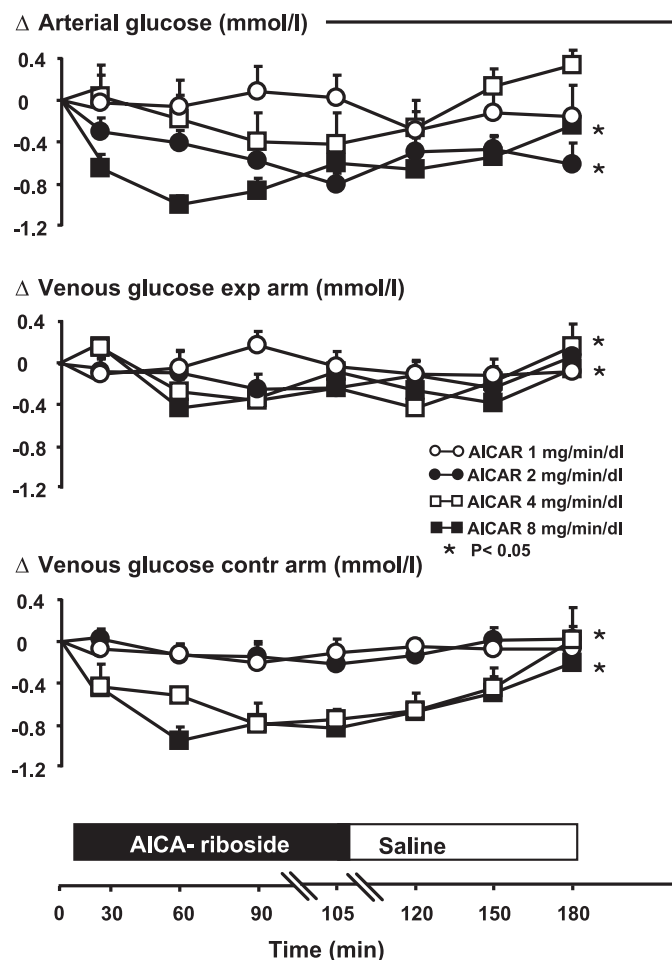


Fig. 3. *Top*: mean ( $\pm$  SE) absolute change in arterial glucose levels (in mmol/l) at the experimental arm. *Middle* and *bottom*: absolute change in venous glucose levels at the experimental and control arms at the 4 doses of AICAR. \*Significant changes over time ( $P < 0.05$ ).

( $P = 0.002$ ). In the other experiments, insulin levels did not change significantly.

During administration of the highest AICA-riboside dose, arterial plasma FFA concentrations decreased significantly. The latter was most pronounced after 60 min of infusion (Fig. 5). Both venous plasma FFA levels decreased in parallel with those of the arterial blood. There was a significant increase in plasma lactate concentrations at both the venous and arterial sites (for all,  $P < 0.05$  compared with baseline values). Peak concentrations were reached at  $t = 150$  min (Fig. 5). Plasma glycerol and total triglyceride concentrations decreased during AICA-riboside infusion ( $P < 0.05$  to baseline for the arterial values).

**AICA-riboside and ZMP concentrations.** During AICA-riboside infusion at the highest dosage of  $8 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ , we observed a strong, significant increase in venous plasma and erythrocyte AICA-riboside concentrations in the experimental arm ( $n = 5$ ; Table 1). On the control side, venous plasma and erythrocyte AICA-riboside concentration increased significantly but was not as pronounced compared with the experimental arm. After discontinuation of AICA-riboside, plasma and erythrocyte AICA-riboside concentrations of both arms decreased. The phosphorylated form of AICA-riboside (ZMP)

increased gradually over time, similarly at both sides. Intracellular ZMP levels remained unchanged up to 70 min after discontinuation of AICA-riboside infusion. At lower dosages of AICA-riboside ( $1, 2, \text{ and } 4 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ), we observed a comparable and dose-dependent pattern of AICA-riboside and ZMP accumulation in erythrocytes (data not shown).

