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Author(s)	Egawa, T; Hamada, T; Ma, X; Karaike, K; Kameda, N; Masuda, S; Iwanaka, N; Hayashi, T
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Caffeine activates preferentially α1-isoform of 5'AMP-activated protein kinase in rat skeletal muscle

Tatsuro Egawa[1], Taku Hamada[2], Xiao Ma[1], Kouhei Karaike[1], Naoko Kameda[1], Shinya Masuda[1], Nobumasa Iwanaka[1], and Tatsuya Hayashi[1]

1 Laboratory of Sports and Exercise Medicine, Graduate School of Human and Environmental Studies, Kyoto University, Kyoto, 606-8501, Japan

2 Department of Health and Sports Management, Osaka University of Health and Sport Sciences, Osaka, 590-0496, Japan

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Address correspondence to:

Tatsuya Hayashi, M.D., Ph.D.

Laboratory of Sports and Exercise Medicine, Graduate School of Human and Environmental Studies, Kyoto University, Yoshida-nihonmatsu-cho, Sakyo-ku, Kyoto, 606-8501, Japan

Telephone, Fax: +81-75-753-6640, E-mail: tatsuya@kuhp.kyoto-u.ac.jp

Abstract

Aim: Caffeine activates 5′AMP-activated protein kinase (AMPK), a signalling intermediary implicated in the regulation of glucose, lipid, and energy metabolism in skeletal muscle. Skeletal muscle expresses two catalytic α subunits of AMPK, α1 and α2, but the isoform specificity of caffeine-induced AMPK activation is unclear. The aim of this study was to determine which α isoform is preferentially activated by caffeine in vitro and in vivo using rat skeletal muscle.

Methods: Rat epitrochlearis muscle was isolated and incubated in vitro in the absence or presence of caffeine. In another experiment, the muscle was dissected after intravenous injection of caffeine. Isoform-specific AMPK activity, the phosphorylation status of AMPKα Thr^{172} and acetyl-CoA carboxylase (ACC) Ser^{79}, the concentrations of ATP, phosphocreatine (PCr), and glycogen, and 3-*O*-methyl-D-glucose (3MG) transport activity were estimated.

Results: Incubation of isolated epitrochlearis muscle with 1 mM of caffeine for 15 min increased AMPKα1 activity, but not AMPKα2 activity; concentrations of ATP, PCr and glycogen were not affected. Incubation with 3 mM of caffeine activated AMPKα2 and reduced PCr and glycogen concentrations. Incubation with 1 mM of caffeine increased the phosphorylation of AMPK and ACC and enhanced 3MG transport. Intravenous injection of caffeine (5 mg kg^{-1}) predominantly activated AMPKα1 and increased 3MG transport without affecting energy status.

Conclusion: Our results suggest that of the two α isoforms of AMPK, AMPKα1 is predominantly activated by caffeine via an energy-independent mechanism and that activation of AMPKα1 increases glucose transport and ACC phosphorylation in

skeletal muscle.

Keywords: acetyl-CoA carboxylase, glucose transport, phosphocreatine, glycogen, Ca^{2+}/calmodulin-dependent protein kinase

Introduction

5′AMP-activated protein kinase (AMPK) is emerging as a signalling intermediary that controls the use of glucose and fatty acids in skeletal muscle. AMPK has been identified as part of the mechanism responsible for exercise-stimulated insulin-independent glucose transport (reviewed in Kahn et al., 2005, Hardie et al. 2006, Fujii et al. 2006) and is also implicated in GLUT4 expression (Zheng et al. 2001, Nakano et al. 2006), glycogen regulation (Jørgensen et al. 2004a, Miyamoto et al. 2007), fatty acid oxidation (Winder and Hardie, 1996, Vavvas et al. 1997), mitochondrial biogenesis (Jager et al. 2007, Garcia-Roves et al. 2008), and enhanced insulin sensitivity (Fiedler et al. 2001, Nakano et al. 2006, Tanaka et al. 2007).

Caffeine (1,3,7-trimethylxanthine) is a xanthine alkaloid compound that acts as a stimulant of skeletal muscle metabolism. Caffeine increases the rate of glucose transport in the absence of insulin in rodent skeletal muscles (Wright et al. 2004, Cantó et al. 2006, Jensen et al. 2007a, Egawa et al. 2009), enhances GLUT4 mRNA and protein expression in cultured myotubes (Ojuka et al. 2002, Mukwevho et al. 2008), and promotes fatty acid metabolism in perfused rat skeletal muscles (Raney and Turcotte, 2008). AMPK is considered part of the mechanism leading to the metabolic activation by caffeine (Wright et al. 2004, Jensen et al. 2007a, Raney and Turcotte 2008, Egawa et al. 2009).

AMPK is a heterotrimeric kinase comprising a catalytic α subunit and two regulatory subunits, β and γ. Two distinct α isoforms (α1 and α2) exist in skeletal muscle, and the Thr^{172} residue in both α1 and α2 catalytic subunits is the primary phosphorylation site responsible for AMPK activation. We demonstrated recently

that caffeine stimulation (≥3 mM for ≥15 min) increases AMPKα Thr^{172} phosphorylation with corresponding increases in both AMPKα1 and α2 activities in incubated rat epitrochlearis and soleus muscles (Egawa et al. 2009). This activation is accompanied by an increased rate of insulin-independent glucose transport, enhanced phosphorylation of acetyl-coenzyme A carboxylase (ACC) Ser^{79}, and decreased phosphocreatine (PCr) concentration (Egawa et al. 2009). These results suggest that caffeine and exercise have similar actions in stimulating skeletal muscle AMPK by reducing the intracellular energy status.

In contrast, Jensen et al. (Jensen et al. 2007a) demonstrated that caffeine stimulation (3 mM for 15 min) acutely stimulates AMPKα1 activity but not α2 activity in incubated mouse and rat soleus muscles. Interestingly, Jensen et al. did not detect any changes in energy status in mouse soleus muscle. Similarly, we previously demonstrated that AMPKα1, but not AMPKα2, is activated in rat epitrochlearis muscle incubated and treated with H_2O_2 and hypoxanthine/xanthine oxidase in the absence of an increase in AMP or a decrease in PCr concentration (Toyoda et al. 2006). We also showed that AMPKα1 is activated in low-intensity contracting muscle, in which AMP concentration is not elevated, whereas AMPKα1 and AMPKα2 are activated in high-intensity contracting muscle, in which the AMP concentration is significantly higher than the resting value (Toyoda et al. 2006).

