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# AMP-activated protein kinase regulates the expression of monocarboxylate transporter 4 in skeletal muscle

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**Abstract**

**Aims**

The aim of this study was to determine the effect of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an AMP-activated protein kinase (AMPK) activator, on monocarboxylate transporter 4 (MCT4) expression in rat skeletal muscle and a prototypic embryonal rhabdomyosarcoma cell line (RD cells).

**Main methods**

We examined the alteration in Glucose transporter 4 (GLUT4) and MCT4 mRNA levels by quantitative real-time PCR. Alteration in GLUT4 and MCT4 protein levels were examined by Western blotting.

**Key findings**

In an in vivo study, AICAR increased MCT4 mRNA and protein levels in a fiber-type specific manner. In an in vitro study, AICAR increased MCT4 mRNA and protein levels. Moreover, AICAR-induced MCT4 expression was blocked by Compound C, an AMPK inhibitor.

**Significance**

In this study, we found that AMPK activation induced expression of MCT4 in RD cells and rat skeletal muscle in a fiber-type specific manner. These results indicate the possible involvement of an AMPK-mediated pathway associated with MCT4 expression in skeletal muscle.

**Keywords**

AMPK; MCT4; up-regulation; L-lactic acid; skeletal muscle

## Introduction

Skeletal muscle is the main site for glucose disposal and the major producer of lactic acid in the body. Lactic acid transport across the plasma membrane is fundamental for metabolism and pH regulation of cells. The transport of lactic acid is mediated by monocarboxylate transporters (MCTs, SLC16). The MCT family now comprises 14 members, of which MCT1-MCT4 catalyze proton-coupled transport of metabolically important monocarboxylates such as L-lactic acid, pyruvate and ketone bodies (Meredith and Christian., 2008). MCT4 is expressed strongly in glycolytic tissues such as white skeletal muscle and is thought to play a major role in the efflux of L-lactic acid (Wilson et al., 1998).

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that has emerged as a master sensor of cellular energy balance in mammalian cells. AMPK is composed of a catalytic α-subunit (α1 or α2) and regulatory subunits β (β1 or β2) and γ (γ1, γ2 or γ3). AMPK is activated in response to an elevation in AMP-to-ATP ratio and phosphorylation, which is mediated by upstream kinases. Skeletal muscle AMPK is activated by diverse stimuli such as exercise, hormones (adiponectin and leptin), and antidiabetic agents (metformin and thiazolidinediones) (Steinberg and Kemp., 2009). Once activated, AMPK phosphorlylates a number of targets including acetyl-CoA carboxylase (ACC), resulting in the induction of various biological reactions. Several studies have shown that 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), an AMPK activator, activates glucose transport (Kurth-Kraczek et al., 1999) and increases the expression of glucose transporter 4 (GLUT4) and several metabolic enzymes

in skeletal muscle (Buhl et al., 2001; Holmes et al., 1999, 2005; Ojuka et al., 2002; Stoppani et al., 2002; Winder et al., 2000; Zheng et al., 2001). It has been reported that an increase in glucose uptake induced by AICAR results in increased lactic acid production in isolated rat skeletal muscle (Miyamoto et al., 2007). However, the effect of AMPK activation on MCT4, which plays a critical role in lactic acid efflux in skeletal muscle, has not been clarified. The aim of this study was to determine the effect of AMPK activation on MCT4 expression in skeletal muscle.

## Materials and methods

### Chemicals

AICAR was obtained from Toronto Research Chemicals (North York, ON, Canada) and Compound C was obtained from Sigma Aldrich (St. Louis, MO).

### Animals

Male Wistar rats, aged 6 to 7 weeks (120-200 g in weight), were obtained from Slc (Hamamatsu, Japan). The housing conditions were described previously (Kobayashi et al., 2008b). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the “Guide for the Care and Use of Laboratory Animals”. To determine the effects of a single AICAR injection, rats were injected subcutaneously with AICAR (0.5 mg/g body wt). Control groups were injected with saline in a volume proportional to the AICAR-treated animals.
To determine the effects of AICAR injections for 5 days, rats were injected subcutaneously with AICAR (0.5 mg/g) or saline for 5 days in succession.

**Blood and muscle tissue collection**

At 1 hour after the subcutaneous AICAR injection, blood was drawn from the tail to assess blood glucose and lactic acid. The blood glucose concentration was determined using a glucometer (Bayer, Osaka, Japan). Plasma lactic acid was determined by Lactate Reagent (Trinity Biotech plc, Wicklow, Ireland) according to the manufacturer's instructions. Rats were anesthetized by intravenous injection of pentobarbital sodium. Gastrocnemius muscles and soleus muscle were quickly removed from the rats. The gastrocnemius muscles were divided into white and red portions. The muscles were frozen in liquid nitrogen and stored at -80˚C until analyses.

**Cell culture**

Prototypic embryonal rhabdomyosarcoma cells (RD cells) were kept in Dulbecco's modified Eagle's medium (Sigma) with 10% fetal bovine serum (ICN Biomedicals, Inc, Aurora, OH) and 1% penicillin-streptomycin (Sigma) at 37˚C under 5% CO2 as described previously (Kobayashi et al., 2005, 2008a).

**Western blotting**

Western blot analysis was performed as described previously (Kobayashi et al., 2006). The muscle tissue samples were homogenized in ice-cold lysis buffer. For protein extracts from cells, RD cells were scraped and washed with ice-cold PBS (containing 1 mM PMSF, 1 mM Na3VO4, 25 mM NaF, 1 μg/mL leupeptin) and centrifuged at 1,500×g for 5 min at 4°C. The resulting pellet was suspended in ice-cold lysis buffer. The suspension was allowed to stand for 5 min on ice and was sonicated for 30 sec at 4°C. The suspension was then centrifuged at 12,000×g for 15 min at 4°C. The supernatant was used for Western

blotting. The lysis buffer used was as follows: RIPA Buffer (Cell Signaling, Beverly, MA) plus 25 mM NaF, 1 mM PMSF (for AMPK, total and phospho-ACC) or 20 mM Tris, 150 mM NaCl, 10 mM Na2EDTA, 10 mM EGTA, 1% Triton X-100, 1 mM Na3VO4, 25 mM NaF, 1 μg/mL leupeptin, and 1 mM PMSF (for GLUT4, MCT4). The protein concentration was determined using a Pierce® BCA Protein Assay Kit (Thermo SCIENTIFIC, Rockford, IL). Proteins were subjected to SDS-PAGE and transferred onto nitrocellulose membranes and then immunoblotted by using antibodies. The primary antibodies used were as follows: goat anti-GLUT4 polyclonal antibody (sc-1608, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-MCT4 polyclonal antibody (sc-50329, Santa Cruz Biotechnology), rabbit anti-ACC (no. 3662, Cell Signaling), rabbit anti-phospho-ACC (no. 3661, Cell Signaling), rabbit anti-AMPKα (no. 2532, Cell Signaling), and mouse anti-actin monoclonal antibody (Chemicon, Temecula, CA). The proteins bound to antibodies were detected using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized by enhanced chemiluminescence (Amersham Biosciences Corp., Piscataway, NJ).

