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A comparison of chronic AICAR treatment-induced metabolic adaptations in red and white muscles of rats

Masataka Suwa · Hiroshi Nakano · Zsolt Radak · Shuzo Kumagai

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Abstract The signaling molecule 5'-AMP-activated protein kinase plays a pivotal role in metabolic adaptations. Treatment with 5-aminoimidazole-4-carboxamide-1- β -D-ribofranoside (AICAR) promotes the expression of metabolic regulators and components involved in glucose uptake, mitochondrial biogenesis, and fatty acid oxidation in skeletal muscle cells. Our aim was to determine whether AICAR-induced changes in metabolic regulators and components were more prominent in white or red muscle. Rats were treated with AICAR (1 mg/g body weight/day) for 14 days, resulting in increased expression levels of nicotinamide phosphoribosyltransferase (NAMPT), peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), glucose transporter 4 proteins, and enhanced mitochondrial biogenesis. These changes were more prominent in white rather than red gastrocnemius muscle or were only observed in the white gastrocnemius. Our results

suggest that AICAR induces the expression of metabolic regulators and components, especially in type II (B) fibers.

Keywords AMP-activated protein kinase · Mitochondrial biogenesis · Nicotinamide phosphoribosyltransferase · SIRT1 · Skeletal muscle

Introduction

Skeletal muscle demonstrates a great degree of metabolic plasticity, with its characteristics subjected to many studies over several decades. Accumulating evidence suggests that a large number of signaling molecules control the metabolic properties of skeletal muscle. The signaling molecule 5'-AMP-activated protein kinase (AMPK) has been shown to play a pivotal role in skeletal muscle cells [1].

AMPK is a heterotrimer comprising catalytic α - and regulatory β - and γ -subunits [2]. Two isoforms exist for the α -subunit (α 1 and α 2) and the β -subunit (β 1 and β 2), with three isoforms for the γ -subunit (γ 1, γ 2, and γ 3). The α -subunit contains the serine/threonine kinase domain, which has been shown to exhibit kinase activity when it is phosphorylated by upstream kinases such as LKB1 and CaMKK [3, 4]. The β -subunit contains a domain that interacts with the α - and γ -subunits and was previously reported to mediate the assembly of the heterotrimeric AMPK complex [5]. The β -subunit also contains a glycogen-binding domain [6]. The γ -subunit binds to AMP following the phosphorylation of threonine 172 in the α -subunit and kinase activation [7]. AMPK is a central energy-sensing master regulator of cellular metabolism and is activated when the cellular AMP/ATP ratio increases [8]. This allosteric regulatory system further promotes the

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phosphorylation of threonine 172 in the α -subunit by upstream kinases [9].

Skeletal muscle AMPK is known to be activated by exercise [10, 11]; secretory factors including leptin [12], adiponectin [13], interleukin-6 [14], and brain-derived neurotrophic factor [15]; and antidiabetic drugs [16, 17]. It is also activated by the adenosine analog 5-aminoimidazole-4-carboxamide-1- β -D-ribofranoside (AICAR); AICAR activation of AMPK stimulates glucose uptake and fatty acid oxidation in skeletal muscle cells [18]. AICAR treatment has also been found to enhance the expression of metabolic components, including glucose transporter 4 (GLUT4) and monocarboxylate transporters 1 and 4 proteins; increase hexokinase activity; and stimulate mitochondrial biogenesis in skeletal muscle [19–22]. Activation of AMPK has been linked to upregulated expression of metabolic regulators, such as silent information regulator of transcription 1 (SIRT1), peroxisome proliferator-activated receptor (PPAR)- γ coactivator-1 α (PGC-1 α), and nicotinamide phosphoribosyltransferase (NAMPT) [21, 23, 24]. It has been proposed that AMPK-induced metabolic adaptations could be mediated, at least partially, by SIRT1 [1].

SIRT1 is an oxidized form of nicotinamide adenine dinucleotide (NAD⁺)-dependent histone deacetylase. It plays an important role in various biological processes via its interactions with and deacetylation of many transcriptional regulators, such as forkhead transcription factor (FOXO) [25], p53 [26], nuclear factor- κ B (NF- κ B) [27], and PGC-1 α [28]. SIRT1 regulates mitochondrial biogenesis and fatty acid oxidation in skeletal muscle cells by deacetylating and functionally activating PGC-1 α [28, 29].