**AICA-riboside uptake experiments (in vitro).** Accumulation of AICA-riboside and ZMP in erythrocytes was completely inhibited by increasing concentrations of dipyridamole, a well-documented inhibitor of the ENT (Fig. 6), confirming that AICA-riboside is taken up by the dipyridamole-sensitive ENT. At a concentration of  $-4.6 \log (= 25 \text{ } \mu\text{M})$  of dipyridamole, the uptake of AICA-riboside is completely inhibited.

**Side effects.** No side effects of AICA-riboside were reported, except for one subject following infusion with the highest AICA-riboside dose. The subject reported minor headache and some nausea after the experiment. These complaints disappeared spontaneously after the subject left the clinical research center. Uric acid, an end product of purine metabolism, increased in plasma ( $P < 0.05$  when compared with baseline values at all 4 dosages; Table 2).

## DISCUSSION

The main findings of our human in vivo study are that 1) intra-arterial AICA-riboside infusion induces a potent time- and dose-dependent vasodilation in the skeletal muscle vascular bed, 2) this vasodilator effect is mediated by endothelial NO release but not by adenosine receptor stimulation, 3) AICA-riboside does

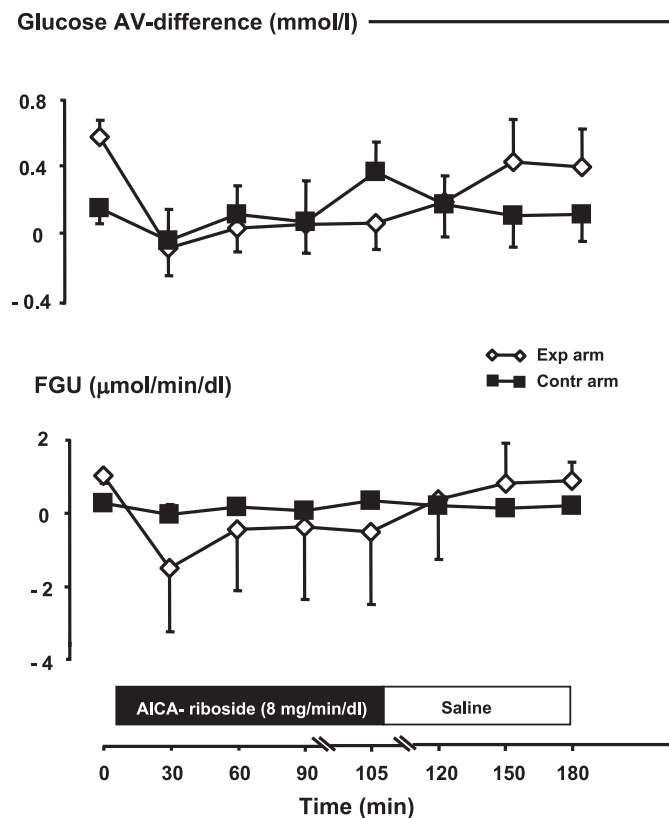


Fig. 4. Mean values ( $\pm$  SE) of arteriovenous (AV) glucose difference (*top*) and of forearm glucose uptake (FGU; *bottom*) before, during, and after infusion of AICA-riboside ( $8 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ).