AMPK is activated in response to energy-depleting stresses such as muscle contraction, hypoxia, and inhibition of oxidative phosphorylation (Hayashi et al. 2000). AMPKα2 is more dependent on AMP than is AMPKα1 with respect to allosteric activation by AMP and covalent activation by upstream kinases (Salt et al. 1998, Stein et al. 2000), indicating that AMPKα2 is more sensitive to energy

depletion than is AMPKα1. We hypothesized that caffeine can act on skeletal muscle and preferentially stimulate AMPKα1 in the absence of energy deprivation. To test this hypothesis, we investigated the effects of caffeine on AMPKα1 and α2 activities in rat skeletal muscles stimulated by caffeine in vitro and in vivo.

Materials and Methods

Caffeine treatment in vitro

Male Sprague-Dawley rats weighing 100 g were obtained from Shimizu Breeding Laboratories (Kyoto, Japan). Animals were housed in an animal room maintained at 22-24°C with a 12:12-h light-dark cycle and fed a standard laboratory diet (Certified Diet MF; Oriental Koubo, Tokyo, Japan) and water ad libitum. Rats were fasted overnight before the experiments and were randomly assigned to the experimental groups. All protocols for animal use and euthanasia followed the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences (Physiological Society of Japan) in accordance with international guidelines, and were reviewed and approved by the Kyoto University Graduate School of Human and Environmental Studies and Kyoto University Radioisotope Research Center.

Muscles were treated as we described previously (Hayashi et al. 1998, Toyoda et al. 2004). Rats were sacrificed by cervical dislocation without anaesthesia, and the epitrochlearis muscles of each side were rapidly removed. Both ends of each muscle were tied with sutures (silk 3-0; Natsume Seisakusho, Tokyo, Japan) and the muscles were mounted on an incubation apparatus with a tension set to 0.5 g. The buffers were continuously gassed with 95% O_2-5% CO_2 and maintained at 37°C. Muscles were preincubated in 7 mL of Krebs-Ringer bicarbonate buffer (KRB) (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.6 mM $NaHCO_3$) containing 2 mM pyruvate (KRBP) for 40 min. The muscles were then incubated for 15 min in 7 ml of fresh buffer containing various concentrations (0-3

mM) of caffeine. The muscles were then used for the measurement of glucose transport, or immediately frozen in liquid nitrogen and subsequently analyzed for ATP, PCr, and glycogen concentrations, and isoform-specific AMPK activity, or used for Western blot analysis.

Caffeine treatment in vivo

We chose male Sprague-Dawley rats weighing 200 g for easy administration of caffeine into the tail vein without anaesthesia. For the dose-response changes in AMPK and ACC phosphorylation, caffeine was dissolved in saline and injected intravenously at various concentrations (0-25 mg kg^{-1} body weight). The injection volume was 1 ml kg^{-1} body weight. Sixty minutes after the injection rats were anaesthetized with intraperitoneal administration of pentobarbital sodium (50 mg kg^{-1} body weight), and then epitrochlearis muscle was rapidly dissected and immediately frozen in liquid nitrogen. Some controls and samples with 5 mg kg^{-1} caffeine stimulation were subjected to the measurement of isoform-specific AMPK activity and glucose transport. For the time-course changes in AMPK and ACC phosphorylation, caffeine (5 mg kg^{-1} body weight) was injected intravenously into the tail vain without anaesthesia. Muscles were dissected under anaesthesia at 30, 60 and 120 min after injection, and then immediately frozen in liquid nitrogen. Control samples were collected immediately after saline injection under anaesthesia. Some controls and samples dissected at 60 min after caffeine injection were subjected to the measurement of ATP, PCr, and glycogen concentrations.

Blood sample analysis

Blood samples were collected from the tail vein using heparinized glass tube at 0 and 60 min after intravenous saline or caffeine injection. Plasma levels for glucose (Glutest-Ace; Sanwa Kagaku Kenkyusyo, Nagoya, Japan), insulin (rat insulin ELISA kit; Morinaga, Yokohama, Japan), caffeine (caffeine/pentoxifylline ELISA kit; Neogen, Lexington, KY, USA) were measured.

Western blot analysis

Sample preparation and Western blot analysis was performed as we described previously (Toyoda et al. 2004). Muscles were homogenized in ice-cold lysis buffer (1:40 wt vol^{-1}) containing 20 mM Tris·HCl (pH 7.4), 1% Triton X, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 2 mM dithiothreitol, 4 mg L^{-1} leupeptin, 50 mg L^{-1} trypsin inhibitor, 0.1 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride and centrifuged at 16,000 *g* for 40 min at 4°C. Lysates were solubilized in Laemmli's sample buffer containing mercaptoethanol and boiled.

The samples (10 μg of protein) were separated on either 10% polyacrylamide gel for AMPK, Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) or 7.5% gel for ACC. Proteins were then transferred to polyvinylidene difluoride membranes (PolyScreen; PerkinElmer, Wellesley, MA, USA) at 100 V for 1 h. Membranes were blocked for 1h at room temperature in TBS-T (TBS with 0.1% Tween 20) containing 5% nonfat dry milk and were then incubated over night at 4°C with phosphospecific AMPKα Thr^{172} (#2531; Cell Signaling Technology, Danvers, MA, USA), AMPKα (#2532; Cell Signaling Technology), CaMKII $Thr^{286\,(287)}$ (#3361; Cell Signaling Technology,

Danvers, MA, USA), phosphospecific ACC Ser^{79} (#07-303; Upstate Biotechnology, Lake Placid, NY, USA), ACC (#3662; Cell Signaling Technology) diluted 1:1000 or CaMKI Thr^{177} (sc-28438-R; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:250. The membranes were then washed, incubated for 1h at room temperature with anti-mouse IgG antibody (GE Healthcare, Buckinghamshire, UK) diluted 1:2500, and developed with enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, UK). The intensity of the signals was quantified using Multi-Analyst software (Bio-Rad, Hercules, CA, USA). The mean intensity of control samples in each membrane was used as reference for controlling gel-to-gel variation. Equal protein loading and transfer was confirmed by Coomassie brilliant blue staining of the membranes.