SIRT1 activity is controlled by NAMPT, also known as pre-B cell colony-enhancing factor (PBEF) or visfatin. NAMPT is the rate-limiting enzyme required for the synthesis of NAD⁺ from nicotinamide, an inhibitor of SIRT1. NAMPT increases the NAD⁺/NADH ratio and decreases nicotinamide concentrations: SIRT1 activity then allosterically increases in skeletal muscle cells [30]. The metabolic capacity of skeletal muscle is thought to be at least partially controlled by the AMPK–NAMPT–SIRT1–PGC-1 α axis. The expression of NAMPT in skeletal muscle has been shown to increase with endurance exercise training and activation of AMPK [23, 31, 32]. NAMPT might play an important role in exercise training-induced metabolic adaptations in skeletal muscle [33].

Skeletal muscle fibers in rodents are categorized as slow-twitch type I and fast-twitch type IIA, IIX and IIB fibers, and express myosin heavy chains I, IIA, IIX, and IIB, respectively [34]. The type I, IIA, and IIX fibers exist in human skeletal muscle [34]. The rank order of maximum contraction velocity in rat skeletal muscle fibers is I < IIA < IIX < IIB [35]; for oxidative capacity it is

IIB < I < IIX < IIA [36] or IIB < IIX < I < IIA [37]. Metabolic responses to some stimuli, such as exercise training [38], detraining [39], and hindlimb suspension [40], appear to differ among the various fiber types. Acute exercise was reported to increase the AMPK phosphorylation in all fiber types, and type IIX fibers exhibited the greatest increase in human skeletal muscle [41].

We previously showed that AICAR induces increases in AMPK phosphorylation levels in type II fiber-rich extensor digitorum longus (EDL) muscle. The extent of this increase appeared to be greater than that in the type I fiber-rich soleus muscle of rats [24]. In another study, it was shown that a single AICAR injection resulted in a 5.5-fold increase in AMPK activity in the white gastrocnemius muscle, but only a 2.9-fold increase in the red gastrocnemius muscle [42]. Collectively, these findings suggested that acute administration of AICAR increases phosphorylation levels of AMPK in type II(B) fibers to a great extent than in type I fibers. Therefore, chronic AICAR treatment might result in greater expression levels of metabolic regulators and components in type II(B) fiber-rich white muscle compared with those in type I fiber-rich red muscles. However, results from previous studies have not clearly revealed the extent of chronic AICAR treatment-induced metabolic adaptations between muscles types. We hypothesized that chronic AICAR treatment would enhance expression levels of metabolic regulators and components. We also believe that these effects would be more prominent in type II(B) fibers than in type I fibers.

Our aims were to determine whether AICAR-induced changes in metabolic regulators, such as NAMPT, SIRT1, and PGC-1 α , and in metabolic components, such as GLUT4 and mitochondrial oxidative enzymes, differed between type I fiber-rich red gastrocnemius and type IIB fiber-rich white gastrocnemius muscles.

Materials and methods

Animal studies

Male Wistar rats (5 weeks old, 136–148 g) from Kyudo (Tosu, Saga, Japan) were used in this study. All rats were handled daily for at least 5 days before the commencement of experiments. Rats were housed in a temperature- (22 \pm 2 $^{\circ}$ C) and humidity-controlled (60 \pm 5 %) room with a 12-h light (07:00–19:00):12-h dark (19:00–07:00) cycle; food and water were provided ad libitum. All experimental procedures were strictly conducted in accordance with the Nakamura Gakuen University guidelines for the Care and Use of Laboratory Animals and were approved by the University Animal Experiment Committee.

Rats were divided into control ($n = 9$) and AICAR treatment ($n = 7$) groups. Rats in the control group were given daily subcutaneous injections of saline, while those in the AICAR group were given daily subcutaneous injections of AICAR (1 mg/g body weight; Toronto Research Chemicals, North York, ON, Canada). This dose of AICAR was previously shown to enhance skeletal muscle AMPK activity and the phosphorylation of threonine 172 in the α -subunit at 60 and 120 min postinjection [19, 24, 43, 44]. Procedures were performed between 08:00 and 10:00 for 14 successive days. Nonfasted rats were intraperitoneally anesthetized with pentobarbital sodium (60 mg/kg body weight) about 24 h after the last injection. The gastrocnemius muscle was dissected, and the deep (red) and superficial (white) portions of the lateral head of the gastrocnemius muscle were isolated. Muscle tissues were immediately frozen in liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ until required. The abdominal fat pads (mesenteric, epididymal and perirenal) were also excised and weighed.

