Effect of AICAR Treatment on Glycogen Metabolism in Skeletal Muscle

William G. Aschenbach, Michael F. Hirshman, Nobuharu Fujii, Kei Sakamoto, Kirsten F. Howlett, and Laurie J. Goodyear

AMP-activated protein kinase (AMPK) is proposed to stimulate fat and carbohydrate catabolism to maintain cellular energy status. Recent studies demonstrate that pharmacologic activation of AMPK and mutations in the enzyme are associated with elevated muscle glycogen content in vivo. Our purpose was to determine the mechanism for increased muscle glycogen associated with AMPK activity in vivo. AMPK activity and glycogen metabolism were studied in red and white gastrocnemius muscles from rats treated with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) in vivo, and also in muscles incubated with AICAR in vitro. In vivo AICAR treatment reduced blood glucose and increased blood lactate compared with basal values. AICAR increased muscle α2 AMPK activity, glycogen, and glucose-6-phosphate concentrations. Glycogen synthase activity was increased in the red gastrocnemius but was decreased in the white gastrocnemius. Glycogen phosphorylase activity increased in both muscles, with an inhibition initially observed in the red gastrocnemius. In vitro incubation with AICAR activated α2 AMPK but had no effect on either glycogen synthase or glycogen phosphorylase. These results suggest that AICAR treatment does not promote glycogen accumulation in skeletal muscle in vivo by altering glycogen synthase and glycogen phosphorylase. Rather, the increased glyceren is due to the well-known effects of AICAR to increase glucose uptake. Diabetes 51:567–573, 2002

The 5’ AMP-activated protein kinase (AMPK) is a member of a metabolite-sensing protein kinase family that is ubiquitously expressed in all mammalian cells (1). AMPK is a heterotrimer that consists of α, β, and γ subunits (2). The α subunit, of which there are two known isoforms (α1 and α2), contains the catalytic domain and a threonine residue (Thr172) that functions as a phosphorylation site for one or more upstream kinases (AMPK kinase) to increase catalytic activity (2). Multiple isoforms of the β (β1 and β2) and γ (γ1, γ2, and γ3) regulatory subunits have been identified and also seem to be critical for enzymatic activity (2) and substrate targeting (3). AMPK activity is elaborately regulated by changes in cellular AMP:ATP and creatine-phosphocreatine ratios (via allosteric mechanisms), Thr172 phosphorylation by AMPK kinase, and reduced susceptibility to dephosphorylation by phosphatases (2).

AMPK has been termed a “master metabolic switch” because it may phosphorylate several key proteins to increase the rates of fat oxidation and glucose metabolism to buffer ATP concentrations in response to low cellular energy charge (2). AMPK is activated in skeletal muscle of exercising humans (4–7) and rat skeletal muscle stimulated to contract in situ (8,9) and in vitro (10,11). Strong evidence suggests that AMPK plays a critical role in increasing glucose uptake by skeletal muscle during contractions (10–14). It is possible that this increased glucose uptake not only could be used for ATP regeneration but also may provide a substrate for glycogen synthesis in skeletal muscle.

Several efforts (10,11,14–18) to elucidate the physiologic roles of AMPK in skeletal muscle have used the compound 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), a pharmacologic activator of AMPK. AICAR treatment results in several adaptations in skeletal muscle that are similar to changes that occur during exercise and exercise training. Acute incubation of isolated rat skeletal muscles with AICAR increases the rate of glucose transport (10–12,14), whereas longer incubations up to 18 h increase the protein expression of GLUT-4 and hexokinase II (16). Long-term daily treatments (5–28 days) of rats with AICAR in vivo increases mitochondrial enzyme activity (18), expression of GLUT-4 (15,17,18) and hexokinase II (17,18), and glycogen content (15,17,18) in skeletal muscle.

