Rapid Publication

Chronic Treatment With 5-Aminoimidazole-4-Carboxamide-1-β-D-Ribofuranoside Increases Insulin-Stimulated Glucose Uptake and GLUT4 Translocation in Rat Skeletal Muscles in a Fiber Type–Specific Manner

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Recent studies have demonstrated that chronic administration of AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside), an activator of the AMP-activated protein kinase, increases hexokinase activity and the contents of total GLUT4 and glycogen in rat skeletal muscles. To explore whether AICAR also affects insulin-stimulated glucose transport and GLUT4 cell surface content, Wistar rats were subcutaneously injected with AICAR for 5 days in succession (1 mg/g body wt). Maximally insulin-stimulated (60 nmol/l) glucose uptake was markedly increased in epitrochlearis (EPI) muscle (average 63%, $P < 0.001$, $n = 18–19$) and in extensor digitorum longus muscle (average 26%, $P < 0.001$, $n = 26–30$). In contrast, administration of AICAR did not maximally influence insulin-stimulated glucose transport in soleus muscle. Studies of EPI muscle with the 4,4′-*O*-[2-[2-[2-[2-[2-[6-(biotinylamino)hexanoyl]amino]ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2,-trifluoroethyl)benzoyl]amino-1,3-propanediyl]bis-D-mannose photolabeling technique showed a concomitant increase (average 68%, $P < 0.02$) in cell surface GLUT4 content after insulin exposure in AICAR-injected rats when compared with controls. In conclusion, 5 days of AICAR administration induces a pronounced fiber type–specific increase in insulin-stimulated glucose uptake and GLUT4 cell surface content in rat skeletal muscle with the greatest effect observed on white fast-twitch glycolytic muscles (EPI). These results are comparable with the effects of chronic exercise training, and it brings the AMP-activated protein kinase into focus as a new interesting target for future pharmacological intervention in insulin-resistant conditions. *Diabetes* 50:12–17, 2001

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Received for publication 22 August 2000 and accepted in revised form 24 October 2000. Posted on the World Wide Web at www.diabetes.org/diabetes on 27 November 2000.

3-OMG, 3-*O*-methylglucose; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride; AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; Bio-LC-ATB-BMPA, 4,4′-*O*-[2-[2-[2-[2-[2-[6-(biotinylamino) hexanoyl]amino]ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2,-trifluoroethyl)benzoyl]amino-1,3-propanediyl]bis-D-mannose; BSA, bovine serum albumin; EDL, extensor digitorum longus; EPI, epitrochlearis; FG, fast-twitch glycolytic; FOG, fast-twitch oxidative glycolytic; HES, hetastarch in saline; KHBB, Krebs-Henseleit bicarbonate buffer; LCFA, long-chain fatty acid; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RG, red part of the gastrocnemius; SO, slow oxidative; WG, white part of the gastrocnemius.

Physical exercise augments insulin sensitivity in skeletal muscle of insulin-resistant animals (1–4) and can also improve insulin sensitivity in type 2 diabetic individuals (5,6). Exercise is therefore pivotal in the treatment of type 2 diabetes. Studies in skeletal muscle from type 2 diabetic and (7) insulin-resistant prediabetic individuals (8) have shown an increased fraction of white type 2b muscle fibers. This change toward fast-twitch fibers in skeletal muscle has further been demonstrated in hyperinsulinemic rats (9) and in fructose-fed rats (an animal model of insulin-resistance) (10). Because it is well established that fast-twitch fibers (in particular type 2b fibers) are less insulin sensitive (11,12), this change in muscle morphology might potentially play a role in the decreased insulin action seen in skeletal muscle of insulin-resistant individuals.

Muscle fibers also seem to differ in their ability to increase insulin action as a response to physical exercise (1–3). The exercise intensity used during exercise programs appears to play an important role for the fiber types recruited during the work performed and thus for their capability to exhibit adaptive changes with respect to insulin action (3) and oxidative capacity (13). Several animal studies featuring treadmill-running programs have shown that predominantly fast-twitch fibers respond with increased insulin-dependent glucose transport capacity (1–3,14). Further, it appears that when the appropriate exercise programs recruiting these fibers are used, the fast-twitch glycolytic muscle fibers (type 2b) gain the greatest relative improvement in insulin action (3).