Preparation of muscle samples

Frozen muscle samples were homogenized in ice-cold homogenization buffer (1:10 w/v; 25 mM HEPES, 250 mM sucrose, 2 mM EDTA, 0.1 % Triton X-100, pH 7.4) supplemented with CompleteTM Protease Inhibitor Cocktail Tablets (Roche Diagnostics, Tokyo, Japan) and centrifuged ($15,000\times g$, $4\text{ }^{\circ}\text{C}$, 25 min). The supernatant was removed and the concentration of proteins determined using a kit (Bio-Rad, Richmond, CA). This muscle homogenate was used for enzymatic assays and in Western blotting analyses. For the Western blotting analyses, the muscle protein homogenate was solubilized in sample loading buffer (50 mM Tris-HCl pH 6.8, 2 % sodium dodecyl sulfate, 10 % glycerol, 5 % β -mercaptoethanol, and 0.005 % bromophenol blue).

Western blotting

Proteins (20 μg) present in homogenates were separated by sodium-dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5 % (SIRT1 and PGC-1 α), 10 % (NAMPT and GLUT4), and 15 % (cytochrome C) resolving gels. Proteins separated by SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride membranes. These membranes were incubated with a blocking buffer of casein solution (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Membranes were incubated with rabbit polyclonal antibodies against PBEF (1:500 dilution; Bethyl Laboratories, Inc., Montgomery, TX, USA), Sir2 (1:1000; Upstate Biotechnology, Lake Placid, NY, USA), PGC-1 α (1:500; AB3242, Chemicon International, Temecula, CA, USA), or

GLUT4 (1:8000; Chemicon International), or with a mouse monoclonal antibody against cytochrome C (1:200; clone 7H8.2C12; Biosource, Camarillo, CA, USA) overnight at $4\text{ }^{\circ}\text{C}$. Membranes were then incubated with anti-rabbit/mouse biotinylated IgG (1:1000; Vector Laboratories) for 30 min. Protein bands were visualized using the avidin and biotinylated horseradish peroxidase macromolecular complex technique (Vector Laboratories). Band densities were determined using NIH Image, version 1.62 (National Institute of Health, Bethesda, MD, USA).

Enzyme activity assays

Enzyme activities were measured spectrophotometrically with assays conducted at $30\text{ }^{\circ}\text{C}$ using saturating concentrations of substrates and cofactors, as previously determined. Citrate synthase (CS) activity was measured at 412 nm to detect the transfer of sulfhydryl groups to 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB). The extinction coefficient for DTNB was 13.6. Hexokinase (HK), pyruvate kinase (PK), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), and β -hydroxyacyl-coenzyme A dehydrogenase (β HAD) activities were measured at 340 nm by following the production or consumption of NADH or NADPH over 3 min. The extinction coefficient for NAD(P)H was 6.22. Further details and procedures have been described previously [45].

Statistical analysis

Values are expressed as the mean \pm SD. We used the unpaired t test to compare data between groups with a P value less than 0.05 considered statistically significant.

Results

Body composition

We summarized the body composition of rats (Table 1); pre- and posttreatment body masses in the AICAR group were not significantly different from those in the control

Table 1 Body composition of rats in the current study

	Control ($n = 9$)	AICAR ($n = 7$)
Pretreatment body weight (g)	140 \pm 3	141 \pm 3
Posttreatment body weight (g)	245 \pm 13	238 \pm 8
Mesenteric fat tissue weight (g)	3.19 \pm 0.41	2.44 \pm 0.52*
Epididymal fat tissue weight (g)	1.81 \pm 0.16	1.32 \pm 0.14*
Perirenal fat tissue weight (g)	0.39 \pm 0.06	0.23 \pm 0.07*