**RT-PCR analysis and quantitative real-time PCR**

RT-PCR analysis and quantitative real-time PCR were performed as described previously (Chisaki et al., 2009). Total RNA was prepared from muscle tissues and RD cells using an ISOGEN (Nippon Gene, Japan) and an RNase-Free DNase Set (QIAGEN, Hilden, Germany). Single-strand cDNA was made from 0.5 to 1.0 μg total RNA by reverse transcription using ReverTraAce (TOYOBO, Japan). PCR was performed using Hot Star Taq PCR (QIAGEN) with gene-specific primers (sequences shown in Table 1)

through 30-40 cycles of 95°C for 30 s, 58-60°C for 30 s, and 72°C for 60 s. The PCR products were subjected to electrophoresis on a 2% agarose gel and then visualized by ethidium bromide staining. Quantitative real-time PCR was performed using an Mx3000TM Real-time PCR System (STRATAGENE) with Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA) and specific primers (sequences shown in Table 1) through 40 cycles of 95°C for 30 s, 56-60°C for 30 s, and 72°C for 60 s. The PCR products were normalized to amplified 18S rRNA, which was the internal reference.

**Uptake study**

Glucose transport was studied using uptake of non-metabolizable glucose analog [3H]-2-deoxy-D-glucose (2-DG). After removal of the growth medium, cells were washed with HBSS buffer (25 mM D-mannitol, 137 mM NaCl, 5.37 mM KCl, 0.3 mM Na2HPO4, 0.44 mM KH2PO4, 4.17 mM NaHCO3, 1.26 mM CaCl2, 0.8 mM MgSO4 and 10 mM HEPES, pH 7.4) and preincubated at 37 °C for 10 min with 0.5 mL of HBSS buffer. Uptake was initiated by applying HBSS buffer containing [3H]-2-DG (0.2 μCi/mL). The uptake study was performed at 37°C for 20 min. Uptake was terminated by suctioning off the applied solution and immersing the plates in ice-cold HBSS buffer.

To quantify the radioactivity of [3H]-2-DG taken up by the cells, the cells were solubilized in 1% SDS/0.2 N NaOH. The remainder of the sample was mixed with 5 mL of scintillation cocktail (ASCⅡ, Amersham Biosciences Corp., Piscataway, NJ) to measure the radioactivity in a liquid scintillation counter (Packard, 1600TR). The cellular protein content was determined by the method of Lowry with bovine serum

albumin as a standard (Lowry et al., 1951).

**Statistical analyses**

Student's t-test or Welch's t-test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was evaluated using ANOVA followed by the Tukey-Krammer test. Statistical significance was defined as $p<0.05$.

**Results**

***Effects of AICAR injection on MCT4 expression in rat skeletal muscle.***

In agreement with results of other studies, rats injected with AICAR were found to have significantly increased plasma lactic acid level and decreased blood glucose level compared with those in controls (Table 2). Muscle can be divided into three different muscle fiber types as follows: slow oxidative (SO), fast-twitch oxidative glycolytic (FOG) and fast-twitch glycolytic (FG). We used white gastrocnemius (WG; ~0% SO, 16% FOG, and 84% FG), red gastrocnemius (RG; ~30% SO, 62% FOG, and 8% FG), and soleus (SOL; ~84% SO, 16% FOG, and 0% FG) (Buhl et al., 2001). Phospho-acetyl CoA carboxylase (P-ACC), a known substrate of AMPK, in whole muscle homogenate was increased at 1 h after AICAR treatment (Fig. 1A). The greatest increase was observed in WG.

Several groups have reported that AICAR treatment increased muscle GLUT4 mRNA and protein in a fiber type-specific manner (Buhl et al., 2001; Zheng et al., 2001). First, we confirmed the effect of AICAR on GLUT4 mRNA level as a positive

control. As shown in Fig. 1B (a), at 12 h after AICAR treatment, GLUT4 mRNA was significantly elevated in WG. On the other hand, AICAR had no effect on GLUT4 mRNA level in SOL. These results are in agreement with results of other studies showing that AICAR increased GLUT4 expression in fast-twitch fibers. Similarly, AICAR treatment increased MCT4 mRNA level in a fiber type-specific manner (Fig. 1B (b)). Furthermore, AICAR injections for 5 days tended to increase GLUT4 and MCT4 protein levels in WG and RG but not in SOL (Fig. 2). These results suggest that AMPK activation induces MCT4 expression in fast-twitch fibers.

