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The Effect of AMP-Activated Protein Kinase and Its Activator AICAR on the Metabolism of Human Umbilical Vein Endothelial Cells

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In several non-vascular tissues in which it has been studied, AMP-activated protein kinase (AMPK) appears to modulate the cellular response to stresses such as ischemia. In liver and muscle, it phosphorylates and inhibits acetyl CoA carboxylase (ACC), leading to an increase in fatty acid oxidation; and in muscle, its activation is associated with an increase in glucose transport. Here we report the presence of both AMPK and ACC in human umbilical vein endothelial cells (HUVEC). Incubation of HUVEC with 2 mM AICAR, an AMPK activator, caused a 5-fold activation of AMPK, which was accompanied by a 70% decrease in ACC activity and a 2-fold increase in fatty acid oxidation. Surprisingly, glucose uptake and glycolysis, the dominant energy-producing pathway in HUVEC, were diminished by 40–60%. Despite this, cellular ATP levels were increased by 35%. Thus activation of AMPK by AICAR is associated with major alterations in endothelial cell energy balance. Whether these alterations protect the endothelium during ischemia or other stresses remains to be determined.

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AMP-activated protein kinase (AMPK) is thought to be a key modulator of the cellular response to ischemia and other stresses (1, 4, 8–10). Among its effects, AMPK phosphorylates and inhibits acetyl CoA carboxylase (ACC), leading to a decrease in malonyl CoA and an increase in fatty acid oxidation, and in liver it also inhibits HMG CoA reductase (9, 10). In muscle, activation of AMPK has been linked to the stimulation of glucose transport during exercise (15) and in a number of cultured cells types to an inhibition of apoptosis (5, 18). Apart from this, the AMPK activator AICAR (5-aminoimidizole-4-carboxamide riboside) has been shown to exert a protective effect in patients undergoing cardiac surgery (14).

AICAR has been used in many studies to assess the effects of AMPK activation on cellular metabolism and function. Upon entering a cell, it is phosphorylated to ZMP, an AMP analog, which allosterically activates both AMPK and an upstream AMPK kinase that causes further activation of AMPK (10). When activated, AMPK phosphorylates and inhibits ACC in all tissues so far studied; however, its other effects on specific tissues may vary. Whether AMPK is present in endothelial cells, let alone its function there, has received little attention. In the present study, these questions were addressed in human umbilical vein endothelial cells (HUVEC). We report here that incubation with AICAR activates AMPK and inhibits ACC in HUVEC and that this is associated with profound alterations in their fuel metabolism and energy balance.

EXPERIMENTAL PROCEDURES

Materials. Cells and culture materials were purchased from Clonetics (San Diego, CA), radioactive chemicals from NEN Life Science Products (Boston, MA), and other reagents from Sigma (St. Louis, MO), unless otherwise indicated.

Cell culture. Human umbilical vein endothelial cells (HUVEC) were grown in EBM2 media obtained from Clonetics in a 37°C, 5% CO_2, 95% air incubator and were used between passages 3 and 5. They were plated in T75 flasks for studies of fuel metabolism and on 100 mm plates for studies of AMPK and ACC.

Incubation with AICAR. Cells were washed with warm phosphate buffered saline (PBS), pre-incubated in an Earle's balanced salt solution (5.5 mM glucose, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5.3 mM KCl, 116 mM NaCl, 1 mM NaH_2PO_4)/20 mM HEPES solution, pH 7.4, for 30 min, and then incubated with Earle's/HEPES solution ± AICAR at 37°C in 5% CO_2 95% air as described in the results. Rates of glucose and fatty acid oxidation and metabolite concentration were measured in cells incubated in media containing 50 μM carnitine (11, 12).

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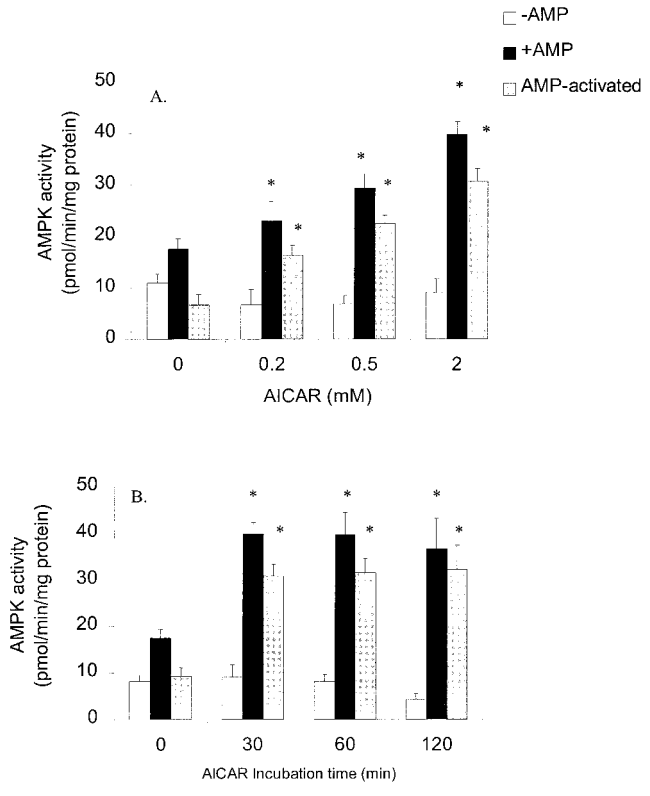