* $P < 0.05$ vs. control group

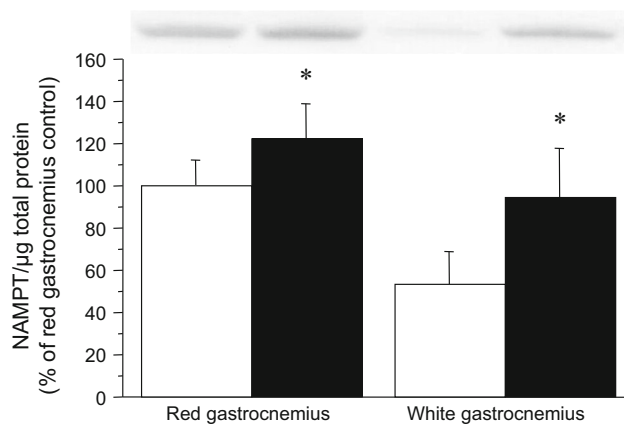


Fig. 1 NAMPT protein expression levels in red and white gastrocnemius muscles of rats in the control (*open columns*) and AICAR (*filled columns*) groups. Values are mean \pm SD; $n = 7$ – 9 muscles per group. * $P < 0.05$ vs. the control group

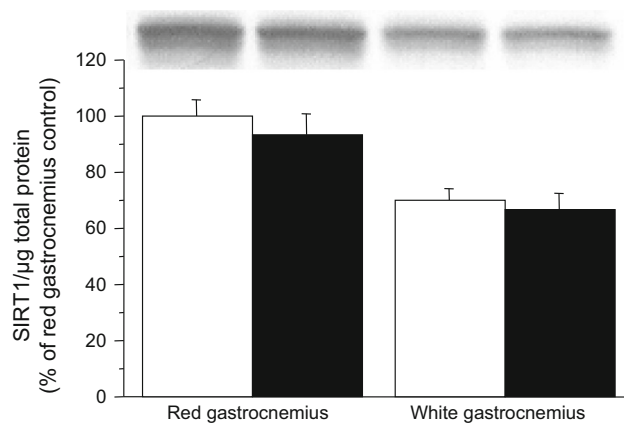


Fig. 2 SIRT1 protein expression levels in red and white gastrocnemius muscles of rats in the control (*open columns*) and AICAR (*filled columns*) groups. Values are mean \pm SD; $n = 7$ – 9 muscles per group

group. Mesenteric, epididymal, and perirenal fat tissue masses in the AICAR group were significantly lower than those in the control group.

Expression of metabolic regulators

NAMPT protein expression levels in red and white gastrocnemius muscles were significantly increased, by 22 and 77 %, respectively ($P < 0.01$), following AICAR treatment (Fig. 1). SIRT1 protein expression levels were unaltered by AICAR treatment in red or white muscles (Fig. 2). PGC-1 α protein expression levels in the white gastrocnemius muscle of rats in the AICAR group were significantly higher than those in the control group (47 %, $P < 0.01$); however, a similar difference was not observed in red gastrocnemius muscle (Fig. 3).

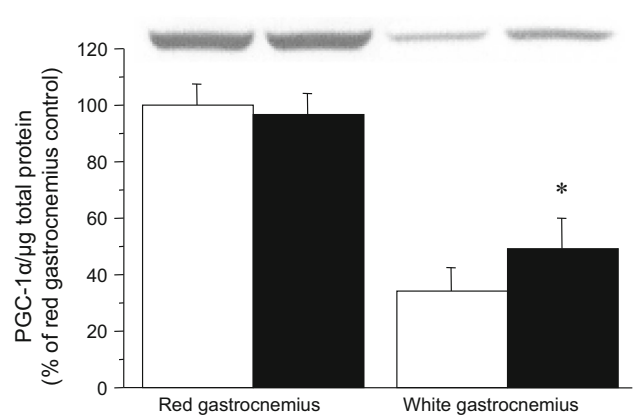


Fig. 3 PGC-1 α protein expression levels in red and white gastrocnemius muscles of rats in the control (*open columns*) and AICAR (*filled columns*) groups. Values are mean \pm SD; $n = 7$ – 9 muscles per group. * $P < 0.05$ vs. the control group

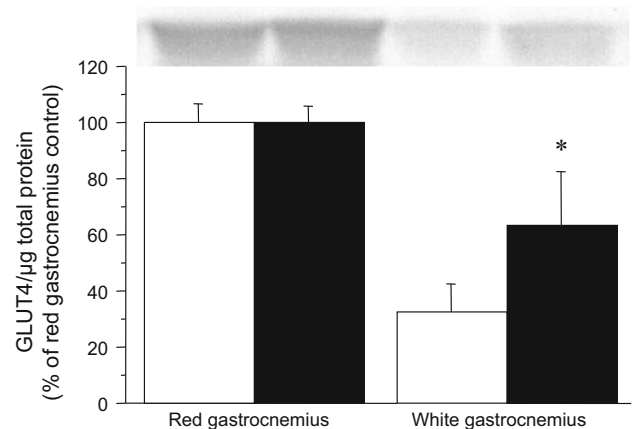


Fig. 4 GLUT4 protein expression levels in red and white gastrocnemius muscles of rats in the control (*open columns*) and AICAR (*filled columns*) groups. Values are mean \pm SD; $n = 7$ – 9 muscles per group. * $P < 0.05$ vs. the control group

