

# Long-Term AICAR Administration Reduces Metabolic Disturbances and Lowers Blood Pressure in Rats Displaying Features of the Insulin Resistance Syndrome

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The insulin resistance syndrome is characterized by several risk factors for cardiovascular disease. Chronic chemical activation of AMP-activated protein kinase by the adenosine analog 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR) has been shown to augment insulin action, upregulate mitochondrial enzymes in skeletal muscles, and decrease the content of intra-abdominal fat. Furthermore, acute AICAR exposure has been found to reduce sterol and fatty acid synthesis in rat hepatocytes incubated in vitro as well as suppress endogenous glucose production in rats under euglycemic clamp conditions. To investigate whether chronic AICAR administration, in addition to the beneficial effects on insulin sensitivity, is capable of improving other phenotypes associated with the insulin resistance syndrome, obese Zucker (*fafa*) rats ( $n = 6$ ) exhibiting insulin resistance, hyperlipidemia, and hypertension were subcutaneously injected with AICAR (0.5 mg/g body wt) daily for 7 weeks. Obese control rats were either pair-fed (PF) ( $n = 6$ ) or ad libitum-fed (AL) ( $n = 6$ ). Lean Zucker rats (*fa/-*) ( $n = 8$ ) served as a reference group. AICAR administration significantly reduced plasma triglyceride levels ( $P < 0.01$  for AICAR vs. AL, and  $P = 0.05$  for AICAR vs. PF) and free fatty acids ( $P < 0.01$  for AICAR vs. AL, and  $P < 0.05$  for AICAR vs. PF) and increased HDL cholesterol levels ( $P < 0.01$  for AICAR vs. AL and PF). AICAR treatment also lowered systolic blood pressure by  $14.6 \pm 4.3$  mmHg ( $P < 0.05$ ), and AICAR-treated animals exhibited a tendency toward decreased intra-abdominal fat content. Furthermore, AICAR administration normalized the oral

glucose tolerance test and decreased fasting concentrations of glucose and insulin close to the level of the lean animals. Finally, in line with previous findings, AICAR treatment was also found to enhance GLUT4 protein expression and to increase maximally insulin-stimulated glucose transport in primarily white fast-twitch muscles. Our data provide strong evidence that long-term administration of AICAR improves glucose tolerance, improves the lipid profile, and reduces systolic blood pressure in an insulin-resistant animal model. The present study gives additional support to the hypothesis that AMPK activation might be a potential future pharmacological strategy for treating the insulin resistance syndrome. *Diabetes* 51:2199–2206, 2002

The beneficial effect of regular physical exercise on critical components of the insulin resistance syndrome (IRS) is well established (1). Thus, regular physical exercise has advantageous effects on blood pressure level in humans (2) and also beneficially influences blood lipid profile and glucose homeostasis in individuals displaying features of IRS (1). These observations are confirmed in animal studies. In obese Zucker (*fafa*) rats characterized by moderate hypertension (3,4), hyperlipidemia (5,6), and insulin resistance/impaired glucose tolerance (7,8), exercise improves insulin sensitivity (9) and reduces blood lipids (10), blood pressure (4), and obesity (11,12).

An important enzyme regarding metabolism in exercising muscles is 5'AMP-activated protein kinase (AMPK), which is widely distributed in different tissues and exhibits several isoforms (13). AMPK is considered to operate as a fuel gain system as the energy stores of the cell are being exhausted (e.g., increases in the AMP/ATP ratio and/or creatine/creatine phosphate ratio) (13,14). In rat skeletal muscle, the alteration in cellular energy charge experienced during contractions leads to AMPK activation. As a consequence, acetyl-CoA carboxylase (ACC) is phosphorylated and inhibited (15,16), and malonyl-CoA decarboxylase is activated (17). These alterations lead to a suppressed cytosolic level of malonyl-CoA (15,18) (an inhibitor of carnitine palmitoyl transferase 1), which in turn leads to an augmented delivery of fatty acids for oxidative processes in the mitochondria (19,20). Importantly, AMPK is also thought to be responsible for the insulin-independent rise in glucose transport and translocation of glucose transporters (GLUT4) seen in relation to

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3-OMG, 3-O-methylglucose; ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside; AL, ad libitum-fed; AMPK, 5'AMP-activated protein kinase; EDL, extensor digitorum longus; EPI, epitrochlearis; FFA, free fatty acid; IRS, insulin resistance syndrome; OGTT, oral glucose tolerance test; PF, pair-fed; RG, red gastrocnemius; sBP, systolic blood pressure; SOL, soleus; UCP, uncoupling protein; WG, white gastrocnemius; ZMP, N<sup>1</sup>-( $\beta$ -D-5'-phosphoribofuranosyl)-5-aminoimidazole-4-carboxamide.

ATP-consuming events, such as hypoxia, and to be at least partially involved in the mechanisms of contraction-induced glucose transport (21,22). Equivalently, recent observations indicate that the AMPK system also plays a role in exercising human muscles (23,24). Hence, AMPK appears to serve as a metabolic master switch (13) that provides the working muscle with additional substrates for ATP-generating processes.

The adenosine analog 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR) is a potent activator of AMPK in intact cells (25). In skeletal muscle, acute administration by *in vivo* injections of AICAR (26) has proven to activate the AMPK and stimulate glucose uptake in a contraction-like manner. Additionally, *in vitro* AICAR exposure inhibited ACC (19) and augmented fatty acid oxidation (20). Thus, the metabolic consequences of acute AICAR exposure mimic the changes experienced in muscles facing an acute bout of physical exercise.