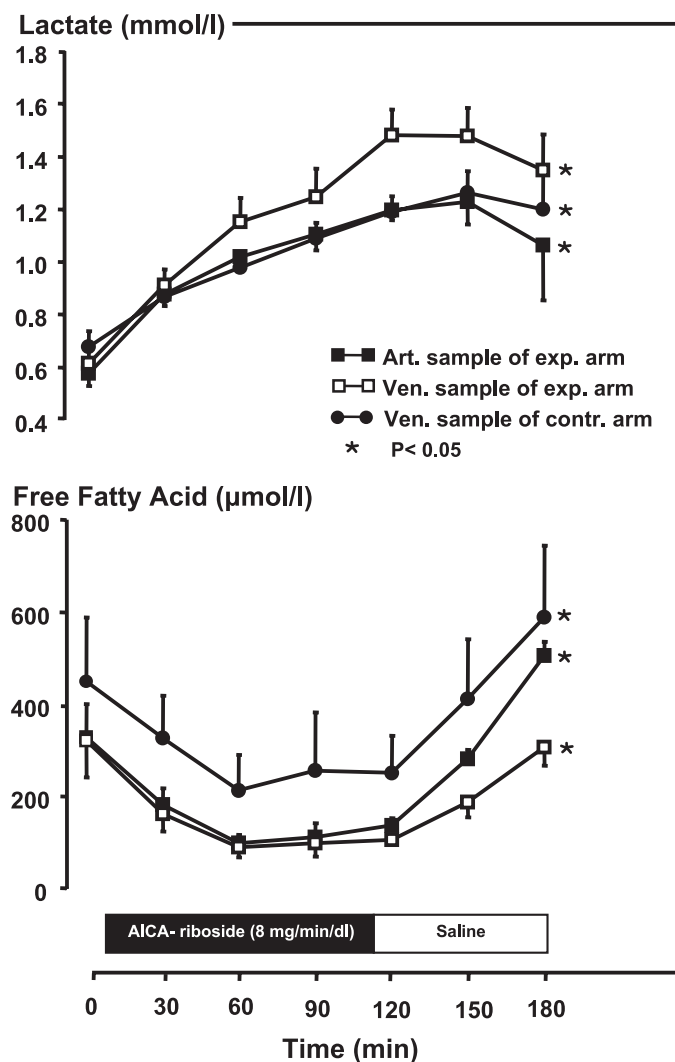


Fig. 5. *Top*: plasma lactate concentrations in mmol/l. *Bottom*: plasma free fatty acid concentrations in  $\mu\text{mol/l}$  in response to AICA-riboside infusion ( $8 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ). \*Significant changes over time ( $P < 0.05$ ).

not increase forearm glucose uptake, and 4) AICA-riboside is rapidly taken up by the dipyridamole-sensitive ENT in erythrocytes (and probably likewise in endothelial and vascular smooth muscle cells).

In the present study, we demonstrate that AICA-riboside induces a strong time- and dose-dependent vasodilation in skeletal muscle tissue. At the highest administration dose, AICA-riboside administration increases blood flow more than sixfold when compared with baseline values. And even at the lowest dosage, this increase is still substantial with a more than twofold increase in blood flow. At low dosages, this vasodilator effect is gradual and plateaus after  $\sim 90$  min, but at higher dosages the maximum effect is reached earlier ( $\sim 30$  min). This time course argues against increased adenosine levels in blood (17) by AICA-riboside or a direct binding of AICA-riboside to the adenosine receptor, since intra-arterial adenosine infusion triggers a prompt vasodilation (30). This finding is in accordance with our observation that the adenosine receptor antagonist caffeine, in the same dose that blunted adenosine-induced vasodilation (28, 29), failed to reduce the vasodilator response to AICA-riboside.

Theoretically, AICA-riboside may be taken up by endothelial or smooth muscle cells by the ENT. Once intracellular, it is phosphorylated to ZMP and may bind to AMPK and subsequently decrease vascular tone. Whether AICA-riboside is taken up by endothelial cells was not determined in the present study. However, in a subset of experiments we were able to measure a dose-dependent accumulation of ZMP in red blood cells consistent with cellular AICA-riboside uptake and subsequent phosphorylation (see Table 1; data not shown for the lower dosages). We observed that the uptake of AICA-riboside was inhibited by dipyridamole, a well-documented inhibitor of the ENT, confirming that AICA-riboside, just as adenosine (16), is taken up by the ENT. The uptake of AICA-riboside and the subsequent phosphorylation to ZMP in endothelial cells may also be important with respect to the slowly progressive vasodilator response after AICA-riboside. During the course of the AICA-riboside infusion, the vasodilator pattern correlated more with the rising intracellular AICA-riboside concentration (and subsequent activation of AMPK) in erythrocytes (and probably *vis à vis* also in locally exposed endothelial cells) than with the intracellular ZMP levels since the FBF was back to baseline levels 70 min after the end of the infusion, whereas the intracellular ZMP levels remained high (see Table 1). In this respect, it is interesting that animal *in vitro* data have shown that AMPK activation by AICA-riboside induces endothelial release of the endogenous vasodilator substance NO (15). With additional experiments, we demonstrate that the