***Effect of AMPK activation on MCT4 expression in RD cells.***

To investigate the mechanism of MCT4 regulation by AMPK activation, we confirmed the effect of AICAR on MCT4 expression in vitro. We used RD cells as an in vitro model of human skeletal muscle. RD cells express a number of muscle-specific proteins (Knudsen et al., 1998). AMPK is composed of a catalytic α-subunit (α1 or α2) and regulatory subunits β (β1 or β2) and γ (γ1, γ2 or γ3). It has been reported that all isoforms of AMPK are present in human skeletal muscle (Wojtaszewski et al., 2005). To determine whether AMPKs are present in RD cells, mRNA expression of AMPKs was investigated by RT-PCR analysis. As shown in Fig. 3A, RT-PCR analysis demonstrated mRNA expression of all isoforms of AMPK. Moreover, Western blotting demonstrated α-subunit expression of AMPK in RD cells (Fig. 3B). Treatment of RD cells with AICAR increased P-ACC level at 1 h, indicating activation of AMPK (Fig. 3C). Since glucose availability for glycolysis is essential to lactic acid production and AMPK activation increases glucose uptake in skeletal muscle, we examined the effect of AICAR on uptake of

2-deoxy-D-glucose (2-DG), which is non-metabolizable glucose analog. As shown in Fig. 3D, AICAR treatment increased 2-DG uptake in RD cells. Next, we examined the effect of AICAR on MCT4 expression in RD cells. As shown in Fig. 4A and B, MCT4 mRNA level was increased from 12 to 48 h. Moreover, AICAR treatment increased MCT4 protein level at 12 and 24 h (Fig. 4C). Treatment with Compound C, an AMPK inhibitor, tended to decrease MCT4 mRNA level, and AICAR-induced MCT4 expression was blocked by Compound C (Fig. 4D). These results suggest that MCT4 expression is regulated by an AMPK-mediated pathway in RD cells.

**Discussion**

AMPK is a key modulator of carbohydrate metabolism and regulates genes involved in muscle metabolism. Pharmacological activation of AMPK by AICAR in skeletal muscle enhances glucose transport and increases expression of GLUT4, hexokinase, mitochondria protein, and several metabolic enzymes. Furthermore, increase in glucose uptake stimulated by AICAR results in increased lactate production in muscle. Transport of lactic acid across the plasma membrane is important for almost all cells. Glycolytic tissues such as white skeletal muscle release lactic acid. Tissues, such as heart and red skeletal muscle, that use lactic acid as a respiratory fuel require the transport of lactic acid into cells. The transport of lactic acid is mediated by MCTs. MCT4 plays a critical role in lactic acid efflux from skeletal muscle and is essential for muscle homeostasis. In rat primary sertoli cells, it has been reported that AICAR treatment increased MCT4 mRNA level and lactic acid production (Galardo et al. 2007). However, the involvement of AMPK in MCT4

regulation in skeletal muscle is not clear. The present study was undertaken to investigate the possible regulation of MCT4 by AMPK in vivo and in vitro.

Firstly, we examined the effect of AICAR on GLUT4 and MCT4 expression in rat skeletal muscle. Similar to previous report, AICAR administration increased GLUT4 mRNA level in a fiber-type specific manner (Fig. 1B (a)). Similarly, AICAR treatment increased MCT4 mRNA level in a fiber-type specific manner (Fig. 1B (b)). Furthermore, AICAR injections for 5 days increased total GLUT4 and MCT4 protein in a fiber-type specific manner (Fig. 2).

In an in vitro study, we used RD cells as in vitro model of human skeletal muscle. The RD cells are tumor cells of skeletal muscle origin affecting children and young adults, and expresse a number of muscle-specific proteins (Knudsen et al., 1998). AMPK is composed of catalytic α-subunit (α1 or α2) and regulatory subunits β (β1 or β2) and γ (γ1, γ2 or γ3). It has been reported that all isoforms of AMPK are present in human skeletal muscle (Wojtaszewski et al., 2005). In RD cells, all isoforms of AMPK (α1, α2, β1, β2, γ1, γ2a and γ3) were present in mRNA level and AMPKα was present in protein level (Fig. 3A and 3B). Furthermore, AMPK activation by AICAR stimulated 2-DG uptake in RD cell (Fig. 3D). AICAR treatment increased MCT4 mRNA and protein levels (Fig. 4A-C). Moreover, AICAR-induced MCT4 expression was blocked by Compound C, an AMPK inhibitor (Fig. 4D). AMPK modulates the expression and function of a number of transcriptional regulators in skeletal muscle (McGee and Hargreaves, 2010). Therefore, AMPK activation may alter promoter activity of the MCT4 gene. However, the detailed downstream pathway of AMPK in the regulation of MCT4 expression remains to be

elucidated. Further investigations to elucidate this pathway are in progress. Among MCT family members, MCT1-MCT4 have been demonstrated experimentally to catalyze the proton-linked transport of lactic acid. Skeletal muscle is known to express MCT1 and MCT4 predominantly. Although MCT2 is expressed abundantly in the liver, it is also present in skeletal muscle. MCT3 has a restricted expression profile, being found on the retinal pigment epithelium and the choroid plexus epithelium. In this study, we also examined the effects of AICAR on MCT1 and MCT2 mRNA levels. In an in vivo study, AICAR treatment increased MCT1 mRNA level in a fiber type-specific manner in agreement with results for MCT4 and GLUT4 (Supplemental Fig. 1). The result suggested that the effect of AICAR on mRNA level is not specific for MCT4. In RD cells, AICAR treatment tended to increase MCT1 mRNA level. However, the alteration of MCT1 expression was modest compared to that of MCT4 expression. On the other hand, AICAR had no effect on MCT2 mRNA level in vivo.

In conclusion, the results of our study indicate the possible involvement of an AMPK-mediated pathway associated with MCT4 expression in skeletal muscle. This is the first study in which the possible regulation of MCT4 in skeletal muscle by AMPK was investigated in vivo and in vitro. Further investigations to clarify the alteration of transport function of MCT4 by induced by AICAR are in progress.

**References**

Buhl ES, Jessen N, Schmitz O, Pedersen SB, Pedersen O, Holman GD, Lund S. Chronic treatment with 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside increases

insulin-stimulated glucose uptake and GLUT4 translocation in rat skeletal muscles in a fiber type-specific manner. Diabetes 2001; 50 (1): 12-7.

Cao H, Hininger-Favier I, Kelly MA, Benaraba R, Dawson HD, Coves S, Roussel AM, Anderson RA. Green tea polyphenol extract regulates the expression of genes involved in glucose uptake and insulin signaling in rats fed a high fructose diet. J Agric Food Chem 2007; 55 (15): 6372-8.

Chisaki I, Kobayashi M, Itagaki S, Hirano T, Iseki K. Liver X receptor regulates expression of MRP2 but not that of MDR1 and BCRP in the liver. Biochim Biophys Acta 2009; 1788 (11): 2396-403.

Enoki T, Yoshida Y, Lally J, Hatta H, Bonen A. Testosterone increases lactate transport, monocarboxylate transporter (MCT) 1 and MCT4 in rat skeletal muscle. J Physiol 2006; 577 (Pt 1): 433-43.