Acute high-intensity exercise has been demonstrated to increase AMPK activity, inhibit ACC, and decrease malonyl-CoA synthesis in rat liver tissue (27). It has also been shown that AMPK activation by AICAR in hepatocytes decreases sterol and fatty acid synthesis because both ACC and 3-hydroxy-methylglutaryl CoA reductase are phosphorylated and inhibited by AMPK (28). As in skeletal muscle, hepatic glucose metabolism is also potentially regulated by the AMPK system. Thus, under *in vitro* conditions, it has been demonstrated that AICAR exposure in rat hepatoma cells and isolated hepatocytes leads to a downregulation of gluconeogenic enzymes and suppressed gluconeogenesis, respectively (29,30). These findings are supported by observations demonstrating a decreased rate of endogenous glucose production after *in vivo* AICAR exposure (26,31). Thus, pharmacological AMPK activation in liver tissue might also mimic the possible acute effects of high-intensity exercise and might potentially decrease the hepatic glucose output as well as reduce sterol and fatty acid synthesis.

Recent studies have demonstrated that chronic AICAR exposure increases glycogen stores, increases total GLUT4, and improves activity of hexokinase and mitochondria oxidative enzymes in rat skeletal muscle (32–34). In addition, 28 days of AICAR administration has proven to lower the amount of abdominal fat in rats (34). Moreover, we recently demonstrated that 5 days of AICAR administration leads to a marked increase in the level of maximally insulin-stimulated glucose transport and GLUT4 translocation in rat skeletal muscle (35).

The presence of several similarities between pharmacological AMPK activation and physical exercise, along with the possible role of the AMPK system in liver and skeletal muscle, could imply that some of the metabolic adaptations exhibited by chronically exercised individuals might be mediated by a repetitive activation of the AMPK system.

Due to the known effects of chronic AICAR administration on muscle metabolism and abdominal fat together with the possible influence of AICAR on liver metabolism, the present study was performed to explore whether long-term AICAR administration in an exercise-like manner improves the major phenotypes associated with IRS. To address this issue, obese Zucker (*fa/fa*) rats were

treated daily with AICAR for a 7-week period. Circulating levels of glucose, insulin, and lipids as well as blood pressure were measured before and after 7 weeks of treatment. Also, an oral glucose tolerance test (OGTT) was performed after treatment. The posttreatment level of retroperitoneal and epididymal fat was determined to evaluate potential changes in fat content. Finally, red gastrocnemius (RG) and white gastrocnemius (WG) muscles were used for evaluation of GLUT4 protein expression, and epitrochlearis (EPI), extensor digitorum longus (EDL), and soleus (SOL) muscles served for estimates of glucose transport capacities under basal and insulin-stimulated *in vitro* conditions.

## RESEARCH DESIGN AND METHODS

**Experimental animals.** The study was approved by the Danish Animal Experiments Inspectorate and complied with the European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes. Male lean Zucker rats (*fa/-*) ( $n = 8$ ) and male obese Zucker rats (*fa/fa*) ( $n = 18$ ) were housed in a temperature-controlled (22–23°C) and light-controlled (12:12-h light/dark cycle) room. Animals were obtained from Mollegaards Breeding Laboratory (Ry, Denmark). Before the experiments, the rats were acclimatized to the environment and trained for tail-cuff blood pressure measurements.

Our previous observations featuring 5 days of AICAR treatment showed a slight decrease in food intake in AICAR-treated animals compared with control animals. Therefore, in addition to an ad libitum-fed control group, a pair-fed control group was introduced to distinguish possible effects of AICAR from effects caused by differences in food intake. Thus, at age 12–14 weeks (mean body weight  $\sim 540.6 \pm 7.0$  g) ( $n = 18$ ), obese animals were randomized into three groups: 1) an AICAR-treated group (AICAR), 2) a group pair-fed (PF) with the AICAR group; and 3) an ad libitum-fed (AL) group. AICAR animals had free access to food. Lean animals (mean body weight  $\sim 350.9 \pm 12.6$  g) ( $n = 8$ ) served as a reference group and were fed ad libitum.

**AICAR injection.** AICAR-treated animals were subcutaneously injected every morning (from 8:00 to 10:00 A.M.) for 7 weeks with 0.5 mg AICAR/g body wt as previously described (32). All control animals were injected with a corresponding volume of 0.9% NaCl every day.

To determine whether a single injection of 0.5 mg AICAR/g body wt increased AMPK activity, a pilot study was performed before the start of the AICAR treatment period. In this experiment, obese Zucker rats at 12 weeks of age were subcutaneously injected with AICAR (0.5 mg AICAR/g body wt) or a corresponding volume of 0.9% NaCl ( $n = 3$ –4 per group). Rats were killed 1 h after the subcutaneous injection. To avoid postmortem changes in intracellular AMP/ATP concentrations, a small portion of the liver was rapidly removed and freeze-clamped according to Davies et al. (36), and portions of RG and WG muscles and epididymal fat were also quickly removed and freeze-clamped for later determination of AMPK activity.