Increased skeletal muscle glycogen content after chronic AICAR treatment represents a physiological paradox because AMPK is proposed to accelerate fuel catabolism to buffer cellular ATP levels. Data in the existing body of literature regarding the actual role of AMPK in glycogen metabolism are controversial. Young et al. (19) observed that incubation of soleus muscle strips (composed mainly of red, slow-twitch fibers) with AICAR increased glycogen phosphorylase activity and lactate release. This observation is indicative of increased glycolytic flux and should be associated with reduced muscle glycogen. Muscle glycogen was not measured in this (19) study, so the effects of acute AICAR treatment on glycogen levels are unknown. Winder et al. (18) and Buhl et al. (15) treated rats for 5–28 days with AICAR and reported that
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mechanism(s) responsible for the paradoxical increase in muscle glycogen after AMPK stimulation with AICAR. First, we determined a 2-h time course of AICAR effects on glycogen accumulation, and glycogen synthesis and glycogen phosphorylase activities in red and white skeletal muscle in vivo. Next, we studied the effects of AICAR treatment in various isolated skeletal muscles in vitro on glycogen synthase and glycogen phosphorylase activities. Our findings support the concept that increased glycogen accumulation in skeletal muscle after AICAR treatment is independent of the activation state of glycogen synthase and glycogen phosphorylase and instead results from the well-known effects of AICAR to stimulate glucose uptake.

RESEARCH DESIGN AND METHODS
In vivo AICAR treatment. Fed male Sprague-Dawley rats were used for all in vivo experiments. Basal blood samples were collected from a tail vein for spectrophotometric determination of glucose (27) and lactate (28) concentrations. Animals were then given an intraperitoneal injection of either AICAR (0.85 mg/g body wt) or a comparable volume of saline and were allowed to remain in their cages. A second blood sample was collected at 30, 60, 90, and 120 min after injections, and rats were then killed via cervical dislocation. Hindlimbs were removed, and red and white gastrocnemius muscles were immediately separated, snap-frozen in liquid N₂, and stored in liquid N₂ until processed for analyses. Protocols for animal use and euthanasia were reviewed and approved by the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines.

In vitro muscle incubation. Fed male rats were killed, and epitroneal muscles (EPI) and flexor digitorum brevis muscles (FDB) were rapidly removed and the ends tied with silk sutures and mounted on an incubation apparatus (Harvard Apparatus, Holliston, MA). These muscles were chosen because of their range in reported fiber-type distributions (type I-type IIα-type IIb%): EPI, 15–20–65% (29); FDB, 7–92–1% (30). Muscles were placed in an in vitro incubation system as previously described (10–12) with modifications. When used, AICAR was added to the buffers at a concentration of 2 mmol/l and was present throughout the entire incubation period. To eliminate possible effects of AICAR-stimulated glucose uptake on glycogen metabolism, we used 2 mmol/l pyruvic acid as an exogenous carbon source instead of glucose. Muscles were also incubated in buffer in the presence of 5.5 mmol/l glucose in control experiments. Immediately following incubation, muscles were re-
frozen, blotted dry, snap-frozen in liquid N₂, and stored in liquid N₂ until determination of AMPK, glycogen synthase, and glycogen phosphorylase activities.

Isoform-specific AMPK activities. Muscles were processed for AMPK determination as previously described (5,7,11,12). AMPK activity was calculated as picomoles of ATP incorporated per milligram of muscle lystate protein per minute.

Western blotting of phosphorylated AMPK and ACC. Eighty micrograms of denatured AMPK lystate protein was separated on either 10% polyacryl-amide gels for p-AMPK or 7% gels for p-ACC. Proteins were then transferred to nitrocellulose membranes at 100 V for 1 h for p-AMPK, or for 2 h for p-ACC. Membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS) with 2.5% nonfat milk and then were incubated with rabbit polyclonal antibodies that recognize either AMPK only when phosphorylated on Thr172 (Cell Signaling Technology, Beverly, MA) or ACC when phosphorylated on Ser27 (Upstate Biotechnology, Lake Placid, NY).

Glycogen synthase and glycogen phosphorylase activities, glycogen content, and glucose-6-phosphate. Frozen muscle samples were pulverized under liquid N₂ and subjected to Polytron homogenization in 19 volumes of buffer consisting of 50 mmol/l Tris-HCl (pH 7.8), 100 mmol/l NaF, and 5 mmol/l EDTA. Glycogen synthase activity was determined in the absence or presence of 6.7 mmol/l glucose-6-phosphate (G-6-P) (31), and glycogen phosphorylase activity was determined in the absence or presence of 3 mmol/l 5’ AMP (32). Muscle glycogen concentrations were measured in homogenates after 2 h of hydrolysis in 2N HCl at 100°C. The resulting free glucose units were determined using a commercial hexokinase-based assay kit (Sigma, St. Louis, MO). G-6-P concentrations were determined in perchloric acid extracts of homogenates according to Lowry and Passonneau (33).