Galardo MN, Riera MF, Pellizzari EH, Cigorraga SB, Meroni SB. The AMP-activated protein kinase activator, 5-aminoimidazole-4-carboxamide-1-b-D-ribonucleoside, regulates lactate production in rat Sertoli cells. J Mol Endocrinol 2007; 39 (4): 279-88.

Holmes BF, Kurth-Kraczek EJ, Winder WW. Chronic activation of 5'-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. J Appl Physiol

1999; 87 (5): 1990-5.

Holmes BF, Sparling DP, Olson AL, Winder WW, Dohm GL. Regulation of muscle GLUT4 enhancer factor and myocyte enhancer factor 2 by AMP-activated protein kinase. Am J Physiol Endocrinol Metab 2005; 289 (6): E1071-6.

Knudsen ES, Pazzagli C, Born TL, Bertolaet BL, Knudsen KE, Arden KC, Henry RR, Feramisco JR. Elevated cyclins and cyclin-dependent kinase activity in the rhabdomyosarcoma cell line RD. Cancer Res 1998; 58 (9): 2042-9.

Kobayashi M, Chisaki I, Narumi K, Hidaka K, Kagawa T, Itagaki S, Hirano T, Iseki K. Association between risk of myopathy and cholesterol-lowering effect: a comparison of all statins. Life Sci 2008a; 82 (17-18): 969-75.

Kobayashi M, Fujita I, Itagaki S, Hirano T, Iseki K. Transport mechanism for L-lactic acid in human myocytes using human prototypic embryonal rhabdomyosarcoma cell line (RD cells). Biol Pharm Bull 2005; 28 (7): 1197-201.

Kobayashi M, Kagawa T, Narumi K, Itagaki S, Hirano T, Iseki K. Bicarbonate supplementation as a preventive way in statins-induced muscle damage. J Pharm Pharm Sci 2008b; 11 (1): 1-8.

Kobayashi M, Otsuka Y, Itagaki S, Hirano T, Iseki K. Inhibitory effects of statins on human monocarboxylate transporter 4. Int J Pharm 2006; 317 (1): 19-25.

Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, Winder WW. 5' AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. Diabetes 1999; 48 (8): 1667-71.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951; 193 (1): 265-75.

McGee SL, Hargreaves M. AMPK-mediated regulation of transcription in skeletal muscle. Clin Sci (Lond) 2010; 118 (8): 507-18.

Meredith D, Christian HC. The SLC16 monocarboxylate transporter family. Xenobiotica 2008; 38 (7-8): 1072-106.

Miyamoto L, Toyoda T, Hayashi T, Yonemitsu S, Nakano M, Tanaka S, Ebihara K, Masuzaki H, Hosoda K, Ogawa Y, Inoue G., Fushiki T, Nakao K. Effect of acute activation of 5'-AMP-activated protein kinase on glycogen regulation in isolated rat skeletal muscle. J Appl Physiol. 2007; 102 (3): 1007-13.

Ojuka EO, Jones TE, Nolte LA, Chen M, Wamhoff BR, Sturek M, Holloszy JO.

Regulation of GLUT4 biogenesis in muscle: evidence for involvement of AMPK and Ca2+. Am J Physiol Endocrinol Metab. 2002; 282 (5): E1008-13.

Steinberg GR, Kemp BE. AMPK in Health and Disease. Physiol Rev 2009; 89 (3): 1025-78.

Stoppani J, Hildebrandt AL, Sakamoto K, Cameron-Smith D, Goodyear LJ, Neufer PD. AMP-activated protein kinase activates transcription of the UCP3 and HKII genes in rat skeletal muscle. Am J Physiol Endocrinol Metab 2002; 283 (6): E1239-48.

Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem. 2006; 281 (14): 9030-7.

Wilson MC, Jackson VN, Heddle C, Price NT, Pilegaard H, Juel C, Bonen A, Montgomery I, Hutter OF, Halestrap AP. Lactic acid efflux from white skeletal muscle is catalyzed by the monocarboxylate transporter isoform MCT3. J Biol Chem 1998; 273 (26): 15920-6.

Winder WW, Holmes BF, Rubink DS, Jensen EB, Chen M, Holloszy JO. Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. J Appl Physiol 2000; 88 (6): 2219-26.

Wojtaszewski JF, Birk JB, Frøsig C, Holten M, Pilegaard H, Dela F. 5'AMP activated protein kinase expression in human skeletal muscle: effects of strength training and type 2 diabetes. J Physiol 2005; 564 (Pt 2): 563-73.

Zheng D, MacLean PS, Pohnert SC, Knight JB, Olson AL, Winder WW, Dohm GL. Regulation of muscle GLUT-4 transcription by AMP-activated protein kinase. J Appl Physiol 2001; 91 (3): 1073-83.

**Figure legends**

Fig. 1. Total phospho-ACC (P-ACC) levels (A) and mRNA levels (B) in muscles of rats treated with AICAR. (A) Rats were injected subcutaneously with AICAR (0.5 mg/g) or saline (control). White gastrocnemius (WG), red gastrocnemius (RG), and soleus (SOL) were removed from rats 1 h after AICAR injection. Data shown are typical results of three independent experiments. (B) Rats were injected subcutaneously with AICAR (0.5 mg/g) or saline. Muscles were removed from rats 12 h after AICAR injection. Each column represents the mean with S.D. of 5-6 measurements. *****; significantly different from the control group at $p<0.05$.

Fig. 2. Total GLUT4 (A) and MCT4 (B) protein levels in muscles of rats treated with AICAR. Rats were injected subcutaneously with AICAR (0.5 mg/g) or saline for 5 days in succession. Muscles were removed from rats 24 h after the last injection. Each column

represents the mean with S.D. of 3 measurements.

Fig. 3. Expression of AMPKs (A, B), and effects of AICAR on P-ACC level (C) and 2-DG uptake (D). (A) Expression of AMPK mRNA. RT-PCR was performed with total RNA isolated from RD cells. Specific primers were used to detect genes as described in Materials and methods. (B) Expression of AMPKα protein. AMPKα protein was detected by Western blotting. (C) Effect of AICAR on P-ACC level in RD cells. RD cells were treated with AICAR (0.1-1 mM) for 1 h. Data shown are typical results of three independent experiments. (D) Effect of AICAR on 2-DG uptake in RD cells. RD cells were treated with AICAR (0.1-1 mM) for 12 h. 2-DG uptake by RD cells was measured at pH 7.4 for 20 min. Each column represents the mean with S.D. of 6-12 measurements. *****; significantly different from the control at $p<0.05$.