At the beginning of the experiments, administration of 0.5 mg AICAR/g body wt lowered blood glucose level (blood samples drawn from the tail) from  $6.8 \pm 0.1$  mmol/l ( $n = 6$ ) to  $4.1 \pm 0.2$  mmol/l ( $n = 6$ ) in nonfasted animals (lowest observed value). After an additional 1 h, blood glucose had returned to preinjection levels. At the end of the treatment period (i.e., after 7 weeks of daily AICAR injection), the same dose was found to decrease blood glucose level in nonfasted animals from  $5.0 \pm 0.3$  mmol/l ( $n = 6$ ) to  $3.4 \pm 0.4$  mmol/l ( $n = 6$ ) (lowest observed value). As before, blood glucose was observed to revert to the preinjection level after an additional 1 h.

**Systolic blood pressure measurement.** The level of systolic tail-cuff blood pressure was monitored before and 3 and 6 weeks after the beginning of AICAR treatment using a plethysmograph (LE 5000; Leticia SA, Barcelona, Spain). Before each measurement, rats were placed in a Plexiglas cage and subsequently heated for 20–30 min at 35°C. Heart rate was assessed directly from the pulse wave of arterial pressure recording to ensure that the animal was calm (heart rate  $< 400$  beats/min). For each animal, the blood pressure level was determined as the average of measurements obtained on 2 successive days. Each day of measurements included 10 single determinations. To avoid any acute interference with blood pressure level, all values were obtained from 8:00 to 10:00 A.M. before the injections with either saline or AICAR were carried out.

**Blood samples.** Before and 2, 4, and 7 weeks after the beginning of AICAR administration, blood samples were drawn from the retro-orbital venous plexus under short halothane anesthesia to determine plasma cholesterol, HDL cholesterol, triglycerides, glucose, insulin, and serum free fatty acids

TABLE 1

AMPK activity and ZMP concentration in liver, RG and WG muscles, and adipose tissue 1 h after injection of a single dose of AICAR (0.5 mg/g body wt) or saline (control)

	Liver tissue		RG muscle		WG muscle		Adipose tissue	
	Control	AICAR	Control	AICAR	Control	AICAR	Control	AICAR
AMPK (pmol · mg <sup>-1</sup> · min <sup>-1</sup> )	48.8 ± 9.9	174.1 ± 27.0*	11.1 ± 3.0	32.3 ± 5.8*	16.4 ± 5.8	90.7 ± 10.2†	15.2 ± 3.4	20.4 ± 3.4
ZMP (μmol/g wet muscle weight)	ND	1.59 ± 0.10	ND	0.70 ± 0.14	ND	0.49 ± 0.17	ND	0.08 ± 0.04

Data are means ± SE. Tissue activities of AICAR-injected animals ( $n = 4$ ) are expressed as picomoles of phosphate incorporated into SAMS peptide per milligram of protein in the postnuclear supernatant per minute.  $n = 4$  for the AICAR group, and  $n = 3$  for the control group. \* $P < 0.05$  for AICAR vs. controls; † $P < 0.01$  for AICAR vs. controls. ND, not detected.

(FFAs). Animals were fasted for 12 h before blood sampling. Because the present study was exploring possible long-term adaptations, blood sampling took place 24 h after the last AICAR injection to exclude potential interference from any remaining acute effects of the last AICAR exposure. Plasma levels of cholesterol, HDL cholesterol, and triglycerides were determined on a Cobas Integra Analyzer (Roche Diagnostics, Rotkreuz, Switzerland). Fasting plasma glucose was measured in duplicate immediately after sampling on a Beckman Glucose Analyzer II (Beckman Instruments, Palo Alto, CA). Serum FFAs were measured enzymatically using a Wako NEFA (nonesterified fatty acid) Test Kit (Wako Chemicals, Richmond, VA). Plasma insulin levels were determined using an ultrasensitive Rat Insulin ELISA (enzyme-linked immunosorbent assay) Kit from DRG Diagnostics (Marburg, Germany).

After 7 weeks of treatment, an OGTT was performed. Animals were tested in the morning after a 12-h fast and 24 h after the last AICAR injection. Blood glucose was measured from samples obtained by tail bleeding before administration of 2.5 g glucose/kg body wt loaded by gavage as well as at 15, 30, 60, 90, and 120 min after the glucose load. Blood glucose levels were determined from all samples using a Hemoglucotest 1-44 and Reflux II reflectance meter (Boehringer Mannheim, Mannheim, Germany). Total area under the curve for OGTT glucose was calculated by the trapezoid method.

Animals were killed by cervical dislocation 48 h after the OGTT, and the epididymal and retroperitoneal adipose tissue were removed and weighed for evaluation of fat content. Further, gastrocnemius muscles were isolated, and the RG (mainly oxidative muscle fibers) and WG (predominantly glycolytic muscle fibers) muscles were separated and snap-frozen in liquid nitrogen. The gastrocnemius muscles served to determine total crude membrane GLUT4 protein content. In addition, SOL (principally a slow-twitch oxidative muscle) and EDL and EPI (both fast-twitch muscles) muscles were carefully removed and used for estimation of glucose transport activity.

**Activity of AMPK and N<sup>1</sup>-(β-D-5'-phosphoribofuranosyl)-5-aminoimidazole-4-carboxamide (AICAR monophosphate, analog of AMP) concentration in tissues.** Gastrocnemius muscles, liver, and adipose tissue were homogenized and centrifuged, and AMPK activity was determined directly on the postnuclear supernatant using an SAMS peptide as previously described (37). N<sup>1</sup>-(β-D-5'-phosphoribofuranosyl)-5-aminoimidazole-4-carboxamide (ZMP) was determined using a high-performance liquid chromatography (HPLC) system on 200-mg perchloric extracts from the individual tissue according to Merrill et al. (19).