Statistical analysis. All data are reported as means ± SE. Mean differences during in vivo experiments were analyzed by ANOVA followed by a Newman-Keuls post hoc analysis. Means in in vitro experiments were compared using a Student’s t test. An α level of 0.05 was selected for all analyses.
RESULTS

AICAR effects on blood metabolites. Figure 1 shows the effects of an intraperitoneal injection of AICAR (0.85 mg/g), and blood was collected for 120 min afterward for determination of glucose (A) and lactate (B) concentrations. Blood glucose was reduced from basal levels of ~111 mg/dl by 25–50 mg/dl throughout the 120-min postinjection period. Blood lactate was also markedly increased after AICAR treatment. *P < 0.05 vs. basal; n = 4–5 per time point.

AICAR increases α2 AMPK activity and AMPK and ACC phosphorylation and alters glycogen metabolism in a fiber-type–specific manner in vivo. Rats were killed at various time points after AICAR treatment, and red and white gastrocnemius muscles were processed for isoform-specific AMPK activity measurements. As shown in Fig. 2A, AICAR treatment had no effect on α1 AMPK activities in either the red or white gastrocnemius. However, α2 AMPK activity was significantly increased in both muscle types (Fig. 2B). In the white gastrocnemius, α2 AMPK reached maximal 2.8-fold activation by 60 min, then was reduced toward basal levels by 120 min. α2 AMPK activity was still elevated above basal values 120 min after AICAR treatment, although the difference was not statistically significant. In the red gastrocnemius, an ~1.8-fold increase in α2 AMPK activity was observed 120 min after AICAR treatment. In agreement with this increased α2 AMPK activity in an in vitro kinase assay, Western blotting of AMPK using a phospho-specific antibody demonstrated that acute treatment of rats with AICAR also increased Thr172 phosphorylation of AMPK at all time points as shown in Fig. 2C (top). Also shown in Fig. 2C is that in vivo AICAR treatment increased phosphorylation of acetyl-CoA carboxylase at Ser79 (bottom). ACC is a downstream substrate of AMPK, and its phosphorylation provides a useful indicator of increased AMPK activity in vivo (9,20,34).

Glycogen synthase and glycogen phosphorylase activities are shown in Fig. 3A and B, respectively. In the red gastrocnemius, glycogen synthase activity was increased after in vivo AICAR treatment. Conversely, glycogen synthase activity was reduced in the white gastrocnemius. Glycogen phosphorylase activity exhibited a temporal response in the red gastrocnemius. The ± AMP activity ratio was significantly reduced below basal levels but was then increased by 90 min after injection. A delayed activation of glycogen phosphorylase was seen in the white gastrocnemius. Despite these different responses in glycogen synthase and glycogen phosphorylase activities, muscle glycogen content was increased in both muscles by 120 min after AICAR treatment and remained elevated throughout the 120-min period (Fig. 4A). G-6-P concentrations were also markedly elevated in both red and white gastrocnemius samples 60 min after AICAR treatment and remained elevated throughout the 120-min period (Fig. 4B).

AICAR stimulates α2 AMPK in vitro but does not alter glycogen metabolism. To determine whether the effects of AICAR on glycogen metabolism in vivo were due to direct effects on glycogen synthase and glycogen phos-
phorylase (10,11), we incubated FDB and EPI muscles with 2 mmol/l AICAR for 50 min. To eliminate any possible effects of glucose uptake, we used pyruvic acid in a set of experiments as a substitute carbon source in the incubation medium. As shown in Fig. 5, incubation with AICAR robustly increased α2 AMPK activity in either muscle studied. However, glycogen synthase (Fig. 6A) and glycogen phosphorylase (Fig. 6B) activities in AICAR-treated muscles were virtually identical to controls when incubated in the presence of either 2 mmol/l pyruvate or 5.5 mmol/l glucose as an exogenous carbon source.

DISCUSSION
The major finding of this study was that short-term AICAR treatment altered the activities of enzymes directly involved in glycogen metabolism and caused glycogen deposition in rat skeletal muscle in vivo. Conversely, AICAR had no effect on muscle glycogen synthase or phosphorylase in an in vitro incubation system. This suggests that the increased muscle glycogen observed in this study and others (15,17,18) after in vivo AICAR treatment is second-

FIG. 3. Glycogen synthase and glycogen phosphorylase activities after in vivo AICAR treatment. A: Glycogen synthase activity was increased ~50% above basal levels in red gastrocnemius samples but was significantly reduced in the white gastrocnemius during the 120 min after AICAR treatment. B: Glycogen phosphorylase demonstrated a delayed increase in activity after AICAR treatment in both muscle samples. Interestingly, the ±AMP activity ratio was significantly reduced by 18% below basal levels in the red gastrocnemius before demonstrating increased activity at 90 min. *P < 0.05; n = 4–5 per time point.