Table 1. AICA-riboside and ZMP concentrations

	Baseline	Intra-Arterial Infusion of AICA-Riboside ( $8 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ), min				
		5	30	60	90	180
<b>Ipsilateral sample</b>						
AICA-riboside (plasma)	0	$2,903 \pm 616$	$1,753 \pm 631$	$1,827 \pm 437$	$2,372 \pm 725$	$85 \pm 16$
AICA-riboside (cells)	0	$1,315 \pm 260$	$991 \pm 299$	$1,114 \pm 231$	$1,320 \pm 264$	$45 \pm 10$
ZMP (cells)	0	$95 \pm 16$	$221 \pm 35$	$396 \pm 37$	$537 \pm 59$	$511 \pm 61$
<b>Contralateral sample</b>						
AICA-riboside (plasma)	0	$41 \pm 6$	$57 \pm 7$	$72 \pm 8$	$91 \pm 10$	$17 \pm 4$
AICA-riboside (cells)	0	$10 \pm 2$	$21 \pm 4$	$28 \pm 2$	$45 \pm 7$	$7 \pm 1$
ZMP (cells)	0	$22 \pm 2$	$153 \pm 12$	$300 \pm 34$	$487 \pm 10$	$528 \pm 37$

Values are means  $\pm$  SE. AICA, 5-aminoimidazole-4-carboxamide; ZMP, AICA-ribotide. Concentrations (in  $\mu\text{M}$ ) in plasma and AICA-riboside and ZMP concentrations (in  $\mu\text{M}$ ) in erythrocytes at the highest dose of AICA-riboside ( $8 \text{ mg} \cdot \text{min}^{-1} \cdot \text{dl}^{-1}$ ). Venous blood samples were taken from the experimental arm (i.e., ipsilateral sample) and the control arm (i.e., contralateral sample). AICA-riboside was infused intra-arterially from  $t = 0$  to 110 min. All values are significantly different from baseline concentrations.

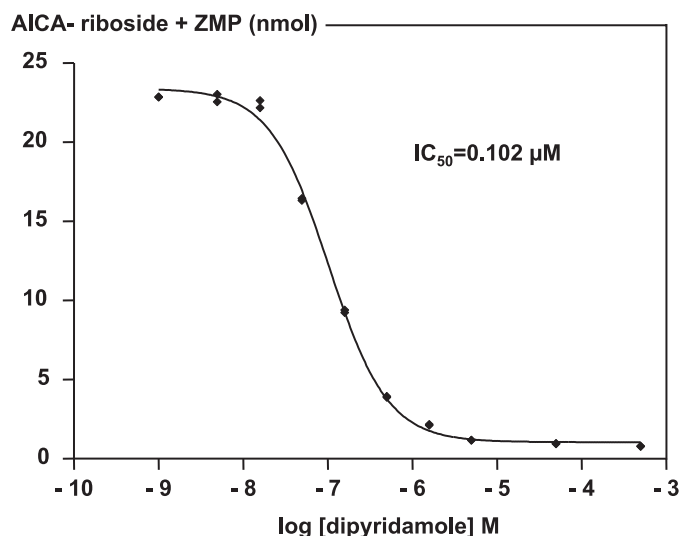


Fig. 6. Effect of the adenosine uptake inhibitor dipyridamole on the uptake of AICA-riboside into erythrocytes. Accumulation of AICA-riboside and AICA-ribotide (ZMP) in erythrocytes can be completely inhibited by increasing concentrations of dipyridamole. At a concentration of  $-4.6 \log (= 25 \mu\text{M})$  of dipyridamole, the uptake of AICA-riboside is completely inhibited.

endothelial NO synthase inhibitor L-NMMA attenuated the vasodilator response of AICA-riboside, which confirms that the vasodilation induced by AICA-riboside is mediated by release of NO.

Besides the strong increase in blood flow, we also show an obvious increase in heart rate, probably reflecting a baroreceptor-mediated compensation to (generalized and skeletal muscle) vasodilation. Another possibility is that AICA-riboside may have a direct effect on the heart (just like adenosine); however, we cannot measure this in the used study setup. In previous clinical studies (10, 24), AICA-riboside was given as an ischemia-protecting agent in patients undergoing cardiac surgery. AICA-riboside was thought to be effective only in ischemic as opposed to nonischemic tissue, which was confirmed by one animal in vivo study (20). In these studies, no vasodilator effect or relevant changes in heart rate or blood pressure were reported. Neither were any hemodynamic effects reported in a previous study that focused on effects of AICA-riboside on skeletal muscle tissue in healthy subjects (9). In short, the present study shows that AICA-riboside administration in vivo in humans induces a strong time- and dose-dependent vasodilation in nonischemic skeletal muscle tissue and also increases heart rate significantly.