**Total crude membrane GLUT4 contents.** Crude membranes (microsomes and plasma membranes) were prepared on 30–35 mg of individual RG and WG muscle. The muscles were homogenized, and the GLUT4 expression was determined after an immunoblotting analysis as previously described (35).

**Measurements of glucose transport in muscles incubated in vitro.** Muscles were split into two strips of ~20 mg and preincubated for 30 min in the absence or presence of insulin (60 nmol/l) in 5 ml oxygenated Krebs-Henselheit bicarbonate buffer ([in mmol/l] 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 118.5 NaCl, 4.7 KCl, 2.5 CaCl<sub>2</sub>, and 1.2 MgSO<sub>4</sub>, pH 7.4) supplemented with 5 mmol/l HEPES, 20 mmol/l mannitol, and 0.1% BSA (Radioimmunoassay Grade; Sigma). All incubations were performed under continuous gassing with 95% O<sub>2</sub>/5% CO<sub>2</sub> at 30°C in a shaking water bath. Glucose transport activity was evaluated under basal and insulin-stimulated conditions with 8 mmol/l 3-O-[methyl-<sup>3</sup>H]glucose and 12 mmol/l [<sup>14</sup>C]mannose as described previously (38). The glucose uptake activities of the individual muscles were calculated as the mean of the two muscle strips.

**Statistical analysis.** All data are presented as means ± SE. Differences between obese groups were evaluated with one-way ANOVA supplemented with Bonferroni's multiple comparison test. An unpaired Student's *t* test was used for comparison between AICAR and lean animals and for the analysis of possible differences in AMPK activity 1 h after injection of AICAR or saline, respectively. A paired Student's *t* test was applied to data of the AICAR-treated animals for evaluation of differences between pre- and posttreatment values ( $P < 0.05$  for significance).

## RESULTS

### AMPK activities and ZMP concentration in tissues.

Table 1 shows AMPK activity 1 h after a single in vivo subcutaneous injection of 0.5 mg AICAR/g body wt or a corresponding volume of saline (0.9% NaCl). In liver tissue, AICAR injection caused an increase in AMPK activity by 3.6-fold on average compared with saline-injected rats ( $P < 0.05$ ). The AMPK activity in WG muscle was increased by 5.5-fold ( $P < 0.01$ ), and, in RG muscle, the AICAR injection caused a 2.9-fold increase ( $P < 0.05$ ) in AMPK activity on average when compared with saline-injected obese animals. In adipose tissue, a nonsignificant 1.3-fold increase ( $P = 0.40$ ) in AMPK activity was detected 1 h after AICAR injection. ZMP concentration 1 h after injection was 1.59 ± 0.10, 0.70 ± 0.14, 0.49 ± 0.17, and 0.08 ± 0.04 μmol/g muscle wet weight in liver tissue, RG muscle, WG muscle, and adipose tissue, respectively. In control animals, ZMP concentrations were undetectable in all tissues.

**Glucose homeostasis.** Fasting plasma glucose was significantly higher in obese Zucker rats ( $n = 18$ ) before treatment compared with the lean controls ( $P < 0.05$ ) (Table 2). Similarly, fasting plasma insulin was ~12-fold higher in obese animals than in lean animals ( $P < 0.01$ ). After 7 weeks of AICAR administration, AICAR animals approached lean animals with respect to fasting levels of circulating glucose and insulin, and the AICAR-treated animals were characterized by markedly lower levels of fasting plasma glucose and insulin compared with AL and PF animals ( $P < 0.01$ , respectively) (Table 2). In addition, the glucose tolerance examined after 7 weeks of treatment was markedly improved in the AICAR group (Fig. 1). The mean area under the curve for blood glucose in the AICAR group (805 ± 24 mmol · l<sup>-1</sup> · min<sup>-1</sup>) was comparable to that of lean animals (861 ± 2.3 mmol · l<sup>-1</sup> · min<sup>-1</sup>) and substantially lower than that of obese AL animals (999 ± 32 mmol · l<sup>-1</sup> · min<sup>-1</sup>) and PF animals (1,056 ± 38 mmol · l<sup>-1</sup> · min<sup>-1</sup>), respectively ( $P < 0.01$  for AICAR vs. AL and PF).

**Plasma lipids.** Before treatment, obese rats exhibited pronounced dyslipidemia compared with lean animals (Table 2). Seven weeks of AICAR administration led to a substantial fall in plasma triglycerides, which were significantly lower than those of obese AL controls ( $P < 0.01$ ). However, the difference between AICAR and PF rats only tended to be statistically significant ( $P = 0.05$ ). Conversely, AICAR treatment induced a twofold rise in HDL cholesterol. This level was clearly different from that of the obese controls ( $P < 0.01$  for AICAR vs. AL and PF). The elevated HDL cholesterol was associated with a concomitant rise in total cholesterol in the AICAR animals