FIG. 1. Activation of AMP-activated protein kinase by AICAR in human umbilical vein endothelial cells (HUVEC). (A) Dose response curve showing phosphorylation of SAMS peptide in the presence and absence of 0.2 mM 5′-AMP and the difference between them (AMPK activity). (B) Time course of AMPK activation in cells incubated with 2 mM AICAR. Values are means ± SD ($n = 6$). *$P < 0.05$ as compared to control cells not treated with AICAR.

AMPK assay. After incubation, cells were washed with cold PBS then placed in 1 ml of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4 at 4°C, 50 mM NaCl, 50 mM NaCl, 50 mM NaF, 30 mM $NaPP_i$, 250 mM sucrose, 10 μN $ZnCl_2$, 100 mM Na-vanadate, 2 mM DTT, 50 μM PMSF, 5 μM pepstatin A, 5 μM leupeptin and 0.4 mg/ml digitonin). The lysed cells were scraped from the plate, homogenized in a Dounce homogenizer (20 strokes) and then centrifuged at 14,000×g for 30 min at 4°C. The supernatants were treated with 35% ammonium sulfate (13), and AMPK was assayed in the precipitate by measuring the phosphorylation of SAMS peptide (QCB, Hopkinton, MA) in the presence and absence of 0.2 mM 5′-AMP (22).

Acetyl CoA carboxylase. After incubation, cells were washed with PBS and then placed in lysis buffer containing 50 mM Tris, pH 7.5, 1 mM DTT, 1 mM EDTA, 50 μM PMSF, 5 μM aprotinin, 5 μM leupeptin, 5 μM pepstatin, 20 mM β-glycerophosphate, 20 mM NaF, 2 mM $NaPP_i$, 1 mM Na-vanadate and 0.1% NP-40. The lysed cells were scraped from the plate, homogenized in a Dounce homogenizer (20 strokes) and then centrifuged for 15 min at 14,000×g. A 50 μl sample was assayed for ACC by the $^{14}CO_2$ fixation method (13).

Studies of fatty acid and glucose oxidation. Fatty acid oxidation was measured on the basis of ^3H_2O formation from [9,10-^{3}H]-palmitate (18 μCi/ml) as described by Moon and Rhead (16). Glucose oxidation was determined from $^{14}CO_2$ production by cells incubated with [U-^{14}C]glucose (10 μCi/ml media). For the latter, 1 ml of media was transferred to a test tube, and capped with a rubber stopper to which a well containing 300 μl 1N NaOH was attached. Two hundred microliters of 10% perchloric acid were injected into the media through the stopper and the released CO_2 collected overnight in the well. In control studies, it was established that recovery of $^{14}CO_2$ when ^{14}C-bicarbonate was added to the empty T75 plates was approximately 82%.

Metabolite and enzyme measurements. Glucose was determined by the hexokinase method using the Sigma Diagnostics Glucose HK kit, lactate and pyruvate enzymatically as described by Passonneau and Lowry (17), and ATP using a bioluminescence kit from Sigma. Glucose uptake was calculated from the difference in its concentration in the media before and after incubation. Lactate dehydrogenase activity was measured with a spectrophotometer (17).

Statistical analysis. Data were analyzed by two tail t-test where $P < 0.05$ is considered statistically significant. The results presented are means ± standard deviation.