Isoform-specific AMPK activity assay

We have raised AMPK polyclonal antibodies in rabbit against isoform-specific peptides derived from the amino acid sequences of rat α1 (residues 339–358) or α2 (residues 490–514) (Toyoda et al. 2004). AMPK activity assay was performed as we described previously (Hayashi et al. 2000, Toyoda et al. 2004). Muscles were homogenized as described in *Western blot analysis*, and resultant supernatants (100 μg of protein) were immunoprecipitated with the α1 or α2 AMPK antibody and protein A-Sepharose beads (Amersham Biosciences, Uppsala, Sweden). Immunoprecipitates were washed twice both in lysis buffer and in wash buffer (240 mM HEPES and 480 mM NaCl). Kinase reactions were performed in 40 mM HEPES (pH 7.0), 0.1 mM SAMS peptide (Hayashi et al. 2000, Toyoda et al. 2004), 0.2 mM AMP, 80 mM NaCl, 0.8 mM dithiothreitol, 5 mM $MgCl_2$, 0.2 mM ATP (2

μCi of [γ-^{32}P] ATP/sample) (PerkinElmer, Wellesley, MA, USA), in a final volume of 40 μl for 20 min at 30°C. At the end of the reaction, a 15-μl aliquot was removed and spotted onto Whatman P81 paper (Whatman International, Maidstone, UK). The papers were washed six times in 1% phosphoric acid and once in acetone. ^{32}P incorporation was quantitated with a scintillation counter, and kinase activity was expressed as fold increases relative to the control samples.

ATP and PCr assay

ATP and PCr concentrations were measured fluorometrically in perchloric acid extracts of epitrochlearis muscle according to the method of Lowry and Passonneau (Lowry and Passonneau, 1972). In brief, each frozen muscle was homogenized in 0.2 M $HClO_4$ (3:25 w/v) in an ethanol-dry ice bath (-20 ~ -30°C) with careful handling of the homogenate being kept unfrozen. After a centrifuge at 16,000 *g* for 2 min at -9°C, the supernatant of the homogenate was neutralized with a solution of 2 M KOH, 0.4 M KCl and 0.4 M imidazole and then centrifuged at 16,000 *g* for 2 min at -9°C, and then subjected to enzymatic analysis (Lowry and Passonneau, 1972). ATP and PCr concentrations were expressed as nanomoles per milligram wet weight of muscle.

Glycogen assay

Glycogen concentration was assayed as we described previously (Nakano et al. 2006, Miyamoto et al. 2007). Each frozen muscle was digested in 1 M NaOH at 85°C for 10 min, and the digestates were neutralized with HCl. The glycogen in the digestates was hydrolyzed by incubated in 2 M HCl for 2 h at 85°C. The digestates

were neutralized with NaOH, and the concentration of hydrolyzed glucose residues was measured enzymatically using Glucose CII Test (Wako, Osaka, Japan). Glycogen concentration was expressed as nanomoles of glucose per milligram wet weight of muscle.

3-O-methyl-D-glucose (3MG) transport

3MG transport assay was performed as we described previously (Hayashi et al. 1998, Toyoda et al. 2004, Nakano et al. 2006). To measure 3MG transport after in vitro caffeine incubation, muscles were transferred to 2 mL of KRB containing 1 mM [^{3}H]3-MG (1.5 μCi mL^{-1}) (American Radiolabeled Chemicals, St. Louis, MO) and 7 mM D-[1-^{14}C] mannitol (0.3 μCi mL^{-1}) (American Radiolabeled Chemicals, St. Louis, MO) at 30°C and further incubated for 10 min. To measure 3MG transport after an injection of caffeine, muscles were incubated for 10 min in 7 mL of KRBP. Muscles were then transferred to 2 mL of transport buffer and incubated for 10 min. The muscles were then blotted onto filter paper, trimmed, frozen in liquid nitrogen, and stored at –80°C. Each frozen muscle was weighed and processed by incubating them in 300 μl of 1 M NaOH at 80°C for 10 min. Digestates were neutralized with 300 μl of 1 M HCl, and particulates were precipitated by centrifugation at 20,000 *g* for 2 min. Radioactivity in aliquots of the digested protein was determined by liquid scintillation counting for dual labels, and the extracellular and intracellular spaces were calculated (Young et al. 1986).

Statistical analysis

Results are presented as means ± SE. One-way ANOVA (Figs 1-7) or two-way

ANOVA (Table 1 and 2) was used to estimate the variance of the dose-response and time-course studies, and statistical significance of difference between control and caffeine-treated groups was evaluated by Scheffe's post hoc test. Student's t test was used to examine the significant differences between control and caffeine-treated groups (Figs 8 and 9). Differences between groups were considered statistically significant at $P < 0.05$.

Results

Caffeine preferentially increased AMPKα1 activity in skeletal muscle incubated in vitro

Our previous study demonstrated that caffeine stimulation in vitro (3 mM for 15 min) activates both AMPKα1 and AMPKα2 in rat epitrochlearis muscle (Egawa et al. 2009). To investigate the dose dependency of isoform-specific AMPK activity stimulated by caffeine at concentrations lower than 3 mM, isolated epitrochlearis muscles were incubated and stimulated with 0.1, 0.5, 1, and 3 mM of caffeine for 15 min. A caffeine concentration of 1 mM clearly increased AMPKα1 by twofold ($P < 0.05$), but AMPKα2 activation required a caffeine concentration of 3 mM (Fig. 1).

Activation of AMPKα1 by caffeine was not associated with energy deprivation

AMPK is activated in response to energy deprivation by allosteric modification and through a mechanism involving phosphorylation of the α subunit by upstream kinases (Hardie and Carling, 1997, Kemp et al. 1999). Our previous study using rat epitrochlearis and soleus muscles demonstrated that in vitro stimulation with 3 mM of caffeine for 15 min decreases PCr concentration by 23% (Egawa et al. 2009). To determine whether caffeine-induced AMPKα1 activation is associated with energy deprivation, we measured concentrations of ATP, PCr, and glycogen in epitrochlearis muscle after stimulation with caffeine for 15 min. The concentrations of ATP, PCr and glycogen were not affected by stimulation with 1 mM of caffeine, but concentrations of PCr and glycogen were significantly decreased by stimulation with 3 mM of caffeine (Fig. 2).

Activation of AMPKα1 by caffeine was associated with increased AMPK and

ACC phosphorylation, and insulin-independent glucose transport

To confirm whether caffeine activates AMPK, we measured the degree of phosphorylation of AMPKα Thr^{172} by Western blot analysis using a phosphospecific antibody in muscle homogenates after stimulation with caffeine. Incubation with caffeine at 1 and 3 mM clearly increased phosphorylation of AMPKα Thr^{172} without changing the total amount of AMPKα (Fig. 3A). ACC is a downstream target of AMPK in skeletal muscle, and the phosphorylation of the Ser^{79} site of ACC reflects the total AMPK activity (Davies et al. 1990, Park et al. 2002). The marked phosphorylation of ACC paralleled the increase in AMPK phosphorylation (Fig. 3B). We also investigated whether the activation of AMPK in skeletal muscle by caffeine is associated with enhanced glucose transport. In the absence of insulin, incubation with 1 mM caffeine increased the rate of 3MG transport by 1.4-fold and incubation with 3 mM increased 3MG transport by 2.3-fold (Fig. 4).