TABLE 2  
Characterization of rats before and after 7 weeks of AICAR treatment

	AICAR	AL	PF	Lean
<i>n</i>	6	6	6	6
Body weight (g)				
Before	537.5 ± 8.1	543.5 ± 18.1	540.8 ± 10.2	350.9 ± 12.6
7 weeks	584.5 ± 10.0	599.0 ± 20.1	583.3 ± 10.0	388.1 ± 12.6
Fat content (g)				
7 weeks	34.61 ± 1.90	40.47 ± 2.14	39.96 ± 1.91	7.39 ± 0.38
Plasma glucose (mmol/l)				
Before	6.6 ± 0.4	6.1 ± 0.2	6.4 ± 0.2	5.6 ± 0.1
7 weeks	5.9 ± 0.1*	7.4 ± 0.4	7.3 ± 0.4	6.0 ± 0.3
Plasma insulin (pmol/l)				
Before	1,234 ± 153	1,333 ± 181	1,249 ± 160	110 ± 10
7 weeks	282 ± 56*	1,119 ± 94	1,209 ± 200	125 ± 12
Plasma triglycerides (mmol/l)				
Before	5.8 ± 0.9	5.7 ± 0.8	4.7 ± 0.7	0.5 ± 0.1
7 weeks	3.6 ± 0.4†	10.4 ± 2.2	7.1 ± 1.9	0.8 ± 0.1
Plasma FFAs, (mmol/l)				
Before	1.19 ± 0.08	1.13 ± 0.08	1.15 ± 0.04	0.58 ± 0.04
7 weeks	0.54 ± 0.04‡	1.05 ± 0.11	0.89 ± 0.06	0.63 ± 0.03
Plasma HDL cholesterol (mmol/l)				
Before	1.39 ± 0.04	1.37 ± 0.07	1.47 ± 0.06	0.87 ± 0.03
7 weeks	2.66 ± 0.05*	1.56 ± 0.09	1.59 ± 0.09	1.02 ± 0.01
Plasma total cholesterol (mmol/l)				
Before	2.30 ± 0.09	2.29 ± 0.12	2.44 ± 0.13	1.31 ± 0.05
7 weeks	5.28 ± 0.13*	3.40 ± 0.19	3.14 ± 0.06	1.95 ± 0.24

Data are means ± SE. \**P* < 0.01 for AICAR vs. AL and PF; †*P* < 0.01 for AICAR vs. AL and *P* = 0.05 for AICAR vs. PF; ‡*P* < 0.01 for AICAR vs. AL and *P* < 0.05 for AICAR vs. PF.

(*P* < 0.01 for AICAR vs. AL and PF). Finally, fasting levels of plasma FFAs were markedly reduced after 7 weeks of AICAR treatment and significantly lower than that of PF and AL animals, respectively (*P* < 0.05 for AICAR vs. PF; *P* < 0.01 for AICAR vs. AL).

**Systolic blood pressure.** Changes in the glucose and lipid profile of AICAR animals were paralleled by a substantial decline in systolic blood pressure (sBP) from 139 ± 4 mmHg before treatment to 124 ± 5 mmHg after 6 weeks of treatment (*P* < 0.05) (Fig. 2). In contrast, in the obese control rats, sBP was either slightly increased or unchanged, and sBP in AL animals was significantly elevated compared with AICAR animals (*P* < 0.01 for AICAR vs. AL). The difference between PF animals and AICAR animals did not reach statistical significance (*P* = 0.11 for AICAR vs. PF).

**Abdominal fat.** Obese animals were, as expected, characterized by vast obesity when compared with lean ani-

mals (Table 2). AICAR treatment was associated with a lower content of abdominal fat (epididymal and retroperitoneal adipose tissue): 34.6 ± 1.9 g (*n* = 6) vs. 40.5 ± 2.1 g (*n* = 6) in AL and 40.0 ± 1.1 g (*n* = 6) in PF, although barely statistically significant (*P* = 0.06 for AICAR vs. AL; *P* = 0.05 for AICAR vs. PF). Obviously, the fat content in lean animals was much lower (7.4 ± 0.4 g; *n* = 8).

**Liver weight.** Seven weeks of AICAR administration led to a slightly increased liver weight compared with pair-fed obese controls: 25.4 ± 0.8 g in AICAR-treated animals (*n* = 6) vs. 21.0 ± 1.4 g (*n* = 6) in PF animals (*P* < 0.05 for AICAR vs. PF). When comparing AICAR animals with ad libitum-fed obese controls (22.3 ± 1.6 g [*n* = 6] in AL), however, no statistically significant difference could be detected. Apparently, the liver weight of the lean animals was much lower (9.8 ± 0.5 g; *n* = 8) when compared with that of their obese littermates.

**Total crude membrane GLUT4 protein contents.** The

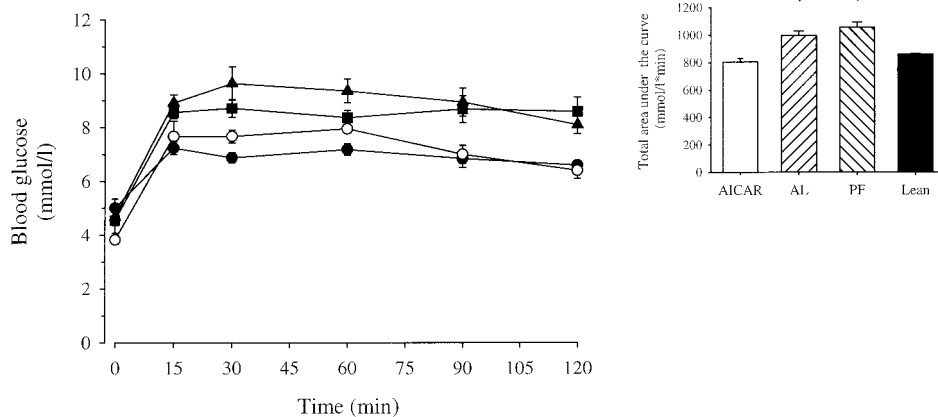


FIG. 1. OGTT performed after 7 weeks of daily AICAR injections. AICAR (●) (*n* = 6); AL (■) (*n* = 6); PF (▲) (*n* = 6); lean (○) (*n* = 4) (means ± SE). Inserted figure: Total area under the curve for OGTT glucose calculated by the trapezoid method. The areas are expressed in millimoles per liter per minute and presented as means ± SE. \**P* < 0.01 for AICAR vs. AL and PF animals, respectively.