Several studies (2, 32) have indicated that NO-dependent vasodilation augments nutritive blood flow and subsequently stimulates glucose uptake. The strong NO-dependent vasodilator effect of AICA-riboside may thus translate into augmented muscle perfusion and thus into an increase in skeletal muscle glucose uptake. In vitro studies (14) and animal in vivo studies (18) show an increase in glucose uptake by AICA-riboside. However, although forearm perfusion was increased in our healthy young subjects, no effects were observed on local skeletal muscle forearm glucose uptake following AICA-riboside infusion. Until now, only three recent in vivo studies have investigated the metabolic effects of AICA-riboside infusion in humans (1, 6, 9). We (6) investigated the effects of AICA-riboside in patients with type 2 diabetes during continuous

intravenous infusion of AICA-riboside ( $0.75 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) and NaCl 0.9% (placebo controlled) for 2 h. Stable isotope methodology and blood and muscle biopsy sampling were applied to assess blood glucose and fatty acid kinetics following AICA-riboside infusion. We observed that AICA-riboside infusion inhibits hepatic glucose output while maintaining whole body glucose output, which results in a decrease in systemic glucose levels. Plasma AICA-riboside concentrations increased to  $161 \pm 11 \mu\text{mol/l}$  during AICA-riboside infusion. Cuthbertson et al. (9) and Babraj et al. (1) investigated the effects of AICA-riboside in healthy subjects and in healthy older subjects with or without type 2 diabetes. They measured 2-deoxyglucose (2-DG) accumulation in skeletal muscle biopsies during systemic 2-DG infusion. Following AICA-riboside treatment ( $10 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ , for 3 h), these authors observed a more pronounced increase in 2-DG in healthy humans (9). Furthermore, euglycemic hyperinsulinemic clamping ( $n = 4$ ; no control experiments) in combination with AICA-riboside infusion revealed an increase of 7% ( $9.3 \pm 0.6$  to  $10 \pm 0.6 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ,  $P < 0.05$ ) in glucose infusion rate during AICA-riboside administration (3–6 h) compared with insulin administration (0–3 h). However, such an increase over time is in complete agreement with the slowly increasing glucose infusion observed during prolonged clamping (5, 31) and does not indicate a major effect on skeletal muscle. In the second study, the 2-DG uptake was attenuated in older men and in male patients with type 2 diabetes during AICA-riboside infusion (low or high dose of 10 or  $20 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ , respectively). No systemic decrease in glucose levels was observed in either study (1, 9). In the first study (9), AICA-riboside plasma levels reached  $180 \pm 40 \mu\text{mol/l}$ . No changes in skeletal muscle AMPK phosphorylation status and/or AMPK activity were observed following AICA-riboside administration in vivo in humans (1, 6, 9). However, Cuthbertson et al. (9) found increased ERK1/2 phosphorylation, and we observed a significant increase in acetyl-CoA carboxylase after AICA-riboside infusion. More studies have to be done to evaluate this difference. It is difficult to compare those three human in vivo studies with this one because we infused AICA-riboside intra-arterially instead of intravenously. Although we used another study setup, we obtained systemic AICA-riboside levels of maximally  $90 \mu\text{mol/l}$  at the highest dose of AICA-riboside ( $8 \text{ mg}\cdot\text{min}^{-1}\cdot\text{dl}^{-1}$ ). The locally reached concentrations were much higher. It is notable that we gave AICA-riboside in four increasing dosages and that we did not observe any effect of AICA-riboside on skeletal muscle at all four dosages.