Caffeine at a concentration of 3mM, but not 1mM, enhanced phosphorylation of CaMKII Thr^{287} in skeletal muscle incubated in vitro

Ca^{2+} has been implicated in the activation of glucose transport through signalling pathways involving AMPK (Wright et al. 2004, Hawley et al. 2005, Wright et al. 2005). CaMKII is directly regulated by Ca^{2+}/calmodulin (Hudmon and Schulman, 2002, Witczak et al. 2010), and has been used as an indicator of elevated cytosolic Ca^{2+} level in skeletal muscle (Blair et al. 2009). We found that 3 mM of caffeine, but not 1mM of caffeine, increased phosphorylation of CaMKII Thr^{287} in incubated muscles (Fig. 5B). Ca^{2+}/calmodulin kinase kinase (CaMKK) has been identified as an AMPK kinase in skeletal muscle (Jensen et al. 2007a, Witczak et al. 2007); CaMKI is an endogenous substrate of CaMKK (Tokumitsu et al. 1995). Incubation

with caffeine did not affect the phosphorylation status of CaMKI Thr^{177} (Fig. 5A).

Intravenous caffeine injection increased phosphorylation of AMPKα Thr^{172} and ACC Ser^{79} in a dose- and time-dependent manner

To determine whether caffeine preferentially activates AMPK in vivo, we measured the degree of phosphorylation of AMPKα Thr^{172} in muscle homogenates after stimulation with intravenous caffeine at various concentrations (0, 2.5, 5, 10, and 25 mg kg^{-1}) and for various times (0, 30, 60, and 120 min). The dose–response study revealed that phosphorylation of AMPKα Thr^{172} increased at caffeine concentrations of 5 mg kg^{-1} or higher in epitrochlearis muscle (Fig. 6A). Phosphorylation of ACC Ser^{79} displayed a pattern similar to that for AMPK phosphorylation (Fig. 6B). The time-course study showed that phosphorylation of AMPKα Thr^{172} and ACC Ser^{79} increased within 60 min of caffeine stimulation in epitrochlearis muscle (Figs 7A and B). The total AMPK and ACC contents of the muscles did not change during the study (Figs 6 and 7).

Intravenous caffeine injection increased AMPKα1 activity and insulin-independent glucose transport without affecting energy status

To determine which catalytic subunit is activated by intravenous caffeine injection, rats were injected with caffeine (5 mg kg^{-1}), and isoform-specific AMPK activity was determined 60 min later in anti-α1 and anti-α2 immunoprecipitates of the muscle. Caffeine increased AMPKα1 activity by 1.7-fold, but had no effect on AMPKα2 activity (Fig. 8). We also investigated whether caffeine-mediated in vivo activation of AMPKα1 in skeletal muscle is associated with enhanced glucose transport. Sixty minutes after the intravenous administration of caffeine, the rate of 3MG transport was increased by 2.0-fold compared with the saline injection group

(Fig. 9). Muscle ATP, PCr, and glycogen concentrations did not differ between the saline and caffeine injection groups (Table 1).

Plasma glucose, insulin, and caffeine levels after intravenous caffeine injection

Intravenous injection of caffeine (5 mg kg^{-1}) did not alter plasma glucose and insulin levels. Plasma caffeine levels 60 min after caffeine (5 mg kg^{-1}) injection were clearly increased compared to the saline injection group (Table 2).

Discussion

Our data show three novel findings relating to the activating effect of caffeine on skeletal muscle AMPK. First, in vitro caffeine treatment at 1 mM activated AMPKα1 but not AMPKα2, and caffeine treatment at 3 mM stimulated both isoforms (Fig. 1), suggesting that AMPKα1 is more sensitive to caffeine than is AMPKα2. The predominant activation of AMPKα1 was also confirmed by in vivo caffeine treatment (Figs 6, 7, and 8). Second, activation of AMPKα1 by caffeine occurred in the absence of any apparent reduction in muscle fuel status (Fig. 2 and Table 1), indicating that AMPKα1 is activated by a mechanism, at least in part, distinct from that of AMPKα2. Third, AMPKα1 activation was associated with increased ACC phosphorylation (Figs 3, 6, and 7) and glucose transport activity (Fig. 4 and 9), suggesting that AMPKα2 activity is not essential for metabolic activation in skeletal muscle.

The physiological importance of AMPKα1 has been elucidated in contraction-stimulated glucose transport using genetic animal models. Jørgensen et al. (Jørgensen et al. 2004b) examined the effect of high-intensity (tetanic) contraction, which markedly stimulated AMPKα2 activity in muscles of a whole-body AMPKα1-knockout mouse. Glucose transport activity was 25% lower in soleus but was normal in extensor digitorum longus muscle compared with muscles from the wild type. More recently, Jensen et al. (Jensen et al. 2008) examined the effect of low-intensity (twitch) contraction and found that glucose transport was markedly lower in muscles from AMPKα1-knockout and kinase-dead AMPK construct (AMPK-KD) mice but not in AMPKα2-knockout muscles. These

results suggest that AMPKα1 plays a major role in activation of low-intensity contraction-stimulated glucose transport, and is required for full activation of high-intensity contraction-stimulated glucose transport in skeletal muscle.

It is notable that most human studies using muscle biopsy samples show that moderate-intensity exercise preferentially activates AMPKα2 (Wojtaszewski et al. 2000, Fujii et al. 2000, Chen et al. 2000, Musi et al. 2001a, Stephens et al. 2002, Wojtaszewski et al. 2002, Wojtaszewski et al. 2003, Birk and Wojtaszewski, 2006, Sriwijitkamol et al. 2007). Similarly, treadmill running (Musi et al. 2001b, Durante et al. 2002, Klein et al. 2007) and electrical stimulation of the sciatic nerve (Vavvas et al. 1997) activate only AMPKα2 in rodent skeletal muscles. However, we demonstrated previously that AMPKα1, but not AMPKα2, is activated immediately as a post-mortem artefact during the dissection procedure (Toyoda et al. 2006). Hardie and Carling (Hardie and Carling, 1997) proposed that any method of cell harvesting that leads to hypoxia or any form of stress is likely to lead a rapid, artefactual activation of AMPK and that rapid cooling is required to preserve the in vivo-activation state of AMPK. Thus, it may be difficult to measure the actual AMPKα1 activity because it is disturbed by additional activation during dissection; only when the activation exceeds that of the dissecting stimuli, would AMPKα1 activity be detectable. For caffeine treatment in vivo, we exposed isolated muscles to caffeine after a preincubation period sufficient to stabilize AMPKα1 activity in oxygenated medium. After caffeine treatment in vivo, we gently and quickly removed muscles from anesthetized rats and immediately froze them in liquid nitrogen. We believe that the muscle preparations used in the present study enabled us to detect modest increases in AMPKα1 activity in muscles treated with caffeine in

vitro and in vivo.