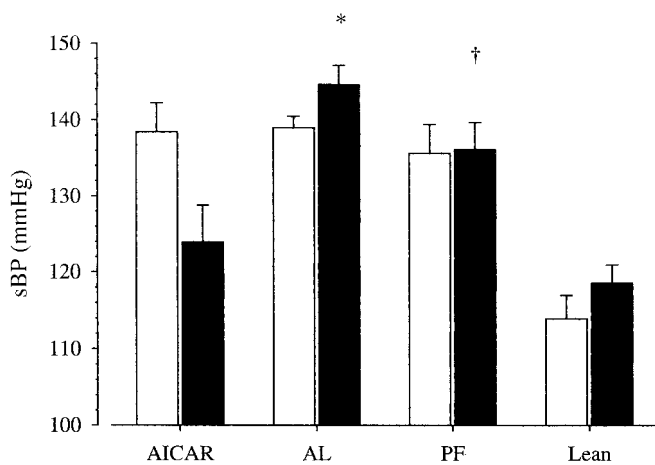


FIG. 2. sBP levels as assessed by the tail-cuff method. Values are obtained before (□) and after (■) 6 weeks of AICAR administration, respectively. AICAR,  $n = 6$ ; AL,  $n = 6$ ; PF,  $n = 6$ ; lean,  $n = 8$ . Data are means  $\pm$  SE. \* $P < 0.01$  for AICAR vs. AL, and † $P = 0.11$  for AICAR vs. PF after 6 weeks of AICAR administration.

crude membrane GLUT4 protein content was evaluated by comparing AICAR-treated rats with obese controls (AL and PF) using the WG and RG muscle (Fig. 3). On average, the total crude membrane GLUT4 protein content of WG muscles in the AICAR-treated group was 73 and 74% higher than that of the AL ( $P < 0.01$ ) and PF animals ( $P < 0.01$ ), respectively. Thus, in AICAR animals, the GLUT4 protein content in WG muscle was close to the level displayed in the WG muscles of the lean reference group ( $P = 0.48$ ).

Although there was a tendency toward a rise in total crude membrane GLUT4 protein content in RG muscles as a consequence of AICAR treatment, these data did not reach statistical significance. Thus, no difference could be detected when comparing obese groups (AICAR, AL, and PF) or comparing AICAR animals with the lean reference group.

**Muscle 3-O-methylglucose transport.** Figure 4 shows the levels of 3-O-methylglucose (3-OMG) uptake on muscles incubated in vitro in the basal and maximal insulin-stimulated (60 nmol/l) state. As expected, the obese control animals (AL and PF) displayed a marked impairment in the level of insulin-stimulated 3-OMG transport when compared with the lean reference group. The muscles of the AICAR-exposed group, however, exhibited a considerably higher capacity for insulin-stimulated 3-OMG transport than the other obese groups (AL and PF, respectively). The most pronounced increase was detected on EPI muscle (mainly composed of type 2b muscle fibers), with an average increase of 73 and 69% when comparing AICAR-treated rats with AL ( $P < 0.01$ ) and PF ( $P < 0.05$ ) animals, respectively. In EDL muscle (predominantly composed of type 2a muscle fibers), an average increase of ~50% was found when comparing the AICAR group with both of the obese control groups ( $P < 0.05$  for AICAR vs. AL, and  $P < 0.05$  for AICAR vs. PF). In the slow-twitch oxidative SOL muscle, however, the differences found when comparing the AICAR-treated rats with the obese control rats did not reach the level of statistical significance. In addition, the glucose transport capacities of the AICAR-treated group approached the level exhibited by the lean group (EPI,  $P = 0.21$ ; EDL,  $P = 0.39$ ; SOL,  $P = 0.29$ ).

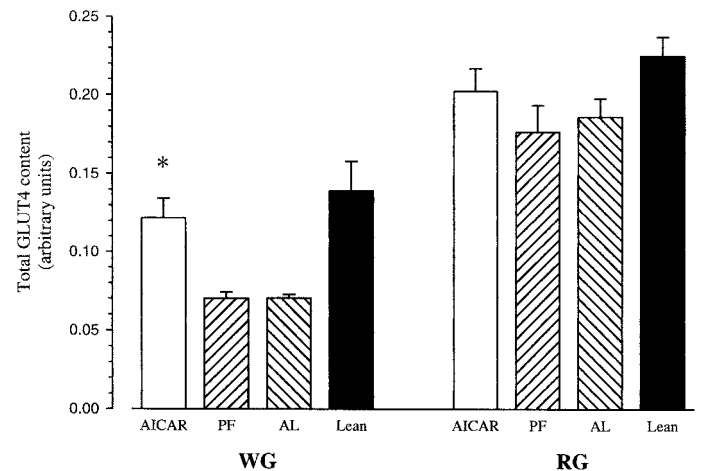


FIG. 3. Total crude membrane GLUT4 content in RG and WG muscles from obese and lean Zucker rats after 7 weeks of daily AICAR administration. Data are means  $\pm$  SE. AICAR,  $n = 6$ ; AL,  $n = 6$ ; PF,  $n = 6$ ; lean,  $n = 8$ . \* $P < 0.01$  for AICAR vs. AL and PF, respectively.