Fig. 4. Effects of AMPK activation on MCT4 mRNA level and protein level in RD cells. (A) Time course of the effect of AICAR on MCT4 mRNA level. RD cells were treated with AICAR (0.5 mM) for various periods (6, 12, 24 and 48 h). Each column represents the mean with S.D. of 3-9 measurements. *****; significantly different from the control at $p<0.05$. (B) Detection of the expression of MCT4 by RT-PCR. RD cells were treated with AICAR (0.5 mM) for 24 h. (C) Effect of AICAR on total MCT4 protein level. RD cells were treated with AICAR (0.1-1 mM) for 12 or 24 h. (D) AMPK inhibition prevents AICAR-induced up-regulation of MCT4 mRNA in RD cells. RD cells were treated for 12 h with AICAR (0.5 mM) alone or in combination with Compound C (20 μM). Each column represents the

mean with S.D. of 3-9 measurements. *****; significantly different from the control at $p<0.05$. †; significantly different from AICAR alone at $p<0.05$.

Supplemental Fig. 1. Effects of AICAR on MCT1 and MCT2 mRNA levels. (a) Rats were injected subcutaneously with AICAR (0.5 mg/g) or saline. Muscles were removed from rats 12 h after AICAR injection. Each column represents the mean with S.D. of 4-5 measurements. *; significantly different from the control group at $p<0.05$. (b) RD cells were treated with AICAR (0.5 mM) for 24 h. Each column represents the mean with S.D. of 6 measurements. *; significantly different from the control at $p<0.05$.

Table 1 Primer information

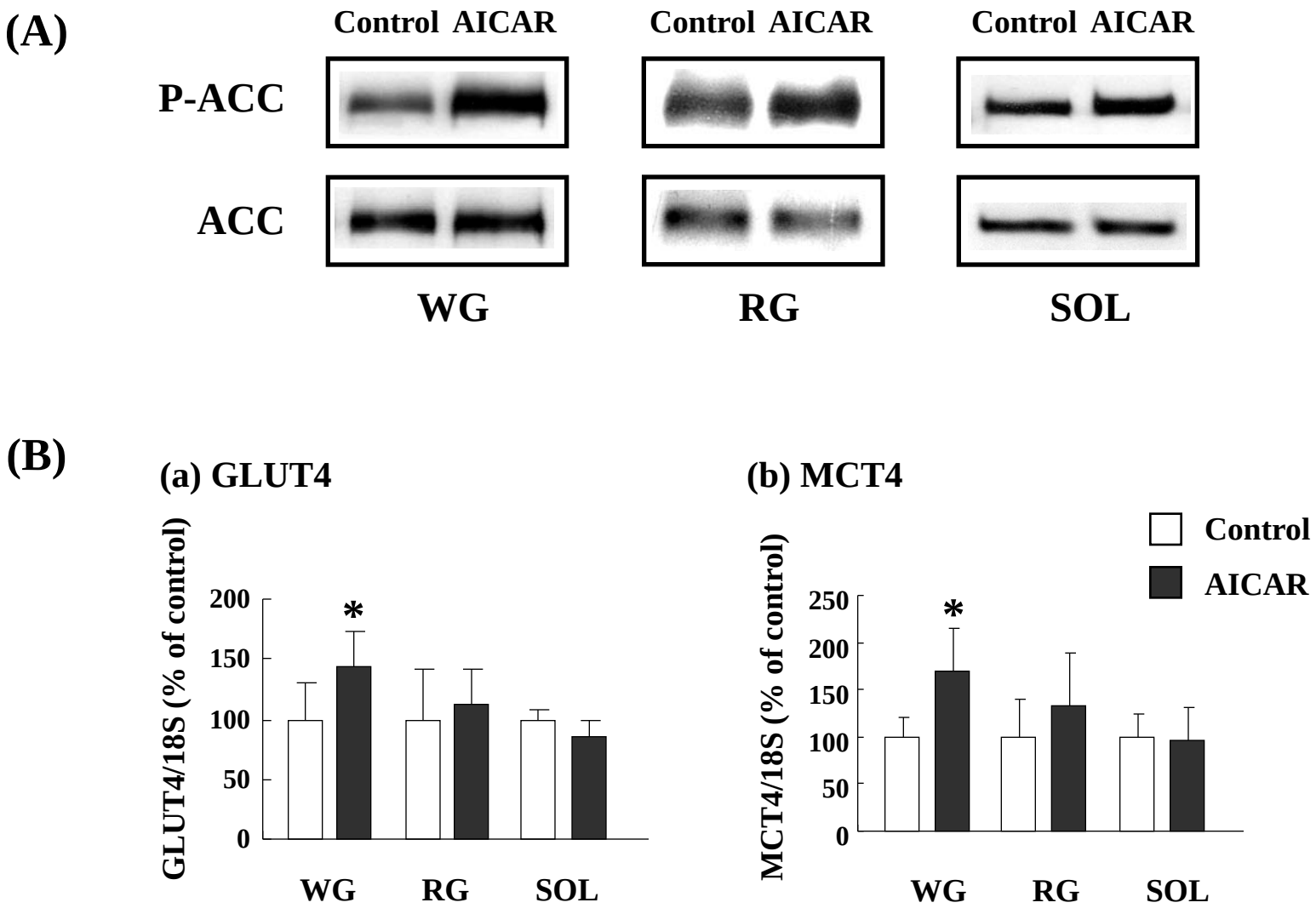
| Gene | | Primer sequence | Product size (bp) | Annealing temperature (°C) | References |
|---|---|---|---|---|---|
| *Human* | | | | | |
| 18S | Forward | 5’-cggctaccacatccaaggaa-3’ | 187 | 58-60 | |
| | Reverse | 5’-gctggaattaccgcggct-3’ | | | |
| AMPKα1 | Forward | 5’-cagggactgctactccacagaga-3’ | 85 | 58 | Wojtaszewski et al., 2005 |
| | Reverse | 5’-ccttgagcctcagcatctgaa-3’ | | | |
| AMPKα2 | Forward | 5’-caactgcagagagccattcactt-3’ | 76 | 58 | Wojtaszewski et al., 2005 |
| | Reverse | 5’-ggtgaaactgaagacaatgtgctt-3’ | | | |
| AMPKβ1 | Forward | 5’-ctttaatggtggattcccaaaagt-3’ | 87 | 58 | Wojtaszewski et al., 2005 |
| | Reverse | 5’-agacgtagggctcctgatggt-3’ | | | |
| AMPKβ2 | Forward | 5’-tggaaagttctgagacatcttgtagaga-3’ | 87 | 58 | Wojtaszewski et al., 2005 |
| | Reverse | 5’-cctcagatcgaaacgcatacatt-3’ | | | |
| AMPKγ1 | Forward | 5’-gttccccaagccagagttca-3’ | 86 | 58 | Wojtaszewski et al., 2005 |
| | Reverse | 5’-tggtagtgcgaaccatagcaat-3’ | | | |
| AMPKγ2a | Forward | 5’-gcggttatggacaccaagaaga-3’ | 119 | 58 | Wojtaszewski et al., 2005 |
| | Reverse | 5’-aaggagctcaggtccggaat-3’ | | | |
| AMPKγ3 | Forward | 5’-ccagatctacatgcgcttca-3’ | 167 | 58 | Present study |
| | Reverse | 5’-agctctgcttcttgctgtcc-3’ | | | |
| MCT4 | Forward | 5’-attggcctggtgctgctgatg-3’ | 243 | 60 | Ullah et al., 2006 |
| | Reverse | 5’-cgagtctgcaggaggcttgtg-3’ | | | |
| *Rat* | | | | | |
| 18S | Forward | 5’-gttggttttcggaactgaggc-3’ | 204 | 56-58 | |
| | Reverse | 5’-gtcggcatcgtttatggtcg-3’ | | | |
| GLUT4 | Forward | 5’-caactggacctgtaacttcatcgt-3’ | 87 | 56 | Cao et al., 2007 |
| | Reverse | 5’-acggcaaatagaaggaagacgta-3’ | | | |
| MCT4 | Forward | 5’-gggtcatcactggcttgggt-3’ | 123 | 58 | Enoki et al., 2006 |
| | Reverse | 5’-ggaacacgggactgcctgc-3’ | | | |