Expression of metabolic components

AICAR treatment significantly increased GLUT4 protein expression levels by 96 % in white gastrocnemius muscle ($P < 0.001$), but not in red gastrocnemius muscle (Fig. 4). Cytochrome C protein expression levels in the white gastrocnemius muscle were also increased by the AICAR treatment (26 %, $P < 0.05$). We did not observe any changes in cytochrome C expression levels in red gastrocnemius muscle (Fig. 5).

Enzyme activities

HK activities in red and white gastrocnemius muscles were significantly increased in the AICAR-treated group, by 52 and 132 %, respectively, compared to the control group

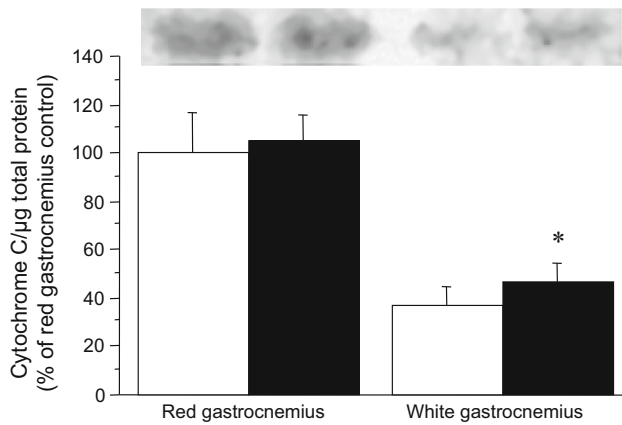


Fig. 5 Cytochrome C protein expression levels in red and white gastrocnemius muscle of rats in the control (*open columns*) and AICAR (*filled columns*) groups. Values are mean \pm SD; $n = 7-9$ muscles per group. * $P < 0.05$ vs. the control group

(Fig. 6a; $P < 0.0001$). PK activity in white gastrocnemius muscle was increased by 26 % because of AICAR treatment ($P < 0.0001$); however, PK activity was relatively unchanged in red gastrocnemius muscle (Fig. 6b). AICAR treatment did not affect LDH activity in either type of muscle (Fig. 6c). CS activity in white gastrocnemius muscle was significantly higher in the AICAR-treated group compared with that in the control group (26 %, $P < 0.0001$). No significant differences were observed for CS activity in red gastrocnemius muscle (Fig. 6d). MDH activities in red and white gastrocnemius muscles were significantly increased by 13 and 38 %, respectively, following AICAR treatment ($P < 0.05$; Fig. 6e) compared

with those seen in the control group. AICAR treatment resulted in a 31 % increase in β HAD activity in white gastrocnemius muscle ($P < 0.01$), with no increase observed in red gastrocnemius muscle (Fig. 6f).

Comparison of AICAR effects in red and white muscles

We have illustrated the relative changes in protein expression levels and metabolic enzyme activities induced by AICAR treatment and compared them with those in the control group (Fig. 7). AICAR induced changes for all parameters relative to the control group, with the exception of SIRT1 protein and LDH activity levels. These were significantly higher in white gastrocnemius muscle compared with that in red gastrocnemius muscle ($P < 0.05$).

Discussion

AMPK has been implicated in several diseases such as diabetes mellitus, hypertension, cardiac hypertrophy, cancer, dementia, and stroke [46]. The control of AMPK activity represents a strategy for preventing these diseases or improving therapies against them. In our current study, we found that treating rats with AICAR over 14 successive days significantly increased expression levels of NAMPT, PGC-1 α , and GLUT4 proteins and enhanced mitochondrial biogenesis in rat skeletal muscle.

We designed our study so as to compare AICAR-induced metabolic alterations in red and white muscles.