RESULTS

In order to determine if AMPK is present in HUVEC and its activity modulated as it is in other cell types, cells were incubated with AICAR. As shown in Fig. 1A, incubation with AICAR for 30 min increased assayable AMPK activity substantially, with about a 5-fold increase with 2 mM AICAR. At this concentration of

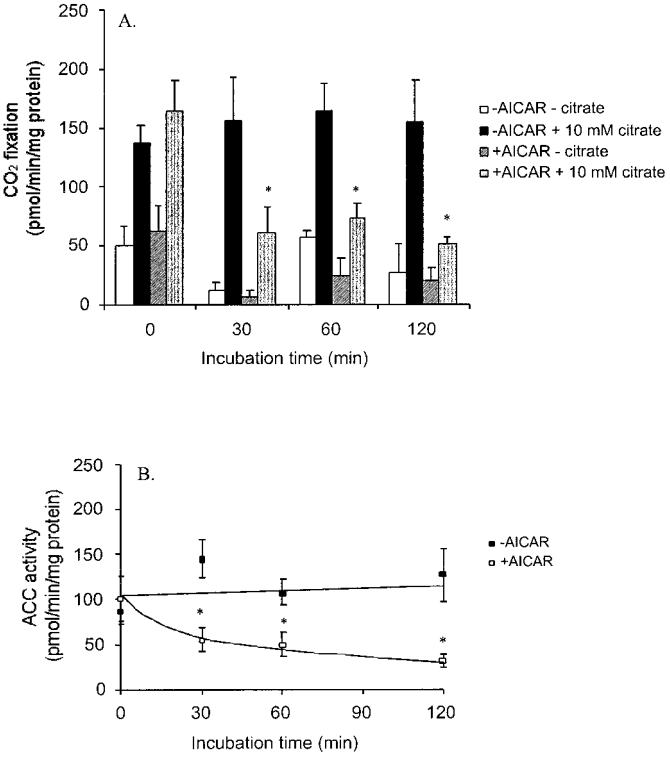

FIG. 2. Effects of AICAR on acetyl CoA carboxylase (ACC) activity in HUVEC. Cells were incubated with 0 or 2 mM AICAR for the indicated time period. ACC activity was determined in 14,000×g supernatants of cell homogenates on the basis of CO_2 fixation in the presence and absence of citrate (see Experimental Procedures). (A) Data for CO_2 fixation. (B) ACC activity calculated from the difference in CO_2 fixation ± citrate. Values are means ± SD ($n = 4$). *$P < 0.01$ as compared to control cells not treated with AICAR.

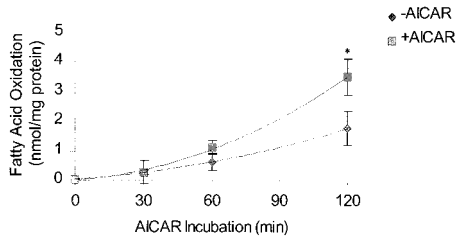

FIG. 3. Fatty acid oxidation. Cells were incubated with 0 or 2 mM AICAR for the indicated time period. Fatty acid oxidation was measured by determining the labeled water produced by cells incubated with [9,10]-^{3}H-palmitate. The incubation media contained 50 μM carnitine and had an initial fatty acid concentration of 0.11 mM. Values are means ± SD (n = 8). *P < 0.01 as compared to control cells not treated with AICAR.

AICAR, the increase in AMPK activity was maximal by 30 min and was sustained for at least 2 h (Fig. 1B).

Figure 2 shows that ACC is present in HUVEC and that its activity was diminished by 65% after incubation with 2 mM AICAR. The decrease in activity was evident within 30 min and appeared to be maximal by 2 h.

The effects of incubation with 2 mM AICAR on cellular fuel metabolism and ATP content were next examined. As expected, fatty acid oxidation was increased by AICAR nearly twofold with a highly significant effect observed after 2 h of incubation (Fig. 3). Surprisingly glucose oxidation was also increased, whereas glucose uptake and glycolysis (lactate + pyruvate production) were diminished by 55% and 37%, respectively (Table 1). Since ATP is primarily generated by glycolysis in HUVEC (3), its calculated production was diminished by 26% (Table 2). Despite this, the concentration of ATP in the cells was not diminished; on the contrary, it was significantly increased by 35% (Table 1). Release of lactate dehydrogenase was the same in all cells (data not shown), suggesting no major effects of AICAR on their integrity.

DISCUSSION

The principal findings of this study are as follows: (i) AMPK and ACC are present in HUVEC, (ii) activation of AMPK by AICAR inhibits ACC and increases fatty acid oxidation, much as it does in the liver and muscle, and (iii) in contrast to its effects on muscle, AICAR decreases glucose uptake and glycolysis in HUVEC. In addition, despite a 26% decrease in calculated ATP generation, AICAR treatment, if anything, increased their content of ATP.

The observation that incubation with AICAR increases AMPK activity suggests the presence in the endothelium of both AMPK and the upstream kinase which phosphorylates and activates it. Our findings are compatible with those of Chen and coworkers (2), who described an AMPK in rat heart that coimmunoprecipitates with the endothelial cell form of nitric oxide synthase (eNOS). In unpublished studies, they found that the "AMPK-α2 isoform is present in capillary endothelial cells in cardiac and skeletal muscle," whereas the "AMPKα1 isoform occurs in cardiac myocytes and vessels" (2). Previously, Vavvas *et al.* (18) had shown that activation of the α2 isoform occurs in skeletal muscle during contraction.