FIG. 4. Effect of in vivo AICAR treatment on muscle glycogen and G-6-P accumulation. A: Muscle glycogen content of both red and white gastrocnemius samples was increased 120 min after treating rats with AICAR. B: Similarly, G-6-P concentrations were robustly increased in both muscles 60–120 min after AICAR treatment. *P < 0.05; **P < 0.001; n = 4–5 per time point.

FIG. 5. Effect of in vitro AICAR treatment on α2 AMPK activity. Isolated FDB and EPI muscles were incubated in a solution containing 2 mmol/l pyruvate in the presence or absence of 2 mmol/l AICAR as described in RESEARCH DESIGN AND METHODS. Incubation with 2 mmol/l AICAR robustly increased α2 AMPK activity above control (CONT) values in all three muscles studied. *P < 0.002; n = 6–14 per group.
ary to the well-known effects of AICAR on stimulating GLUT-4 translocation (15,35,36) and glucose transport (10–12,14,36) rather than to direct effects on glycogen synthase and glycogen phosphorylase by upstream kinases and/or phosphatases. An increased glucose uptake by skeletal muscle is indirectly supported by the fact that in vivo AICAR treatment caused a marked, prolonged reduction in blood glucose with corresponding increases in muscle G-6-P and blood lactate concentrations.

AMPK is a metabolite-sensing enzyme proposed to facilitate fat and carbohydrate catabolism to buffer cellular ATP levels in response to changes in energy charge. However, pharmacologic activation of AMPK (15,17,18) and a single amino acid mutation in the enzyme’s subunit (24) are associated with increased muscle glycogen content. In this study, we suggest a mechanism by which AMPK stimulation via AICAR causes this paradoxical increase in muscle glycogen content in vivo. AMPK has been shown to phosphorylate proteins directly involved in glycogen metabolism in vitro, such as glycogen synthase and phosphorylase kinase (20), the latter being the immediate upstream effector of glycogen phosphorylase. A recent in vitro study using four different phosphorylase kinase substrates demonstrated that it is not a substrate of AMPK (37).

In the current study, muscle glycogen phosphorylase activity and blood lactate concentrations were increased after in vivo AICAR treatment, suggesting stimulation of the glycogenolytic pathway. Muscle G-6-P concentrations were also markedly increased by AICAR treatment, which could be due to increased rates of glycogenolysis and/or increased glucose uptake and phosphorylation by hexokinase-II. These observations are consistent with previous reports of increased glycogen phosphorylase activity (19) and G-6-P concentrations (14) in muscle and of increased lactate concentrations in muscle (14) and blood (17,18) after AICAR treatment. When taken up by skeletal muscle, AICAR is converted to 5-aminoimidazole-4-carboxamide ribonucleotide (ZMP), a monophosphorylated derivative that mimics the effects of AMP inside the cell (38). AMP is a known allosteric effector of both AMPK (2) and glycogen phosphorylase (39). It has been suggested that AICAR increases glycogen phosphorylase activity via activation of phosphorylase kinase and by direct effects of ZMP on glycogen phosphorylase (19). Our isolated muscle incubation experiments showed that glycogen phosphorylase activity was not altered by AICAR and was greatest during incubations with 5.5 mmol/l glucose. This suggests that the glycogen accumulation and changes in glycogen synthase and glycogen phosphorylase activities observed in vivo were due to increased glucose uptake and accumulation of intermediary metabolites such as G-6-P within the muscle cells. Therefore, the observed increase in blood lactate after AICAR treatment is likely a result of the increased glucose uptake by muscle contributing to glycolytic flux and was not due to glycogen breakdown.