An important enzyme concerning glucose metabolism in exercising skeletal muscle is the 5′-AMP–activated protein kinase (AMPK), which is a widely distributed intracellular enzyme with several isoforms that are thought to be the main fuel gain systems activated as the cell is exposed to metabolic stress (15,16). As a response to an increased AMP/ATP ratio, the AMPK switches on ATP-generating metabolic pathways (15,17). Exercise (18) and in situ muscle contractions (19) have been shown to increase the activity of AMPK. Further, AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) is proved to be a potent activator of the AMPK in intact cells (20), and acute activation of the AMPK system by AICAR is known to induce increases in glucose transport capacity (21–23) and GLUT4 translocation (24). Therefore, AMPK-activation may be responsible for the contraction-induced glucose uptake seen in skeletal muscle (25).

Long-term activation of AMPK with AICAR increases glycogen content, hexokinase activity, and total GLUT4 protein content in skeletal muscle (26,27). Moreover, a recent study has shown increases in several mitochondrial enzymes after 28 days of AICAR treatment (28). These and similar effects are well known as adaptive phenomena found in chronically exercising muscles (2,13,14,29) and support the idea that repetitive AMPK stimulation could be involved in mediating these long-term adaptive changes. However, the possible effect of long-term AICAR administration on insulin-stimulated glucose uptake is unsettled.

Consequently, the present study was undertaken to investigate whether chronic AICAR treatment could induce similar adaptive changes, as chronic exercise is known to provide, in insulin-induced glucose uptake and insulin-stimulated GLUT4 translocation. Furthermore, we assessed whether observed changes were fiber type–specific, as seen in physical exercise.

RESEARCH DESIGN AND METHODS

Treatment of rats. All animal experiments were 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 Wistar rats (weight ~100 g) were housed in a temperature- (22–23°C) and light-controlled (12/12-h light/dark cycle) room. AICAR animals were treated for 5 days in succession with AICAR (Sigma, St. Louis, MO) 1 mg/g body weight administered through subcutaneous injections as previously described (26). Animals were injected every morning from 8 to 10 A.M. and had free access to food. Control rats were injected similarly with a corresponding volume of 0.9% NaCl. Pilot studies showed a tendency toward a slight decrease in food intake and weight gain in the AICAR-treated group compared with control rats. Therefore, control animals were pair-fed and the mean weight after the period of treatment did not differ significantly (AICAR mean weight 124.4 ± 1.9 g, control mean weight 127.7 ± 1.3 g, $P = 0.14$, $n = 52$). Animals were fasted 16–17 h before all experiments, and ~24 h after the last injection, animals were killed by cervical dislocation. Mammalian muscle can be divided into three different muscle fiber types as follows: *1*) slow oxidative ([SO] type 1), *2*) fast-twitch oxidative glycolytic ([FOG] type 2a), and *3*) fast-twitch glycolytic ([FG] type 2b). Thus, soleus (~84% SO, 16% FOG, and 0% FG), extensor digitorum longus (EDL) (~3% SO, 57% FOG, and 40% FG) and epitrochlearis (EPI) (~15% SO, 20% FOG, and 65% FG) muscles were used in the experiment (30). Gastrocnemius muscles were removed and the red (RG) (~30% SO, 62% FOG, and 8% FG) and white (WG) (~0% SO, 16% FOG, and 84% FG) parts were carefully isolated (31). EPI, EDL, and soleus muscles were used for estimation of glucose transport activity. A subgroup of EPI muscles was chosen for cell surface labeling of GLUT4. Muscles for glucose uptake measurements and photolabeling of GLUT4 were incubated in vitro immediately after removal. RG and WG muscles were snap-frozen in liquid nitrogen directly after removal and were used to determine total crude membrane GLUT4 protein contents, glycogen contents, and expressions of GLUT4 mRNA.