The results also show that ACC is present in HUVEC and that, as in muscle and liver, its activity decreases when AMPK is activated (Fig. 2). In other studies, we have found that it is the liver isoform (ACCα) that is present in these cells (Dagher *et al.*, unpublished data). In both liver and muscle, the decrease in ACC activity caused by AICAR is associated with a decrease in malonyl CoA, and secondary to this an increase in fatty acid oxidation. Malonyl CoA was not measured in the present study; however, the effects

TABLE 1

Effects of a 2 Hour Incubation with AICAR on Fuel Metabolism

	0 mM AICAR (nmol/h/mg protein)	2 mM AICAR (nmol/h/mg protein)
Glucose uptake (8)	1450 ± 400	650 ± 210***
Lactate production (9)	1630 ± 90	1010 ± 100**
Pyruvate production (6)	100 ± 10	70 ± 10*
Glucose oxidation (6)	1550 ± 350	2310 ± 200**
Fatty acid oxidation (8)	880 ± 290	1750 ± 310*
ATP content (8) (nmol/mg protein)	69 ± 7	93 ± 6**

Note. Results are means ± SD with number of observations in parentheses. Rates of glucose and fatty acid oxidation assume labeled substrate taken up by cells is not diluted with endogenous substrate prior to oxidation. Results significantly different from those of cells incubated in the absence of AICAR: *P < 0.02, **P < 0.01, ***P < 0.001.

TABLE 2

Effects of a 2 Hour Incubation with AICAR on Calculated ATP Production

	0 mM AICAR (nmol/h/mg protein)	2 mM AICAR (nmol/h/mg protein)
Glycolysis	1730 ± 100	1080 ± 110**
Glucose oxidation	59 ± 13	88 ± 8**
Fatty acid oxidation	122 ± 40	243 ± 43*
Total	1911 ± 153	1411 ± 161***

Note. Calculations are based on the data in Table 1. It is assumed that 129 and 38 mole of ATP are generated from the mole of fatty acid and glucose, respectively, and 1 mole ATP from each mole of lactate and pyruvate released into the media: *P < 0.02, **P < 0.01, ***P < 0.001 vs. cells incubated with no AICAR added to media.

of AICAR on both ACC activity and fatty acid oxidation suggest that its concentration was probably diminished. Direct effects of AMPK on CPT1 in isolated liver cells have been reported by Velasco *et al.* (20). Whether they occur in endothelium was not examined.

The most unexpected finding of this study was that incubation with AICAR led to decreases in glucose uptake and glycolysis and a 26% decrease in the calculated rate of ATP production. Furthermore, despite the latter, the measured concentration of ATP in AICAR treated cells was increased by about 35%. To explain these findings, two possibilities need to be considered. The first is that activation of AMPK by AICAR leads to a decrease in energy-requiring processes. Such a hypothesis was first proposed by Hardie and Carling (10) who suggested that in response to a low fuel situation (i.e., increased AMP/ATP ratio), "activation of AMPK protects the cell by switching off ATP-consuming pathways (e.g., cholesterol and fatty acid synthesis) and turning on alternative pathways for ATP generation (e.g., fatty acid oxidation)." Whether activation of AMPK diminished ATP-consuming processes in endothelium to a sufficient extent to account for the maintenance of ATP levels in the face of the apparent 26% decrease in ATP generation remains to be determined. A second possibility is that the calculated value for ATP generation grossly underestimates the contribution of fatty acid oxidation. This could result from dilution of label by fatty acid derived from the hydrolysis of cellular triglycerides and phospholipids. Such dilution would be even more of a problem, if, as suggested in skeletal muscle, exogenous fatty acids enter an intracellular triglyceride pool prior to their oxidation (7). A net dilution of the labeled fatty acid by a factor of 5 would bring the rate of ATP generation in the AICAR-treated cells (which have a higher rate of free acid oxidation) to the same level as that of untreated cells (Table 2).

Finally, the possibility that AICAR has effects not mediated by AMPK requires consideration. In this context, AICAR, also called acadesine, has been shown to increase adenosine levels in ischemia-reperfusion myocardium (6), a finding which has led to an evaluation of its therapeutic efficacy in patients following cardiac surgery and other stresses (14). In addition, in isolated hepatocytes AICAR has been shown to cause inhibition of 6-phosphofructo-2-kinase, leading to a drop in fructose-2,6-bisphosphate, a potent activator of the key glycolytic enzyme phosphofructokinase, and thus resulting in diminished glycolysis (21). Whether either of these effects is relevant to the findings reported here will require further study.

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