A study by Jensen et al. (Jensen et al. 2007a) demonstrated the causal relationship between AMPK and caffeine-stimulated glucose transport in skeletal muscle. They found that caffeine-stimulated glucose transport was reduced markedly in soleus muscle isolated from a transgenic mouse with muscle-specific expression of an AMPK-KD construct. However, because both AMPKα1 and AMPKα2 activities were suppressed in muscles of the AMPK-KD mouse (Jensen et al. 2007b), their findings do not allow one to draw conclusions about the relative importance of these isoforms to glucose transport regulation. Moreover, in contrast to the wild-type mouse, the AMPK-KD mouse showed significantly higher AMP concentration in soleus muscle after caffeine stimulation (Jensen et al. 2007a), and this result does not confirm the association between energy deprivation and glucose transport activity. Our study demonstrates clearly that AMPKα1 activation occurs in the absence of AMPKα2 activation and in the absence of any apparent reduction in muscle energy status, whereas AMPKα1 and AMPKα2 activation is accompanied by a significant reduction in muscle energy status.

The finding that caffeine increased AMPKα Thr^{172} phosphorylation in skeletal muscle (Figs 3, 6, and 7) provides evidence that caffeine induces covalent modification via an AMPK kinase. Jensen et al. (Jensen et al. 2007b) showed that the CaMKK inhibitor, STO-609, inhibits activation of AMPKα1 and AMPKα2 as well as AMPKα Thr^{172} phosphorylation in incubated mouse skeletal muscles after a low-intensity tetanic contraction in vitro. Jensen et al. (Jensen et al. 2007a) also showed that caffeine-induced AMPKα1 activation and 2-deoxyglucose transport in mouse skeletal muscle is blocked by STO-609. However, we did not detect any

increases in the phosphorylation status of CaMKI, a downstream target of CaMKK (Fig. 5A), indicating that CaMKK is not involved in caffeine-induced AMPK activation in skeletal muscle. Moreover, 1 mM of caffeine did not significantly affect the phosphorylation status of CaMKII (Fig. 5B), indicating that Ca^{2+} signalling is not involved in the increase in glucose transport. The LKB1 complex (Sakamoto et al. 2004, Sakamoto et al. 2005) is the main kinase that regulates AMPKα2 activity in mouse skeletal muscle during tetanic contraction in situ and in vitro (Sakamoto et al. 2005). The LKB1 complex is constitutively active and is not activated directly by AMP; binding of AMP to AMPK facilitates the phosphorylation of AMPK by the LKB1 complex (Hawley et al. 2003, Sakamoto et al. 2004). Thus, LKB1 is believed to be a crucial AMPK kinase in the response to energy deprivation in skeletal muscle during intense contraction. In the present study, AMPKα1 activation by 1 mM caffeine was not accompanied by energy depletion (Figs 1 and 2), suggesting that LKB1 is not the main AMPK kinase involved in activation of AMPKα1 by 1 mM caffeine. Thus, our results raise the possibility that other enzymes involved in the regulation of AMPK, such as transforming growth factor-β-activated kinase 1 (Momcilovic et al. 2006) and protein phosphatase 2C (Davies et al. 1995), are involved in caffeine-induced AMPKα1 activation in skeletal muscle.

In summary, our results suggest (1) that caffeine preferentially activates AMPKα1 in the absence of energy deprivation, (2) that caffeine activates both AMPKα1 and AMPKα2 in the presence of energy deprivation, and (3) that activation of the α1 isoform leads to enhanced glucose transport and ACC phosphorylation in rat skeletal muscle. We propose that both AMPKα2 and AMPKα1 play important roles in regulating muscle metabolism and that AMPKα2 is not essential for metabolic

activation in skeletal muscle.

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Conflicts of interest

The authors state that there are no conflicts of interest.

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Tables

Table 1 ATP, PCr, and glycogen concentrations in muscle after saline (Control) or Caffeine 5mg kg^{-1} injection

		Time after injection	
		0 min	60 min
ATP (nmol mg^{-1})	Control	5.29 ± 0.31	5.04 ± 0.23
	Caffeine 5 mg/kg^{-1}	5.48 ± 0.25	5.41 ± 0.23
PCr (nmol mg^{-1})	Control	15.9 ± 0.72	14.9 ± 0.38
	Caffeine 5 mg/kg^{-1}	15.1 ± 0.35	14.9 ± 0.66
Glycogen (nmol mg^{-1})	Control	23.4 ± 1.34	24.6 ± 2.09
	Caffeine 5 mg/kg^{-1}	23.9 ± 1.85	24.7 ± 0.98

Values are mean ± SE; n = 6-8 per group.

Table 2 Plasma glucose, insulin, and caffeine levels after saline (Control) or Caffeine 5mg kg^{-1} injection

		Time after injection	
		0 min	60 min
Glucose (mg dL^{-1})	Control	56.7 ± 1.7	59.0 ± 1.9
	Caffeine 5 mg/kg^{-1}	60.1 ± 2.0	62.5 ± 2.3
Insulin (ng mL^{-1})	Control	0.51 ± 0.10	0.41 ± 0.07
	Caffeine 5 mg/kg^{-1}	0.61 ± 0.11	0.47 ± 0.05
Caffeine (μM)	Control	ND	ND
	Caffeine 5 mg/kg^{-1}	ND	54 ±4

Values are mean ± SE; n = 4-8 per group. ND; not detectable.

Legends to figures

Figure 1

Caffeine activates preferentially AMPKα1 activity in incubated rat epitrochlearis muscle. Isolated muscles were incubated in the absence (Control) or presence of caffeine for 15 min. Isoform-specific AMPK activity was determined in anti-AMPKα1 and -AMPKα2 immunoprecipitates. Fold increases are expressed relative to the activity of muscles in the control group. Values are mean ± SE; n = 6-14 per group. *P<0.05, **P<0.01 vs. Control.

Figure 2

ATP, PCr and glycogen concentrations in incubated rat epitrochlearis muscle. Isolated muscles were incubated in the absence (Control) or presence of caffeine for 15 min. ATP (A), PCr (B), and Glycogen (C) concentrations were measured enzymatically. Values are mean ± SE; n = 5-10 per group. *P<0.05, **P<0.01 vs. Control.