Muscle preincubations. All intact muscles except the gastrocnemius muscles were initially preincubated for 30 min (glucose transport measurements) or 20 min (photolabeling) in the presence or absence of insulin (60 nmol/l) in 5 ml of oxygenated Krebs-Henseleit bicarbonate buffer (KHBB) ([mmol/l] 1.2 KH_2PO_4, 25 $NaHCO_3$, 118.5 NaCl, 4.7 KCl, 2.5 $CaCl_2$, and 1.2 $MgSO_4$, pH 7.4) supplemented with 5 mmol/l HEPES, 20 mmol/l mannitol, and 0.1% bovine serum albumin (BSA) (radioimmunoassay, Grade, Sigma). All incubations were carried out at 30° C under continuous gassing with 95% O_2/5% CO_2 in a shaking water bath.

Photolabeling. After preincubation, muscles were transferred to a dark room and incubated in 1 ml KHBB containing 400 µmol/l Bio-LC-ATB-BMPA (4,4′-*O*-[2-[2-[2-[2-[2-[6-(biotinylamino)hexanoyl]amino]ethoxy]ethoxy]ethoxy]-4-(1-azi-2,2,2,-trifluoroethyl) benzoyl]amino-1,3-propanediyl]bis-D-mannose) (32). After 8 min of incubation at 18°C, muscles were irradiated for 6 min in a Rayonet photochemical reactor using 300-nm lamps (Southern New England Ultraviolet Company, Branford, CT). After irradiation, muscles were trimmed and quickly frozen in liquid nitrogen and stored at –80°C. Two frozen EPI muscles were pooled and weighed (≈30 mg for the 2 muscles). The samples were then homogenized in ice-cold hetastarch in saline (HES) buffer (20 mmol/l HEPES, 5 mmol/l NaEDTA, 255 mmol/l sucrose, 1 µg/ml antipain, aprotinin, pepstatin, leupeptin, and 100 µmol/l AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride], pH 7.2) and later centrifuged at 320,000*g* for 60 min at 4°C to obtain a total membrane fraction. This pellet was resuspended and solubilized in a phosphate-buffered saline (PBS) buffer containing 3% (wt/vol) of Thesit and proteinase inhibitors (antipain, aprotinin, pepstatin, and leupeptin each at a concentration of 1 µg/ml and 100 µmol/l AEBSF). The homogenate was rotated for 1 h at 4°C and then subjected to centrifugation at 30,000*g* for 30 min at 4°C. Biotinylated proteins in the supernatants were precipitated with 100 µl of 50% slurry of immunopore immobilized streptavidin on 6% agarose (Pierce Chemical, Rockford, IL). After the samples had been incubated at 4°C for ~12 h, the crude membrane precipitates were washed as follows: 4 times in 1% Thesit PBS buffer, 4 times in 0.1% Thesit PBS buffer, and finally in PBS buffer. Electrophoresis sample buffer (62.5 mmol/l Tris, pH 6.8, 2% SDS, and 10% glycerol) was added to the pellet, and the sample was heated to 95°C for 30 min. Mercaptoethanol was added to 10% and the supernatant fraction was subjected to SDS-PAGE (10% gel). Protein was transferred to nitrocellulose membranes, blocked with 5% nonfat milk, and incubated with a polyclonal anti–COOH-terminal peptide GLUT4 antibody (33) diluted 1/4000 (vol/vol) in Tris-buffered saline and 0.1% (vol/vol) Tween 20. Labeled proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL), and autoradiograms were quantified by scanning densitometry.

Measurements of glucose transport in in vitro incubated muscles. After preincubation, glucose transport was assessed under basal or insulin-stimulated conditions with 8 mmol/l 3-*O*-[methyl-^{3}H]glucose and 12 mmol/l [^{14}C]mannitol as described previously (34).

Total crude membrane GLUT4 contents. Crude membranes (plasma membranes and microsomes) were prepared from 30–35 mg of individual RG and WG muscles. The muscles were homogenized in ice-cold HES-buffer containing proteinase inhibitors and centrifuged at 320,000*g* for 60 min at 4°C to obtain a total membrane fraction. The pellet was resuspended and solubilized in a PBS buffer containing 3% (w/v) of Thesit and proteinase inhibitors. The homogenate was rotated for 1 h at 4°C and then centrifuged at 30,000*g* for 30 min at 4°C. Aliquots of the solubilized crude membranes (20 µg) were subjected to SDS-PAGE, followed by immunoblotting analysis. GLUT4 expression was determined and quantified as described above.