Table 2 Blood glucose and plasma lactic acid concentration of rats treated with AICAR for 1 h.

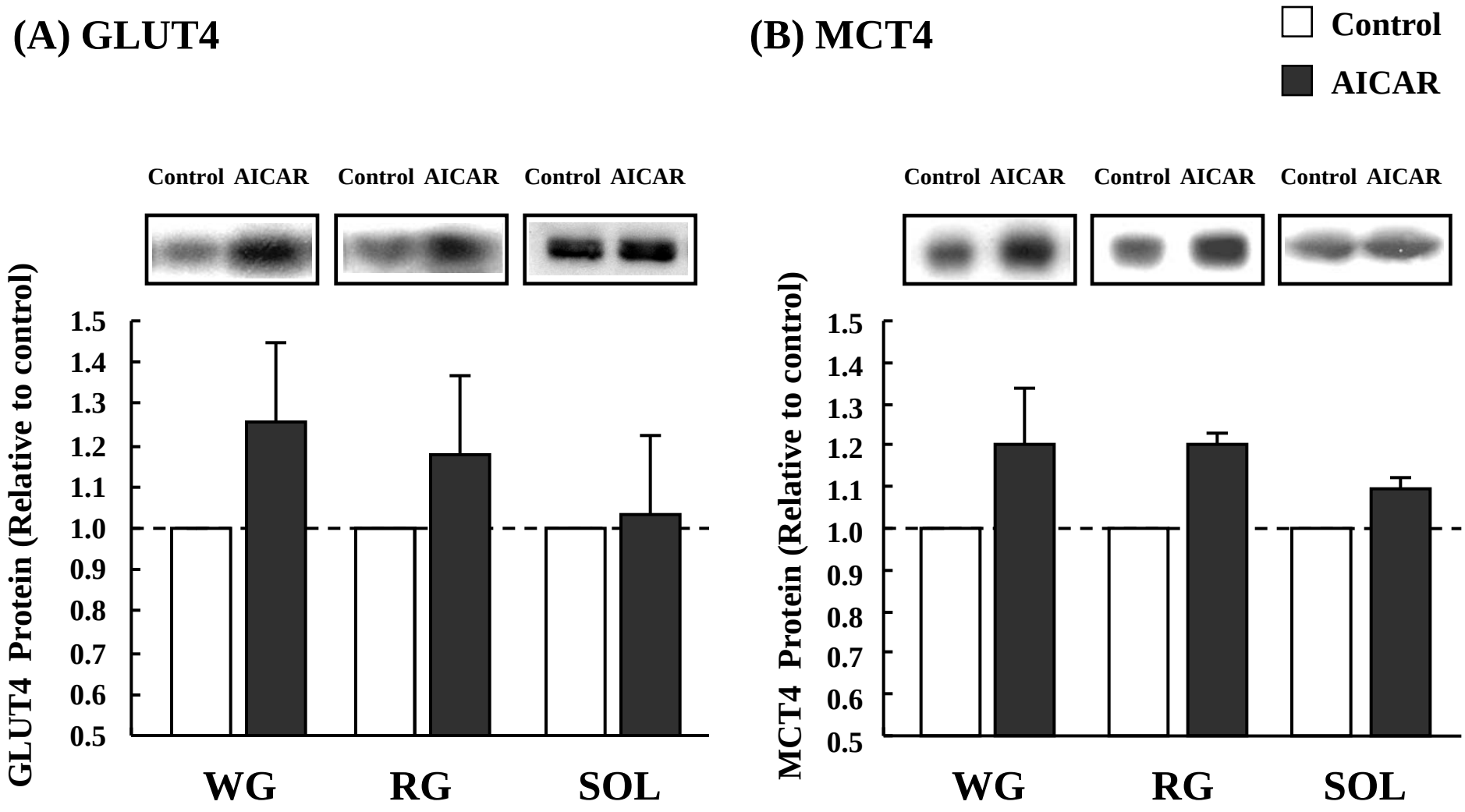
| | Control | AICAR |
|---|---|---|
| Blood glucose (mg/dL) | 137 ± 19 | 65 ± 12***** |
| Blood lactate (mM) | 1.8 ± 0.6 | 5.3 ± 0.6***** |

Rats were injected subcutaneously with AICAR (0.5 mg/g) or saline (control). Values are means ± S.D. of 3 measurements. *****; significantly different from the control at $p<0.05$.