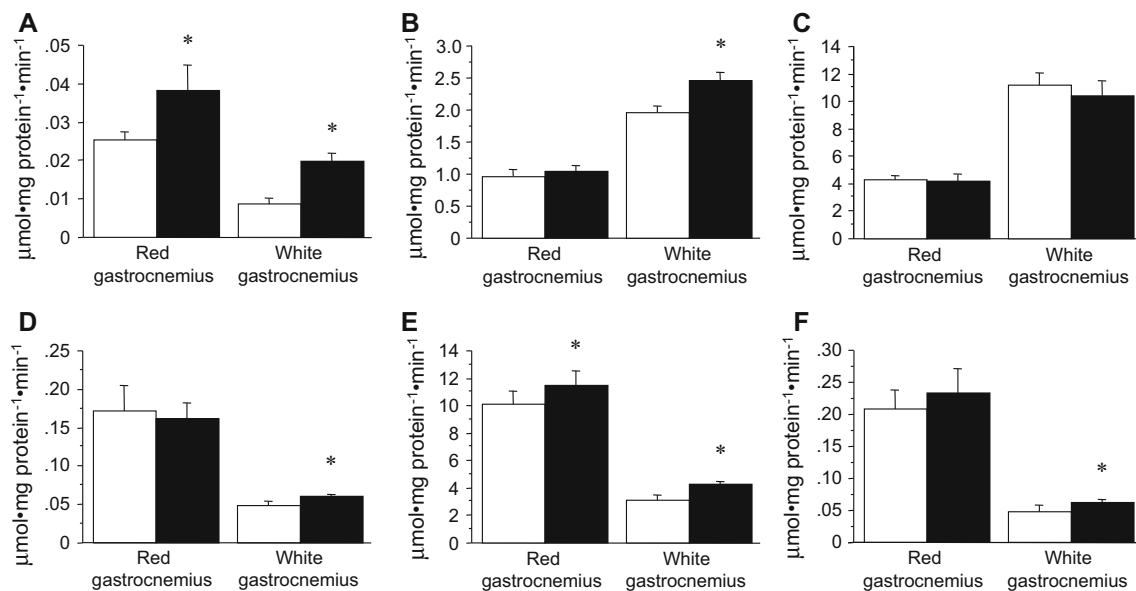


Fig. 6 Metabolic enzyme activities in red and white gastrocnemius muscles. **a** Hexokinase, **b** pyruvate kinase, **c** lactate dehydrogenase, **d** citrate synthase, **e** malate dehydrogenase, and **f** β -hydroxyacyl-

coenzyme A dehydrogenase in rats of the control (*open columns*) and AICAR (*filled columns*) groups. Values are mean \pm SD; $n = 7-9$ muscles per group. * $P < 0.05$ vs. the control group

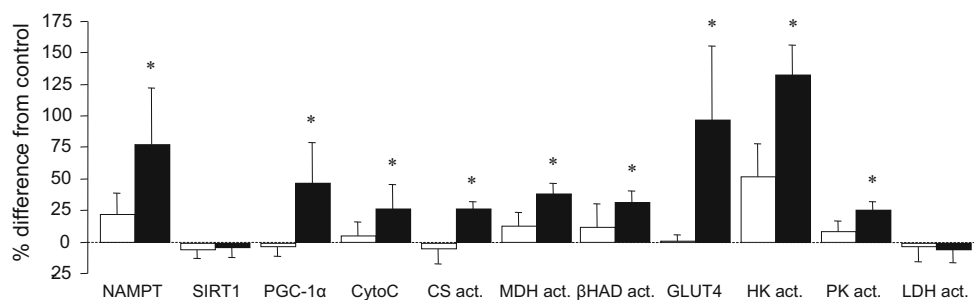


Fig. 7 Proportional differences in protein expression levels and metabolic enzyme activity levels between rats in the control and AICAR groups for red (*open columns*) and white (*filled columns*) gastrocnemius muscles. Differences in these measurements were

determined as follows: % difference = $100 \times (\text{AICAR group}/\text{mean control group}) - 100$. Values are mean \pm SD; $n = 7$ per group. * $P < 0.05$ vs. red gastrocnemius muscle. *CytoC* cytochrome C, *act.* activity

Our results showed that the expression levels of metabolic regulators, such as NAMPT and PGC-1 α proteins, following the AICAR treatment were higher in the white gastrocnemius muscle than in the red gastrocnemius muscle. Furthermore, the expression of the GLUT4 and cytochrome C proteins as well as CS, MDH, β HAD, HK, and PK activities were higher in the white gastrocnemius muscle than in the red gastrocnemius muscle following the AICAR treatment. The red gastrocnemius muscle contains ~50 % type I fibers and less than 1 % type IIB fibers. The white gastrocnemius muscle contains ~90 % type IIB and ~10 % of type IIX fibers, with low levels but a minimal amount of type I or IIA fibers in rats [37, 47]. Based on these results, AICAR-induced metabolic improvements were suggested to be more prominent in type II(B) fibers than in type I fibers.