GLUT4-mRNA expressions

Isolation of RNA. Total RNA was isolated from 20–25 mg of individual RG and WG muscles using the TriZol reagent (Gibco BRL, Life Technologies).

Real-time reverse transcriptase–polymerase chain reaction. cDNA was made using random hexamer primers as described by the manufacturer (GeneAmp RNA PCR Kit; PerkinElmer Cetus, Norwalk, CT). Afterwards, polymerase chain reaction (PCR) mastermix containing the specific primers and AmpliTaq GOLD DNA polymerase was added. GLUT4 primers (GCACAGC CAGGACATTGTTG and CCCCCTCAGCAGCGAGTGA) produced an amplicon of 318 bp. Real-time quantitation of GLUT4 mRNA relative to β-actin mRNA was performed with a SYBR-Green real-time PCR assay using an ICycler PCR machine (Bio-Rad, Richmond, CA). Briefly, GLUT4 and β-actin mRNA were amplified in separate tubes, and the increase in fluorescence was measured in real time. The threshold cycle, which is defined as the fractional cycle number at which the fluorescence reaches 10 × the standard deviation of the baseline was calculated, and the relative gene expression was calculated essentially as described in PerkinElmer's User Bulletin No. 2 covering the aspect of relative quantitation of gene expression. All samples were amplified in duplicate. A similar set-up was used for negative controls except that the reverse transcriptase was omitted and no PCR products were detected under these conditions.

Total glycogen contents. Pieces of RG and WG muscles (30–40mg) were homogenized in 1.0 mol/l KOH at 70°C for 20 min. After a short centrifugation at 4°C, the glycogen was enzymatically digested into glucose by amyloglycosidase

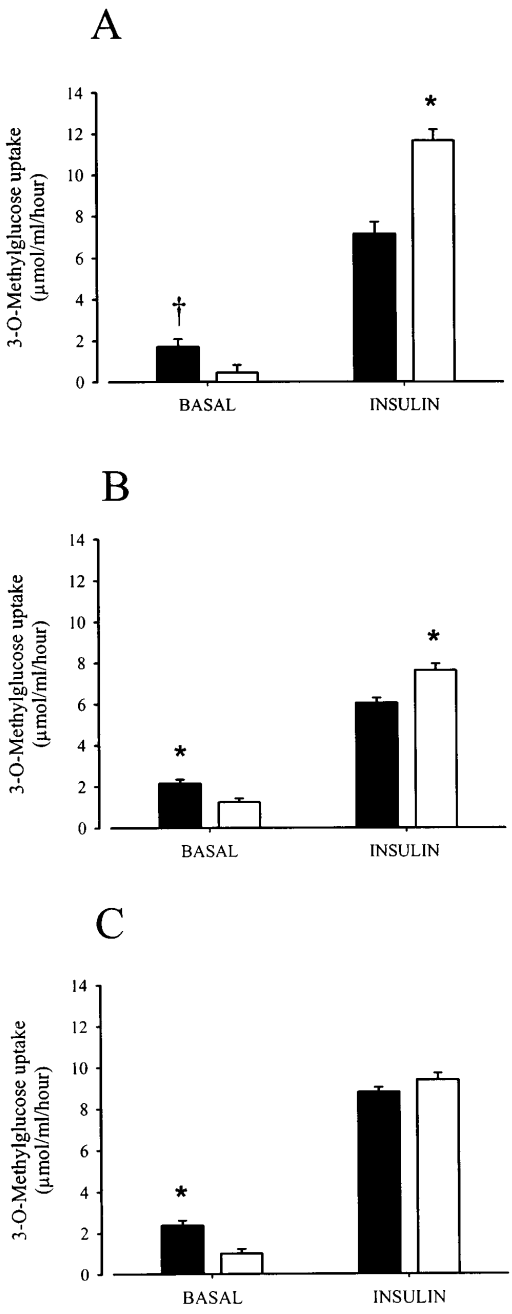

FIG. 1. 3-OMG transport in three different muscles during 10 min of incubation in a basal and a maximally insulin-stimulated (60 nmol/l) condition *A*: EPI (insulin n = 18, basal n = 17); *B*: EDL (insulin n = 26, basal n = 28); *C*: Soleus (insulin n = 28, basal: n = 28). Data are means ± SE. *P < 0.001, †P < 0.05 comparing AICAR data (□) with control data (■).