Figure 3

Caffeine increases phosphorylation of AMPKα Thr^{172} (A) and ACC Ser^{79} (B) in incubated rat epitrochlearis muscle. Isolated muscles were incubated in the absence (Control) or presence of caffeine for 15 min. Muscle lysates were then analyzed for phosphorylation of AMPKα Thr^{172} (pAMPK) and ACC Ser^{79} (pACC) by Western blot. Fold increases are expressed relative to the level of muscles in the control group. Representative immunoblots are shown. Values are mean ± SE; n = 6-12 per

group. *P<0.05, **P<0.01 vs. Control.

Figure 4

Caffeine increases 3-*O*-methyl-D-glucose (3MG) transport in incubated rat epitrochlearis muscle. Isolated muscles were incubated in the absence (Control) or presence of caffeine for 15 min, and then 3MG transport activity was determined. Values are mean ± SE; n = 9-13 per group. *P<0.05, **P<0.01 vs. Control.

Figure 5

Caffeine does not change CaMKI Thr^{177} phosphorylation (A), but increases CaMKII Thr^{287} phosphorylation (B) in incubated rat epitrochlearis muscle. Isolated muscles were incubated in the absence (Control) or presence of caffeine for 15 min. Muscle lysates were then analyzed for phosphorylation of CaMKI Thr^{177} (pCaMKI,) and CaMKII Thr^{287} (pCaMKII) by Western blot. The phosphorylation of CaMKII isoforms migrated between 50 to 75 KDa was summed (Rose et al. 2007). Fold increases are expressed relative to the level of muscles in the control group. Representative immunoblots are shown. Values are mean ± SE; n = 4-9 per group. *P<0.05 vs. Control.

Figure 6

Intravenous caffeine injection increases phosphorylation of AMPKα Thr^{172} (A) and ACC Ser^{79} (B) in a dose-dependent manner in rat epitrochlearis muscle. Muscles were dissected 60 min after injection, and then analyzed for phosphorylation of AMPKα Thr^{172} (pAMPK) and ACC Ser^{79} (pACC) by Western blot. Fold increases

are expressed relative to the level of muscles in the saline injection (control) group. Representative immunoblots are shown. Values are mean ± SE; n = 8-17 per group. *P<0.05, **P<0.01 vs. Control.

Figure 7

Intravenous caffeine injection increases phosphorylation of AMPKα Thr^{172} (A) and ACC Ser^{79} (B) in a time-dependent manner in rat epitrochlearis muscle. Muscles were dissected at indicated time points after caffeine injection (5 mg kg^{-1}). Control samples were dissected immediately after saline injection. Muscles were then analyzed for phosphorylation of AMPKα Thr^{172} (pAMPK) and ACC Ser^{79} (pACC) by Western blot. Fold increases are expressed relative to the activity of muscles in the control group. Representative immunoblots are shown. Values are mean ± SE; n = 7-15 per group. *P<0.05, **P<0.01 vs. Control.

Figure 8

Intravenous caffeine injection activates AMPKα1 activity without changes of energy status in rat epitrochlearis. Muscles were dissected 60 min after caffeine (5 mg kg^{-1}) or saline injection. Isoform-specific AMPK activity was determined in anti-AMPKα1 and -AMPKα2 immunoprecipitates. Fold increases are expressed relative to the activity of muscles in the saline injection (control) group. Values are mean ± SE; n = 5-8 per group. **P<0.01 vs. Control.

Figure 9

Intravenous caffeine injection increases 3-*O*-methyl-D-glucose (3MG) transport in

rat epitrochlearis muscle. Muscles were dissected 60 min after caffeine (5 mg kg^{-1}) or saline injection (control), and 3MG transport activity was determined. Values are mean ± SE; n = 6-7 per group. **P<0.01 vs. Control.

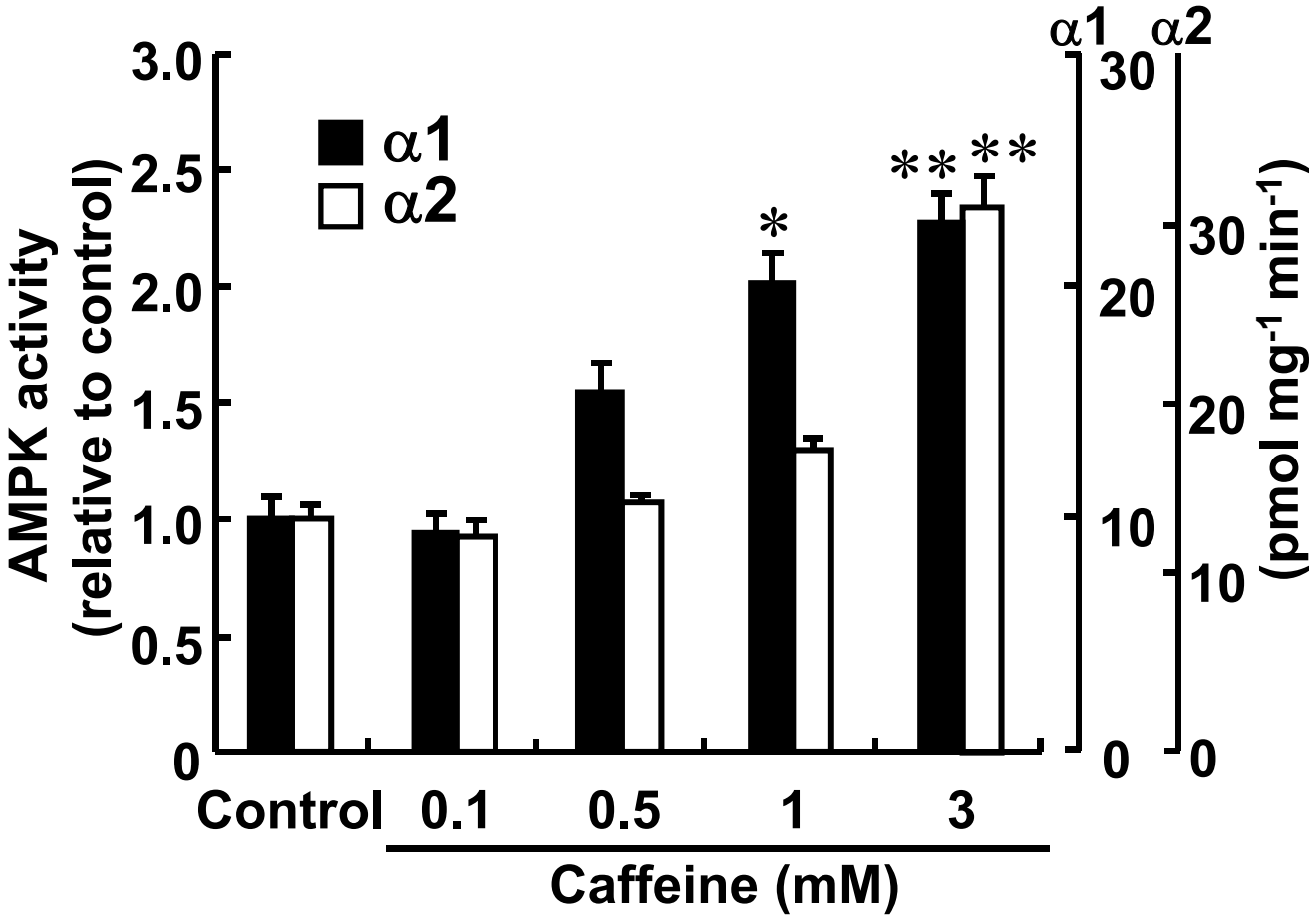
AMPK activity
(relative to control)
3.0
2.5
2.0
1.5
1.0
0.5
0
α1
α2
*
**
α1
α2
30
20
10
0
30
20
10
0
(pmol mg-1 min-1)
Control
0.1
0.5
1
3
Caffeine (mM)