To understand the discrepancy between the effects of AICA-riboside on blood flow vs. glucose uptake, we have explored extensively the local pharmacokinetics after intra-arterial

Table 2. Uric acid levels (in mmol/l) in the 4 different doses of AICA-riboside

AICA-Riboside, $\text{mg}\cdot\text{min}^{-1}\cdot\text{dl}^{-1}$	Baseline	$t = 120 \text{ min}$	$t = 180 \text{ min}$
1	$0.22 \pm 0.03$	$0.26 \pm 0.03^*$	$0.26 \pm 0.03^*$
2	$0.19 \pm 0.01$	$0.31 \pm 0.02^*$	$0.31 \pm 0.02^*$
4	$0.21 \pm 0.03$	$0.43 \pm 0.03^*$	$0.44 \pm 0.03^*$
8	$0.21 \pm 0.02$	$0.59 \pm 0.02^*$	$0.67 \pm 0.02^*$

Values are means  $\pm$  SE. Reference value for uric acid is 0.15–0.4 mmol/l. \* $P < 0.05$  compared with baseline values.

AICA-riboside infusion. We demonstrate that AICA-riboside is rapidly taken up by the dipyridamole-sensitive ENT in erythrocytes. As such, erythrocytes can be considered a representative model for the cellular uptake of AICA-riboside in endothelial and vascular smooth muscle cells, which also contain the same ENT (16). The ENT is also responsible for transport of adenosine across membranes (16). Recent human *in vivo* reports suggest that intra-arterially infused adenosine might not reach skeletal muscle because erythrocytes and endothelial and vascular smooth muscle layers (in which the ENT is abundantly expressed) function as a sink for adenosine (16). They might act as an effective barrier to adenosine, impeding its diffusion from the intravascular compartment to the interstitial space. As such, AICA-riboside, being transported by the same dipyridamole-sensitive ENT as adenosine (16), may not reach the skeletal muscle tissue either despite direct intra-arterial infusion. This is also consistent with the findings of a pharmacological study about AICA-riboside in humans (13). They observed that ZMP was trapped in the erythrocytes for several days and could not be metabolized nor diffuse to the extracellular space (13). This might explain the vasodilator effects and the failure of AICA-riboside administration to increase glucose uptake, because no adequate muscle tissue concentrations of AICA-riboside can be obtained despite local plasma AICA-riboside concentrations being 4–5 times higher compared with animal *in vivo* studies (4).

Despite the absence of a measurable impact of AICA-riboside administration on skeletal muscle glucose uptake *in vivo* in humans, we observed a substantial decline in systemic plasma glucose concentrations (Fig. 3). This seems consistent with previous findings showing a substantial reduction in plasma glucose concentration following AICA-riboside administration *in vivo* in animals (4, 18, 36) and in patients with coronary artery disease (10) and type 2 diabetes (6). This has been shown to be attributed to the inhibitory effects of AICA-riboside administration on hepatic glucose output (6, 36). A possible explanation for the potent effect of AICA-riboside administration on hepatic tissue as opposed to muscle tissue might be related to the fact that hepatic endothelium is unique because it has fenestrae, lacks a basal lamina, and can transfer molecules and particles by endocytosis (23). This might allow a substantial uptake of AICA-riboside in hepatic as opposed to muscle tissue, resulting in greater AMPK stimulation in hepatic tissue.

Our study has some limitations. Although we could not correlate an increase in FBF in a subsequent rise in glucose uptake, we cannot exclude that the increase in FBF in itself can stimulate glucose uptake in skeletal muscle, as described previously (2, 32). Another (possible) limitation of our study is that overactivity of the sympathetic nervous system can influence glucose uptake negatively. The observed significant increase in heart rate may also be an expression of this. Unfortunately, no catecholamines have been measured in our study. Third, for safety and ethical reasons, we did not take local muscle biopsies of the forearm, and thus we were unable to measure AICA-riboside uptake and subsequent AMPK phosphorylation in skeletal muscle. Although muscle biopsies might have been very informative about phosphorylation of AMPK and any signaling in skeletal muscle tissue, it should also be realized that the changes might be too limited or too temporary to be detectable.

In conclusion, intra-arterial AICA-riboside in humans induces a potent time- and dose-dependent vasodilation in the skeletal muscle vascular bed. This vasodilation is mediated by release of NO and not by adenosine receptor stimulation. Despite stimulating skeletal muscle blood flow, AICA-riboside administration does not substantially augment skeletal muscle glucose uptake *in vivo* in humans.

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