## DISCUSSION

In the current study, the obese (*fa/fa*) Zucker rat was used as an experimental model of IRS. AICAR administration induced several beneficial effects on the metabolic abnormalities of these animals. Even though the animals were continuously monitored during the whole treatment period, the present study focuses on evaluating the end point conditions displayed after 7 weeks of AICAR treatment. Nevertheless, metabolic alterations were already detectable as early as after 2 weeks (data not shown). At this point, however, the pair-fed group could not be clearly distinguished from the AICAR group because the small differences seen were not statistically significant. Later, after an additional 2 weeks of treatment (i.e., at 4 weeks) (data not shown), the same situation was emerging as that seen after 7 weeks, but the alterations were still not as pronounced as those after 7 weeks.

Long-term AICAR administration induced a considerable decrease in fasting plasma levels of insulin and glucose in this animal model for insulin resistance. These changes were accompanied by a normalization of glucose tolerance when compared with non-insulin-resistant lean animals. Further, a muscle fiber type-related increase in GLUT4 protein expression and insulin-stimulated 3-OMG transport in muscle after long-term AICAR administration was demonstrated. The latter is equivalent to our previous findings in lean Wistar rats, where we found marked increased insulin-stimulated glucose transport and GLUT4 protein expression in skeletal muscle after short-term AICAR exposure (35). The alterations in insulin action in skeletal muscle are likely to be involved in the mechanism of our findings on whole-body glucose homeostasis. Moreover, it has previously been shown that acute in vivo AICAR exposure can decrease endogenous glucose production in normal rats (26). Recently, this decrease has also been demonstrated in insulin-resistant obese Zucker rats (31). These observations are consistent with the potential influence of AICAR on the regulation of hepatic gluconeogenesis, as suggested by in vitro studies of isolated hepatocytes and hepatoma cells (29,30). Therefore, an altered endogenous glucose production may contribute to the observed changes in glucose and insulin homeostasis.

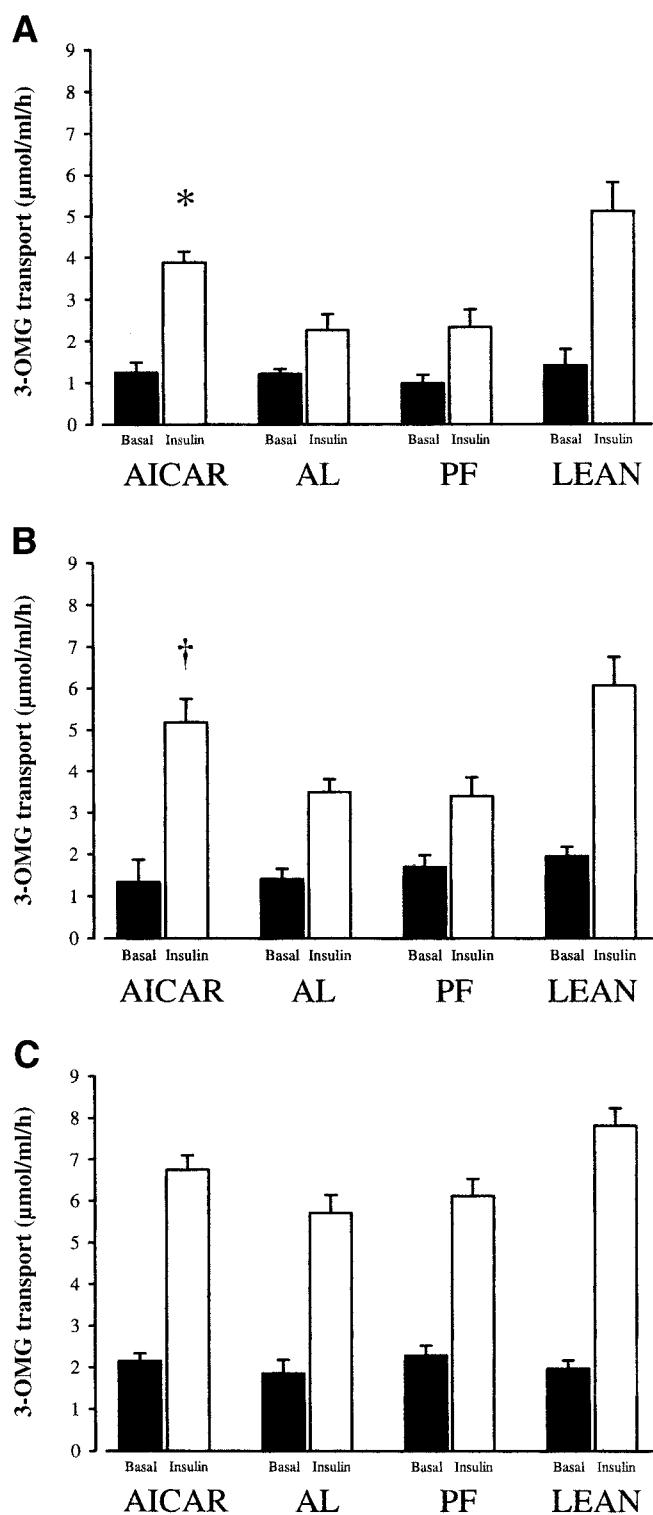


FIG. 4. 3-OMG transport in three different muscles during 10 min of incubation in a basal and maximally insulin-stimulated condition (60 nmol/l), respectively. Data are means  $\pm$  SE. A: EPI ( $n = 6$ ),  $*P < 0.01$  for AICAR vs. AL and  $P < 0.05$  for AICAR vs. PF. B: EDL ( $n = 6$ ),  $\dagger P < 0.05$  for AICAR vs. AL and PF. C: SOL ( $n = 6$ ).