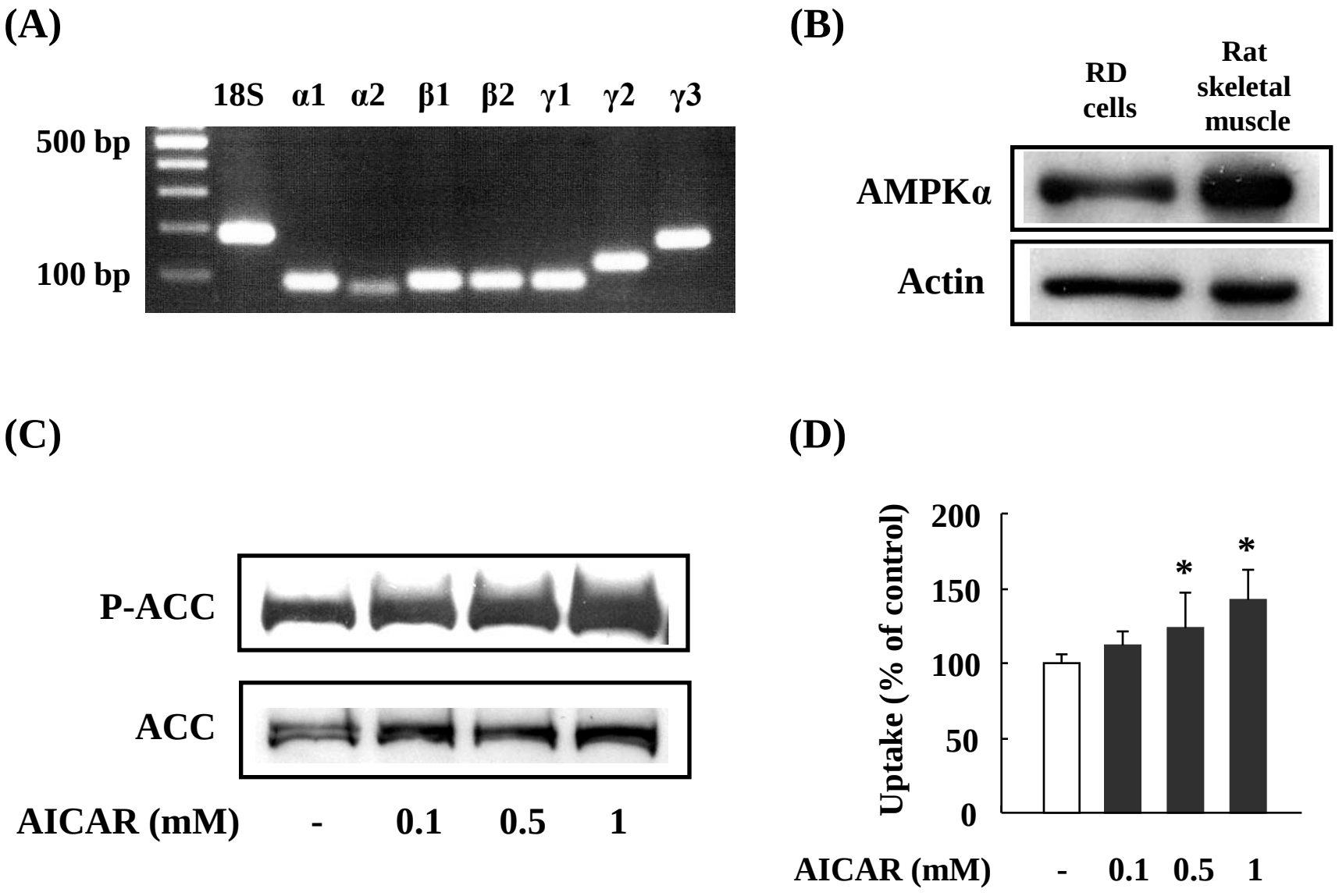
# Fig. 1


(A)
Control AICAR
Control AICAR
Control AICAR
P-ACC
ACC
WG
RG
SOL
(B)
(a) GLUT4
GLUT4/18S (% of control)
200
150
100
50
0
*
WG
RG
SOL
(b) MCT4
Control
AICAR
MCT4/18S (% of control)
250
200
150
100
50
0
*
WG
RG
SOL