The fiber type-dependent effects of AICAR on muscle metabolic profiles observed in our present study could be partially associated with different AMPK phosphorylation levels among fiber types following AICAR treatment. We previously showed that acute AICAR treatment-induced increases in AMPK phosphorylation levels appeared to be greater in type II fiber-rich EDL muscle than in type I fiber-rich soleus muscle [24]. Additionally, phosphorylation levels of acetyl-coenzyme A carboxylase (ACC), a downstream target of AMPK, in the EDL muscle appeared to be enhanced to a great extent than in the soleus muscle following AICAR treatment [24]. Previous results indicate that a single AICAR injection resulted in a 5.5-fold increase in AMPK activity in white gastrocnemius muscle, but only a 2.9-fold increase in red gastrocnemius muscle [42]. These fiber type-dependent effects of AICAR on the phosphorylation of AMPK might be responsible for the different results we observed in red and white muscles.

Another possible cause of these differences could be the varying expression patterns of the AMPK γ subunit isoforms among fiber types. In rodents, the AMPK γ 3 isoform is primarily expressed in muscles that are rich in type IIB fibers, but is rarely expressed in type I fiber-rich muscles

[48, 49]. The skeletal muscles of AMPK γ 3 mutant (R225Q) transgenic mice exhibit enhanced mitochondrial biogenesis, fatty acid oxidation, and glycogen synthesis as well as increased expression levels of genes encoding the corresponding regulatory proteins [48, 50, 51]. In contrast, AMPK γ 3 isoform knockout mice have decreased gene expression levels [50]. Cantó et al. [52] reported that fasting-induced increases in the expression of NAMPT, PGC-1 α , and GLUT4 mRNA and exercise-induced increases in NAMPT mRNA expression and PGC-1 α deacetylation were ameliorated in AMPK γ 3 knockout mice. Incubating EDL muscle isolated from wild-type mice with AICAR promoted the phosphorylation of the AMPK downstream targets, such as ACC and the Akt substrate of 160 kDa (AS160); however, these changes were ablated in EDL muscles isolated from AMPK γ 3 isoform knockout mice [53]. The AMPK γ 3 isoform appears to be necessary for AICAR-induced AMPK signaling in skeletal muscle and controls AICAR-stimulated metabolic adaptations, especially in type IIB fibers.

Consistent with our results, AICAR treatment for 28 successive days was previously shown to increase NAMPT protein and mRNA expression levels in the skeletal muscle of mice [23]. The effects of AICAR on the accumulation of the NAMPT protein were not apparent in AMPK α 2 kinase dead (nonfunctional enzyme) mice, whereas NAMPT mRNA levels were maintained [23]. The AICAR-induced increases observed in the expression of the NAMPT protein we observed can be attributed to regulation by AMPK signaling at the posttranscriptional or translational level. Results from a previous study demonstrated that AMPK controlled intracellular NAD⁺ concentrations and SIRT1 activity [54]. Collectively, these results suggest that AMPK regulates SIRT1 activity by modulating the quantity of the NAMPT protein, followed by PGC-1 α deacetylation and transcriptional activation.

The activated form of PGC-1 α can interact with several transcription factors, including myocyte enhancer factor 2 (MEF2), GLUT4 enhancer factor (GEF), cAMP response

element binding protein (CREB), estrogen-related receptor α (ERR α), PPAR α and γ , and nuclear respiratory factor 1 (NRF1). NRF promotes the expression of GLUT4 and PGC-1 α , and also promotes mitochondrial biogenesis [1]. The AMPK-NAMPT-SIRT1-PGC-1 α axis possibly mediates the AICAR-induced metabolic improvements we observed in skeletal muscle.