(Boehringer Mannheim, Germany) at 37°C for 3 h. Free glucose concentration was estimated using a glucose assay reagent kit (GAGO 20; Sigma Chemical).

Statistical analysis. Differences between the AICAR group and control animals were analyzed statistically using the unpaired Student's t test. All data are reported as means ± SE.

RESULTS

Muscle 3-*O*-methylglucose uptakes. Figure 1 shows the effect of maximal insulin (60 nmol/l) stimulation on 3-*O*-methylglucose (3-OMG) uptake on in vitro incubated muscles from AICAR-treated and control rats. Maximal insulin stimulation of 3-OMG uptake was found to be markedly increased in AICAR-treated rats when compared with untreated rats. The most pronounced effect was found in the white FG muscle, EPI, in which the maximally insulin-stimulated glucose uptake was increased (average 63%, P < 0.001, n = 18–19) (Fig. 1*A*) when compared with untreated rats. Further, a significant (on average 26%, P < 0.001, n = 26–30) increase in maximally insulin-stimulated glucose uptake was found in the white EDL muscle (Fig. 1*B*). In contrast, insulin-stimulated 3-OMG–transport did not differ between the groups in the red slow-twitch oxidative soleus muscle (P = 0.15, n = 28–30) (Fig. 1*C*). 3-OMG uptake in the basal state in AICAR treated group was significantly reduced in all three muscle groups (average by 74% [P < 0.03, n = 17–20] in EPI, 43% [P < 0.001, n = 28] in EDL, and 58% [P < 0.001, n = 28] in soleus when compared with controls).

Cell surface GLUT4 levels. To assess the effect of 5 days of AICAR administration on GLUT4 cell surface content in EPI muscles from treated and untreated rats, muscles were labeled with the Bio-LC-ATB-BMPA photolabeling technique in the presence and absence of insulin (60 nmol/l) (Fig. 2). Cell surface GLUT4 content was considerably increased (average 68%, P < 0.02, n = 5) after insulin stimulation in AICAR-treated rats when compared with controls. However, the GLUT4 cell–surface content in the basal state was not different in AICAR-treated and control rats.

Total crude membrane GLUT4 protein contents and GLUT4-mRNA expressions. Total crude membrane GLUT4 protein content was compared between AICAR-treated and

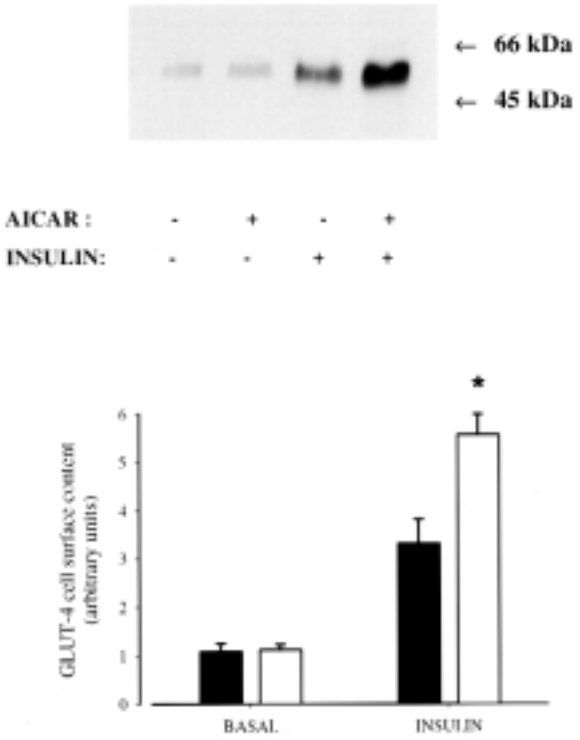

FIG. 2. Effect of 5 days of AICAR administration on insulin-stimulated (60 nmol/l) cell surface GLUT4 content in EPI muscle measured by the Bio-LC-ATB-BMPA photolabeling technique (upper panel). Representative SDS-PAGE of Bio-LC-ATB-BMPA labeled cell surface GLUT4 immunoblotted with a polyclonal anti-GLUT4 antibody (lower panel). Data are means ± SE (n = 5) and are expressed in arbitrary units. *P < 0.02 comparing AICAR data (□) with control data (■).