AICAR treatment vastly decreased circulating levels of triglycerides and FFAs. This result might in part be explained by the improvements in glucose and insulin homeostasis, but a possible inhibition of hepatic synthesis of fatty acids exerted by AMPK (28) is also likely. Further, a

recent study has shown that acute in vivo AICAR infusion in awake obese Zucker rats suppresses plasma concentrations of triglycerides and fatty acids and decreases glycerol turnover, suggesting a potential antilipolytic effect as well as a possible reduction in hepatic lipogenesis (31). In addition, AMPK activation is known to acutely increase the rate of muscular (19,20) and hepatic (39) fatty acid oxidation. Thus, AICAR administration may have facilitated the catabolism of FFAs and triglycerides, contributed to a decreased level of whole-body lipolytic activity, and reduced the hepatic synthesis of these metabolites.

We found a considerable rise in the plasma level of HDL cholesterol (91% on average) after 7 weeks of AICAR administration, and total plasma cholesterol was also increased—this increase, however, was proportional to the increase displayed by HDL cholesterol. The exact significance of the increased level of cholesterol is not clear, and these observations are puzzling given that AMPK usually is considered to inhibit hepatic sterol synthesis (28). Additional research in this matter is required.

In our experiment, long-term AICAR administration tended to lead to a decrease in the epididymal and retroperitoneal fat contents. This result suggests an increased degradation of adipose tissue and thus supports previous long-term AICAR experiments performed on lean rats demonstrating a reduction in intra-abdominal fat content (34). AICAR did not significantly activate AMPK in the adipose tissue of our animals. Even if a minor but barely detectable activation occurs, the decrease in fat mass is most likely ascribable to events taking place outside the adipose tissue, keeping the proposed antilipolytic effect of AICAR in mind (31). Instead, an increased rate of whole-body fatty acid oxidation (e.g., in liver and skeletal muscle) induced daily by AICAR injection could be a partial explanation for our findings. Also of note is that two previous studies on rat skeletal muscles have shown that acute AICAR exposure upregulates mRNA of uncoupling protein (UCP)-2 (40) and UCP-3 (40,41). An enhanced level of UCPs might lead to increased energy use and fat degradation. Alternatively, our data on retroperitoneal and epididymal fat mass might reflect a simple redistribution of the adipose tissue with no quantitative change in total fat content. Further investigation in this matter is necessary.

The liver weight was moderately increased in AICAR-treated animals. This finding is consistent with previous findings after 28 days of AICAR exposure in nonobese rats (34). Still, the mechanism involved in this observation is not clear. In the context of possible future long-term administration of AICAR-related compounds, this is an important issue to address when considering the potential side effects of AMPK-stimulating agents.

sBP level decreased during the period of AICAR injections. After 3 weeks, a decline was already indicated (data not shown), but the decline in blood pressure level was clearly more evident after 6 weeks of AICAR administration. Accordingly, insulin levels were also decreased early during AICAR exposure (i.e., after 2–4 weeks; data not shown) and further diminished after 7 weeks. However, AICAR administration normalized several metabolic dysfunctions, and it is likely that these alterations indirectly

also contributed to the lowered sBP level. A direct effect of blood pressure regulation, however, cannot be excluded.

We administered AICAR in an in vivo experiment. AICAR given this way has previously been shown to activate AMPK in skeletal muscle (32). AICAR activation of AMPK in liver tissue, however, has so far only been demonstrated under in vitro circumstances. In the present study, we were able to demonstrate that in vivo AICAR administration, as described above, sufficiently activates the AMPK system in liver tissue as well as in RG and WG muscles. In addition, the enhanced AMPK activity detected in liver and muscle tissues was accompanied by a concomitant increase in the concentration of the nucleotide ZMP. In contrast, only a modest and statistical nonsignificant increase in AMPK activity after AICAR injection could be detected in adipose tissue, and, correspondingly, the level of ZMP in this tissue was nearly undetectable. Still, the present study demonstrates an activation of AMPK in liver as well as in skeletal muscle as a consequence of in vivo AICAR administration, suggesting that this enzyme system is potentially involved in the mechanism of our findings. Although it is most likely that the chemical compound AICAR exerts its effects through activation of the AMPK, it cannot be ruled out that other cellular pathways are influenced by AICAR administration. For instance, a direct inhibitory effect on fructose-1,6-bisphosphatase and thereby suppression of hepatic gluconeogenesis by ZMP (AICAR monophosphate) has previously been proposed (30,42).

In summary, our results are comparable to the effects of long-term exercise. The current findings lend additional support to the notion that pharmacological AMPK activation can be a possible new intervention strategy to improve the scenario of metabolic abnormalities associated with IRS. Thus, the AMPK system could be a future target for pharmacological therapies to reduce atherogenic risk factors closely associated with IRS.

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