In a previous study, it was reported that SIRT1 protein expression levels were increased in EDL muscle following administration of a single dose of AICAR to rats [24]. In humans, one short period of intense activity promotes the phosphorylation of AMPK α and SIRT1 protein expression in the vastus lateralis muscle. The same level of activity with glucose ingestion did not lead to an increase in either AMPK phosphorylation or SIRT1 protein content [55]. Based on these findings, it appears likely that AMPK signaling regulates the SIRT1 protein content in skeletal muscle.

We demonstrated that 14 successive days of AICAR treatment did not alter the expression of SIRT1 in either red or white gastrocnemius muscle in rats. Chronic administration of AICAR (30 days) to *mdx* mice also failed to enhance the expression of SIRT1 mRNA [56]. Furthermore, 5 days of a successive AICAR treatment decreased SIRT1 protein expression levels in rats [57]. These findings suggest that the effects of AICAR treatment on SIRT1 expression in skeletal muscle in vivo differed depending on duration of the treatment period. Although administration of a single dose of AICAR was shown to promote AMPK activity [22, 24], long-term (28 days) AICAR treatment abolished the activation of AMPK in rat skeletal muscle [22, 58]. These results indicate treatment period-specific effects of AICAR that lead to the activation of AMPK and inconsistent SIRT1 expression patterns.

Another possibility is that chronic AICAR administration results in an excess of nutrients in skeletal muscle fibers. These are manifested as elevated glycogen stores and inhibit accumulation of the SIRT1 protein, thus masking the effects of AICAR. Caloric restriction was previously reported to increase SIRT1 protein expression levels in skeletal muscle [59], suggesting that the expression of the SIRT1 protein is inversely associated with energy stores. The concentration of glycogen at 22–25 h after a final AICAR injection in the skeletal muscle of rats treated with AICAR over 4 weeks was higher than that in saline-treated rats [22]. This finding suggests that long-term administration of AICAR results in constantly elevated glycogen concentrations in skeletal muscle that might interfere with accumulation of SIRT1. Further investigations are required to elucidate the mechanism(s) responsible for varying different results obtained between short- and long-term AICAR treatments with respect to SIRT1 expression.

Although AICAR stimulates skeletal muscle metabolism via the direct activation of AMPK [60], other indirect mechanisms might also mediate metabolic adaptations. As an example, AICAR can activate other AMP-sensing enzymes [61, 62]. AICAR also controls the secretion or production of hormones/cytokines that regulate metabolism, including insulin [63], adiponectin [64], interleukin 6 (IL-6) [65], and tumor necrosis factor- α (TNF- α) [64]. Adiponectin and IL-6 are activators of AMPK [13, 14], whereas TNF- α is an inhibitor of AMPK [66, 67]. Therefore, in vivo AICAR treatment-induced metabolic modifications in skeletal muscle could be attributed to the direct and indirect activation of AMPK and AMPK-independent mechanisms. The administration of AICAR to mice over-expressing $\alpha 2$ kinase-dead AMPK led to an increase in the expression of NAMPT mRNA but not the corresponding protein [23], suggesting that AICAR affects the expression of NAMPT at the transcriptional level via an AMPK-independent mechanism.

In this study, we demonstrated that chronic AICAR treatment significantly decreased the mass of abdominal fat pads with concomitant increases in the expression of metabolic regulator proteins and mitochondrial components. Although the reasons for such reductions currently remain unclear, it is possible that increases in fatty acid oxidation and oxidative capacity in skeletal muscle induced by AICAR enhanced whole-body energy expenditure. Alternatively, it is possible that treatment with AICAR results in a slightly reduced food intake. However, slight caloric restriction should not affect metabolic modifications caused by AICAR. We previously demonstrated that caloric restriction of around 65 % in ad libitum-fed rats for 14 days did not affect expression of metabolic regulator proteins or mitochondrial components in skeletal muscle [45].

In conclusion, chronic AICAR treatment of rats for 14 successive days significantly increased NAMPT and PGC-1 α protein expression levels in red and white gastrocnemius muscles. GLUT4 protein expression levels were also increased, and mitochondrial biogenesis was enhanced in skeletal muscles following AICAR treatment. These increases in the expression of metabolic regulators and components in white gastrocnemius muscle were more prominent than those in red gastrocnemius muscle, suggesting that AICAR-induced metabolic adaptations occurred, particularly in type II(B) fibers. In contrast, long-term administration of AICAR did not affect SIRT1 expression.

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Conflict of interest We have no conflicts of interest to declare.

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