After AICAR treatment in vivo, glycogen synthase activation state was increased in red gastrocnemius samples. Glycogen synthase activity is under complex regulation in vivo by multisite phosphorylation and dephosphorylation of serine residues and also by G-6-P (40). G-6-P may increase glycogen synthase activity via allosteric stimulation and also by covalently binding to the enzyme and causing a conformational change in the protein structure that makes the regulatory serine residues more susceptible to dephosphorylation by phosphatases (41). In yeast, both G-6-P and fructose-6-phosphate (F-6-P) also promote dephosphorylation of glycogen synthase via inhibition of glycogen synthase kinase (42). It is unknown whether this occurs in mammalian cells, but could represent another potential mechanism to promote glycogen synthesis. Stimulation of glucose uptake by AICAR would be expected to result in an accumulation of G-6-P (14) and possibly F-6-P within the muscle, particularly if AICAR failed also to increase glycolytic flux at the level of phosphofructokinase-I (43). An increase in the G-6-P and F-6-P levels then may explain the dephosphorylation and activation of glycogen synthase observed in the red gastrocnemius, because the ± G-6-P activity ratio reflects the phosphorylation state of the enzyme. It was shown recently that readministration of glucose to cultured human muscle cells after glucose deprivation induced profound increases in glucose uptake and glycogen synthase activity (44). Accumulation of G-6-P and F-6-P also would be expected to inhibit glycogen phosphorylase activity in vivo via feedback inhibition (39), which would not be reflected in the in vitro assay used in this investigation. This could explain how muscle glycogen is accumulated despite an observed coactivation of glycogen synthase and glycogen phosphorylase in this study and others (45).

An apparent conundrum in the current investigation was the dissociation between glycogen accumulation and glycogen synthase activity in skeletal muscle after in vivo AICAR treatment. For example, glycogen content was increased above basal levels in the white gastrocnemius despite nearly a 25% reduction in the glycogen synthase ± G-6-P activity ratio measured in the same samples. This is not the first investigation to report such a dissociation between in vivo glycogen deposition and glycogen synthase activity measured in vitro. Mandarino et al. (46) studied arteriovenous glucose balance across human legs and determined glucose storage (taken to represent glycogen synthesis) during a hyperinsulinemic clamp at 6 and 12 mmol/l plasma glucose concentration. Leg glucose storage was increased twofold by the higher plasma glucose load despite no change in the measured fractional velocity (0.1/10 mmol/l G-6-P activity ratio) of glycogen synthase in muscle biopsy samples. Similar findings have been observed in rats studied during clamp procedures (47). Increasing plasma glucose concentrations under conditions of constant insulinemia resulted in large increases in glycogen synthesis with no changes in glycogen synthase ± G-6-P activity ratio. These results demonstrate that the phosphorylation state of glycogen synthase, as reflected by an in vitro ± G-6-P activity ratio, does not always correlate with glycogen synthesis in vivo and suggest a potent role for substrate (glucose) supply in regulating glycogenic flux that may be independent of the phosphorylation state of glycogen synthase.

The reasons for the divergent responses of glycogen synthase in red and white gastrocnemius are unknown. GLUT-4 content of red skeletal muscle is greater than white in humans (22) and rats (15,21,48). After treadmill exercise in rats, the GLUT-4 content of sarcolemma puri-
fied from red muscles was approximately twofold higher than sarcocollema from white muscle (21). Because a substantial body of evidence suggests that AMPK is critical for increasing GLUT-4 translocation and glucose uptake during exercise (49), it is possible that in the current study, AICAR treatment increased glucose uptake to a greater extent in red versus white muscle, as has been shown previously (14). A greater glucose uptake in the red gastrocnemius may explain why dephosphorylation of glycogen synthase was observed only in this muscle. However, the current results suggest that the increase in glucose uptake was great enough in both muscle fiber types to promote glucose flux into glycogen, regardless of the phosphorylation state of glycogen synthase. Furthermore, measured G-6-P concentrations were actually greater in the white rather than red gastrocnemius samples.

In conclusion, the results of this study suggest that the major mechanism by which in vivo AICAR treatment causes net glycogen deposition in skeletal muscle is by its well-known effect of increasing glucose uptake, and not by affecting kinases and phosphatases that lead to dephosphorylation and activation of glycogen synthase. Our observation of increased glycogen accumulation in skeletal muscle is in contrast to a previous study (17) that showed no change in muscle glycogen after acute AICAR treatment. This is most likely explained by the fact that muscle glycogen concentrations were measured for 2 h after AICAR treatment, as opposed to 1 h in a previous study (17). On the basis of the data in this study, we speculate that in vivo, AICAR promotes glycogen accumulation in skeletal muscle by increasing glucose uptake, which secondarily results in an accumulation of intracellular G-6-P. This G-6-P may then serve as a feedback inhibitor of glycogen phosphorylase, an activator of glycogen synthase, and a primary substrate for glycogen synthesis and may also enter the glycolytic pathway.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (AR42238 and AR45670) and American Diabetes Association to L.J.G. W.G.A. was supported by a National Research Service Award (DK5769).

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