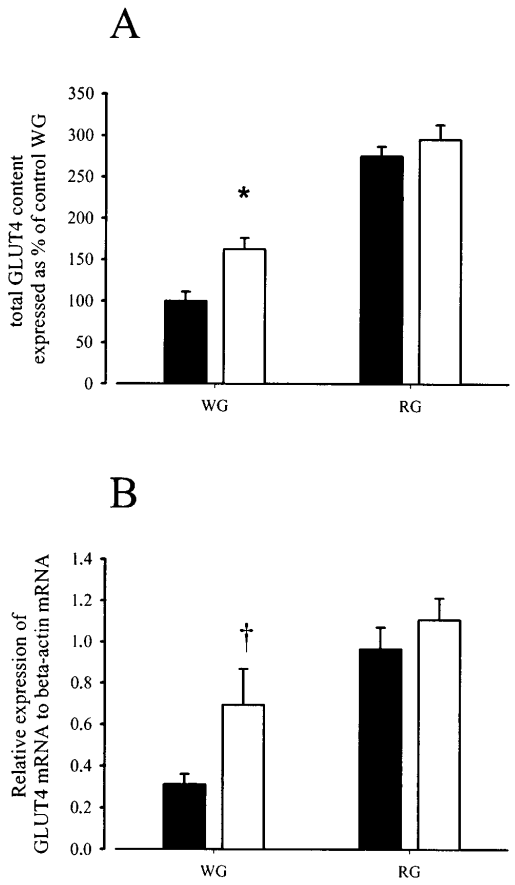

FIG. 3. Total crude membrane GLUT4 content and GLUT4-mRNA expression in RG and WG muscle after 5 days of AICAR administration. Data are means ± SE (n = 8). *A*: Crude membrane GLUT4 data are expressed in arbitrary units as percent of control level. *P < 0.005 comparing AICAR data (□) to control data (■). *B*: GLUT4-mRNA level are expressed as the ratio between GLUT4-mRNA and β-actin mRNA expression in the muscle. †P < 0.05 comparing AICAR data (□) to control data (■).

control rats using the WG and RG muscle (Fig. 3*A*). Five days of AICAR administration resulted in a 63% increase (P < 0.005, n = 8) in GLUT4 content in WG muscles when compared with controls. In contrast, although RG muscles had a higher initial content of GLUT4, AICAR administration did not lead to a further rise in GLUT4 content (P = 0.36, n = 8). Consistent with the changes observed on crude membrane GLUT4 protein content, 5 days of AICAR exposure increased the GLUT4-mRNA expression with 123% on average (P < 0.05, n = 8) in WG and had no significant effect on the GLUT4-mRNA expression in RG (P = 0.37, n = 8). Similar to the protein levels, the mRNA expression of GLUT4 was higher in RG muscle compared with the WG (Fig. 3*B*).

Glycogen contents. AICAR-treated rats also had a fiber type–specific increase in glycogen content when compared with controls (Table 1). WG muscles exhibited an average increase of 105% (P < 0.001, n = 10), whereas RG muscles displayed only a 24% increase (P < 0.02, n = 10) in glycogen content.

DISCUSSION

The present study is the first to demonstrate that chronic AICAR administration increases maximally insulin-stimulated glucose transport capacity and GLUT4 cell surface content in skeletal muscle in a fiber type–specific manner. The effect was only present in white muscles and in particular the EPI, which is composed primarily of FG muscle fibers. AICAR did not modify maximally insulin-stimulated glucose transport in slow-twitch oxidative muscles (soleus). Interestingly, several exercise studies have demonstrated that the exercise-induced increase in skeletal muscle insulin sensitivity in a similar fiber type–specific process, with white muscles having a higher response to exercise compared with red muscles (2,3).

The low basal glucose uptake observed in all AICAR-treated muscles could be associated with the large increase in muscle glycogen content seen in the muscles after chronic AICAR treatment. Several previous studies have found an inverse relationship between muscle glycogen content and glucose transport capacity (35–37). Despite the fact that high glycogen content will result in a decreased glucose uptake, the AICAR-treated rats exhibited a prominent increase in insulin action after maximal insulin stimulation.