Figure 1

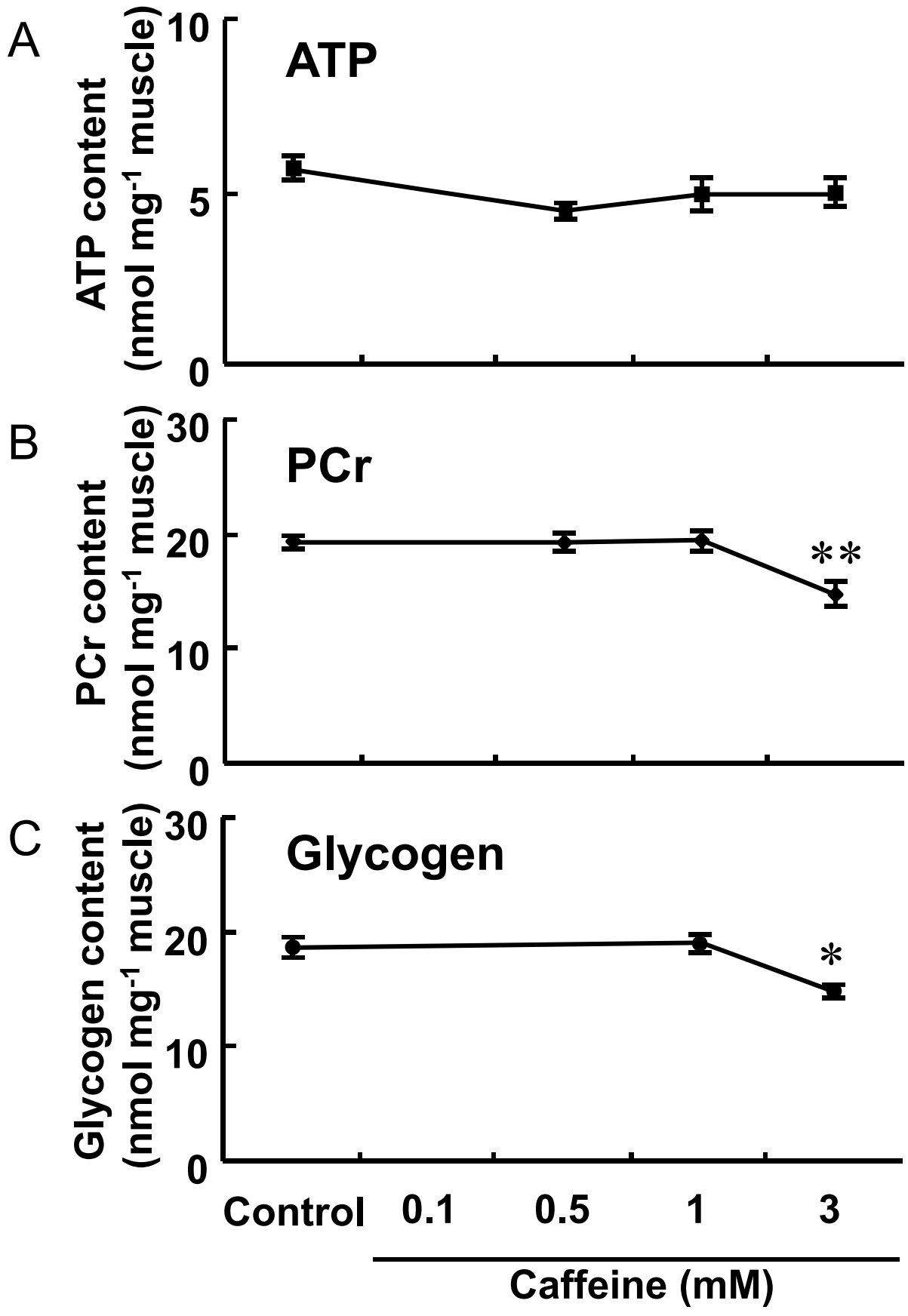
A
ATP
ATP content (nmol mg-1 muscle)
10
5
0
B
PCr
PCr content (nmol mg-1 muscle)
30
20
10
0
**
C
Glycogen
Glycogen content (nmol mg-1 muscle)
30
20
10
0
*
Control
0.1
0.5
1
3
Caffeine (mM)