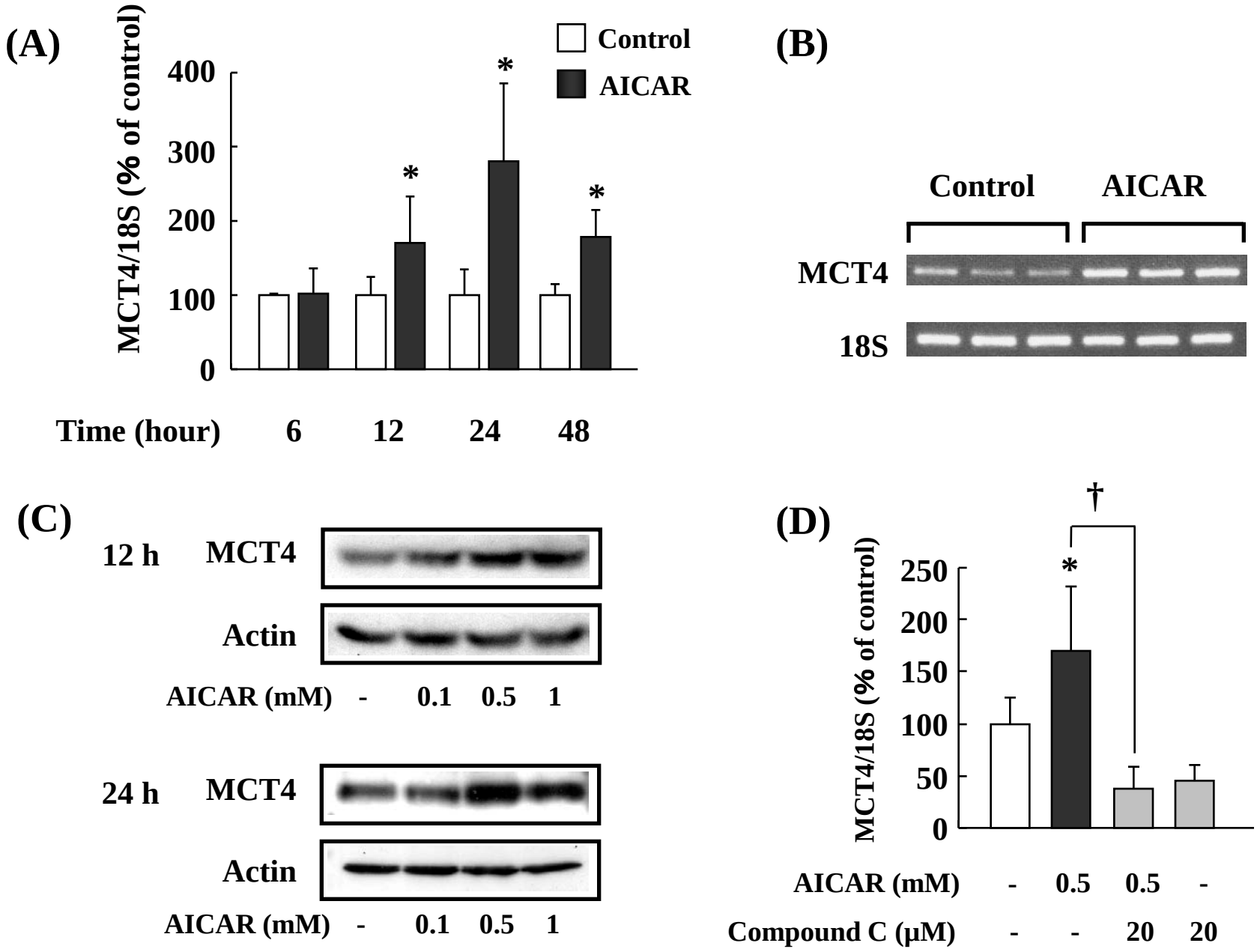
# Fig. 2


(A) GLUT4
(B) MCT4
Control
AICAR
Control AICAR Control AICAR Control AICAR
GLUT4 Protein (Relative to control)
MCT4 Protein (Relative to control)
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
WG
RG
SOL

# Fig. 3

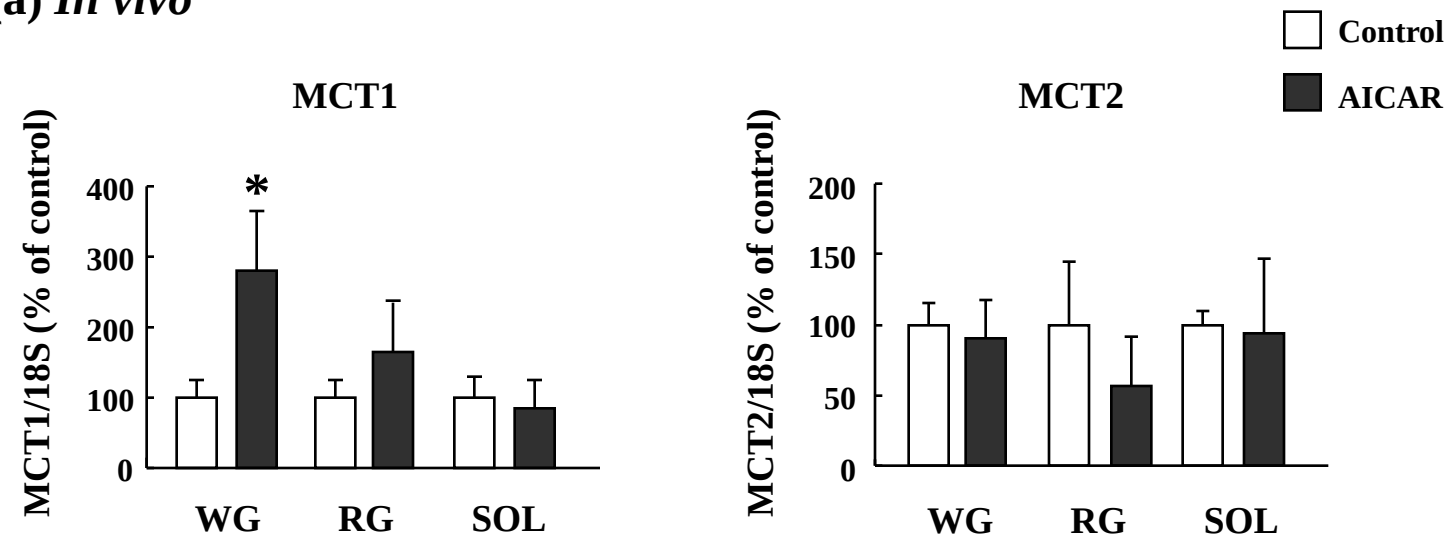

(A)
18S
α1
α2
β1
β2
γ1
γ2
γ3
500 bp
100 bp
(B)
RD cells
Rat skeletal muscle
AMPKα
Actin
(C)
P-ACC
ACC
AICAR (mM)
-
0.1
0.5
1
(D)
Uptake (% of control)
200
150
100
50
0
*
*
AICAR (mM)
-
0.1
0.5
1

# Fig. 4


(A)
Control
AICAR
MCT4/18S (% of control)
400
300
200
100
0
*
*
*
Time (hour)
6
12
24
48
(B)
Control
AICAR
MCT4
18S
(C)
12 h
MCT4
Actin
AICAR (mM)
-
0.1
0.5
1
24 h
MCT4
Actin
AICAR (mM)
-
0.1
0.5
1
(D)
†
*
MCT4/18S (% of control)
250
200
150
100
50
0
AICAR (mM)
-
0.5
0.5
-
Compound C (μM)
-
-
20
20

# Supplemental Fig. 1

## (a) *In vivo*




## (b) *In vitro*