The decreased basal glucose uptake seen in AICAR muscles emphasizes that the observed maximal insulin stimulation is not a consequence of an additive effect between any residual acute AICAR stimulation and insulin. Instead, the observed changes appear to be due to a long-term effect of AICAR improving the ability of these muscles to respond to insulin.

However, the lower basal glucose uptake was not reflected in an equivalent decrease in cell surface GLUT4 content measured by the Bio-LC-ATB-BMPA technique. Methodological limitations in measuring cell surface GLUT4 content (which is already very low in the basal state) may have caused a failure to detect a potential further reduction in cell surface GLUT4 labeling in AICAR-treated rats under basal condition. Alternatively, the photolabeling technique, which involves irradiation of the muscles with high-energy ultra violet light, may have stimulated the basal muscle cell and thus created a discrepancy between the surface labeling and transport activity in the basal state. It is also possible that activation of AMP kinase selectively lowers basal (but not insulin-stimulated) transport catalytic activity without altering the number of exposed exofacial hexose binding sites.

In line with recent reports (26–28), we found that chronic AICAR administration resulted in an increase of total crude membrane GLUT4 and glycogen content in skeletal muscles. As previously shown, this effect is also fiber-type specific (26,28), with the largest effect in white muscles. The observed AICAR-induced changes in crude membrane GLUT4 content of the WG muscles were reflected in a significant rise in GLUT4-mRNA expression.

Long-term exercise is known to induce an increase in total GLUT4 content (1,2,14) and has also been found to enhance

TABLE 1
Effect of 5 days of AICAR injections on glycogen content in RG and WG muscle

WG/control	WG/AICAR	RG/control	RG/AICAR
5.8 ± 0.8	11.9 ± 0.6*	8.1 ± 0.5†	10.0 ± 0.6‡

Data are means ± SE and are expressed in mmol glucose/g muscle (wet wt). *P < 0.001 vs. control WG muscle; †P < 0.03 vs. control WG muscle; ‡P < 0.02 vs. control RG muscle (n = 10).

the activity of key proteins involved in the insulin-signaling pathway (38–40). Further investigation is needed to show whether the increase in insulin-dependent glucose transport after AICAR treatment also involves changes in the insulin-signaling pathway.

Alternatively, it is possible that the improved insulin action after repetitive AICAR exposure could be due to an improved fatty acid oxidation. The long-chain fatty acid (LCFA)-CoA/malonyl-CoA hypothesis postulates that increased LCFA-CoA content in skeletal muscles may lead to insulin resistance (17). AMPK in muscle cells is known to phosphorylate and inhibit acetyl CoA-carboxylase (18,19), and this is a potential mechanism underlying the decrease in intramuscular malonyl-CoA seen in exercising rat muscles (18,41) and in rat skeletal muscles exposed to AICAR (21). A decrease in intramuscularly malonyl-CoA, which is an inhibitor of the carnitine palmitoyl transferase, will in turn lead to an increased rate of fatty acid oxidation (21,42) and will be accompanied by a decrease in cellular LCFA-CoA content (42). Such repetitive decreases in intracellular LCFA-CoA associated with long-term exercise or chronic AICAR administration could be one potential mechanism enhancing the sensitivity of skeletal muscles to insulin.

In summary, chronic AICAR administration increases maximally insulin-stimulated glucose transport in rat skeletal muscles in a fiber type–specific manner as seen in long-term exercise. This effect may be mediated through a repetitive activation of AMPK, and the current results suggest that AMPK might be considered a relevant target for future pharmacological intervention to improve insulin action in skeletal muscles, with insulin insensitive white muscles as the main target.

ACKNOWLEDGMENTS

This study work was supported by grants from the Institute of Experimental Clinical Research, University of Aarhus; the Danish Medical Research Council; the Danish Diabetes Association; the Novo Nordisk Foundation; the Medical Research Council (U.K.) and Diabetes (U.K.); Carl J. Beckers Fond; and the Torben Frimodt and Alice Frimodt Foundation.

We thank H. Petersen and E. Hornemann for their excellent technical assistance. Prof. H. Ørskov and A. Flyvbjerg are thanked for stimulating discussions.

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