Figure 2

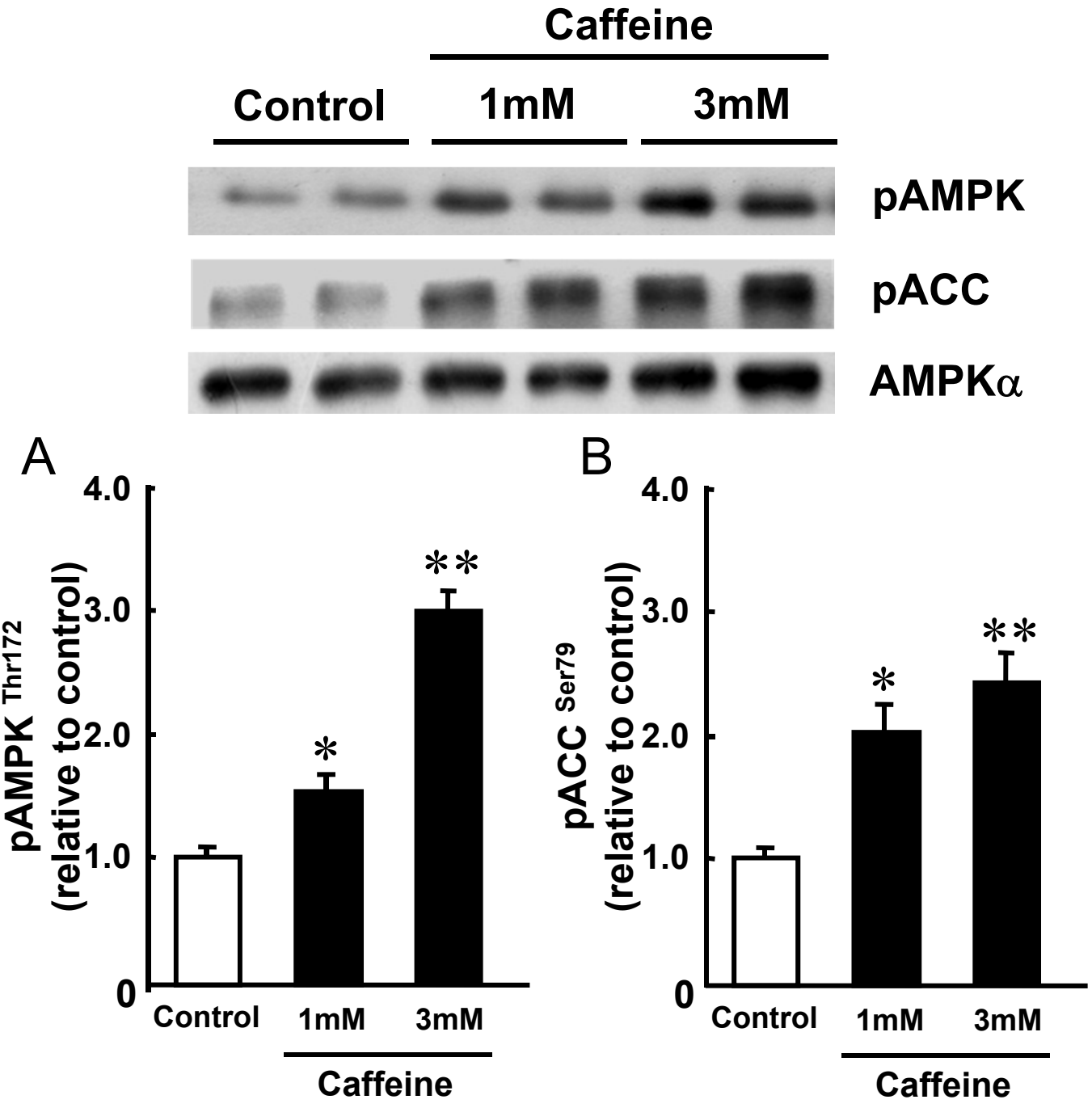
Caffeine
Control
1mM
3mM
pAMPK
pACC
AMPKα
A
B
pAMPK Thr172
(relative to control)
pACC Ser79
(relative to control)
4.0
3.0
2.0
1.0
0
*
**
Control
1mM
3mM
Caffeine

Figure 3

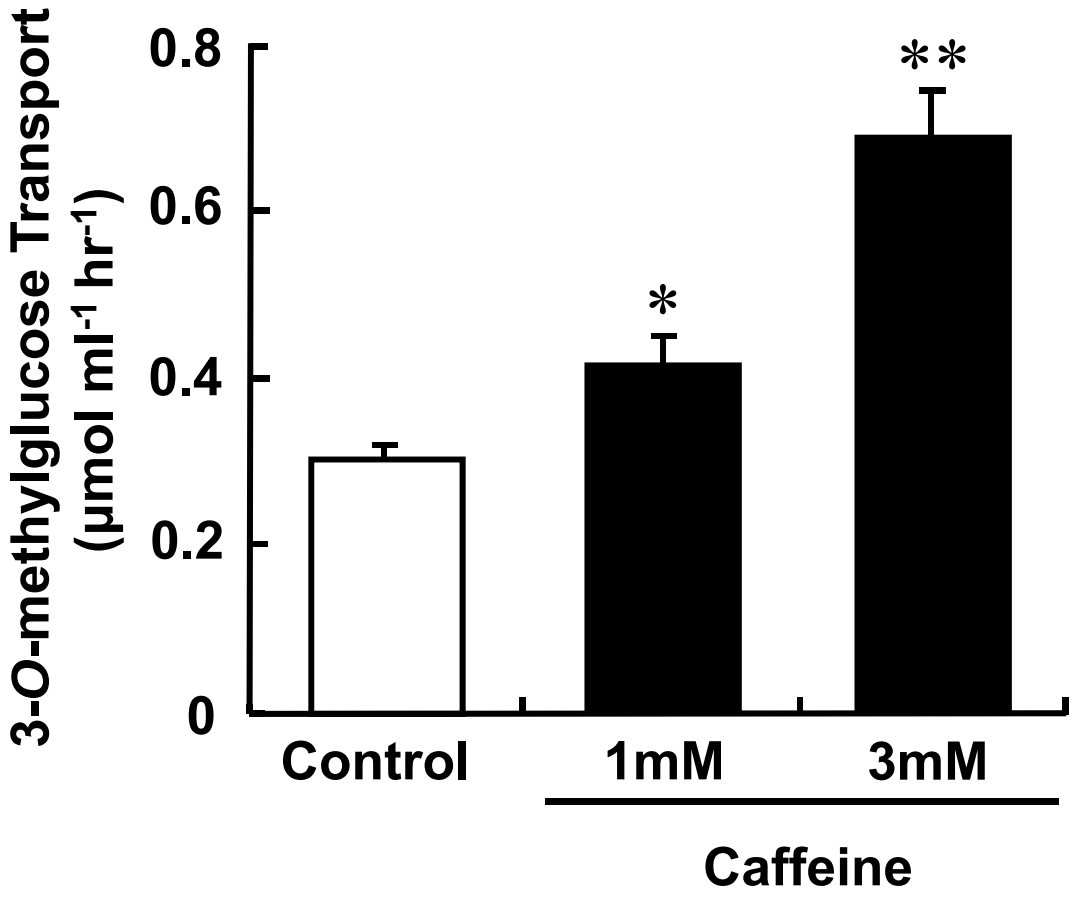
3-O-methylglucose Transport
(µmol ml-1 hr-1)
0.8
0.6
0.4
0.2
0
*
**
Control
1mM
3mM
Caffeine

Figure 4

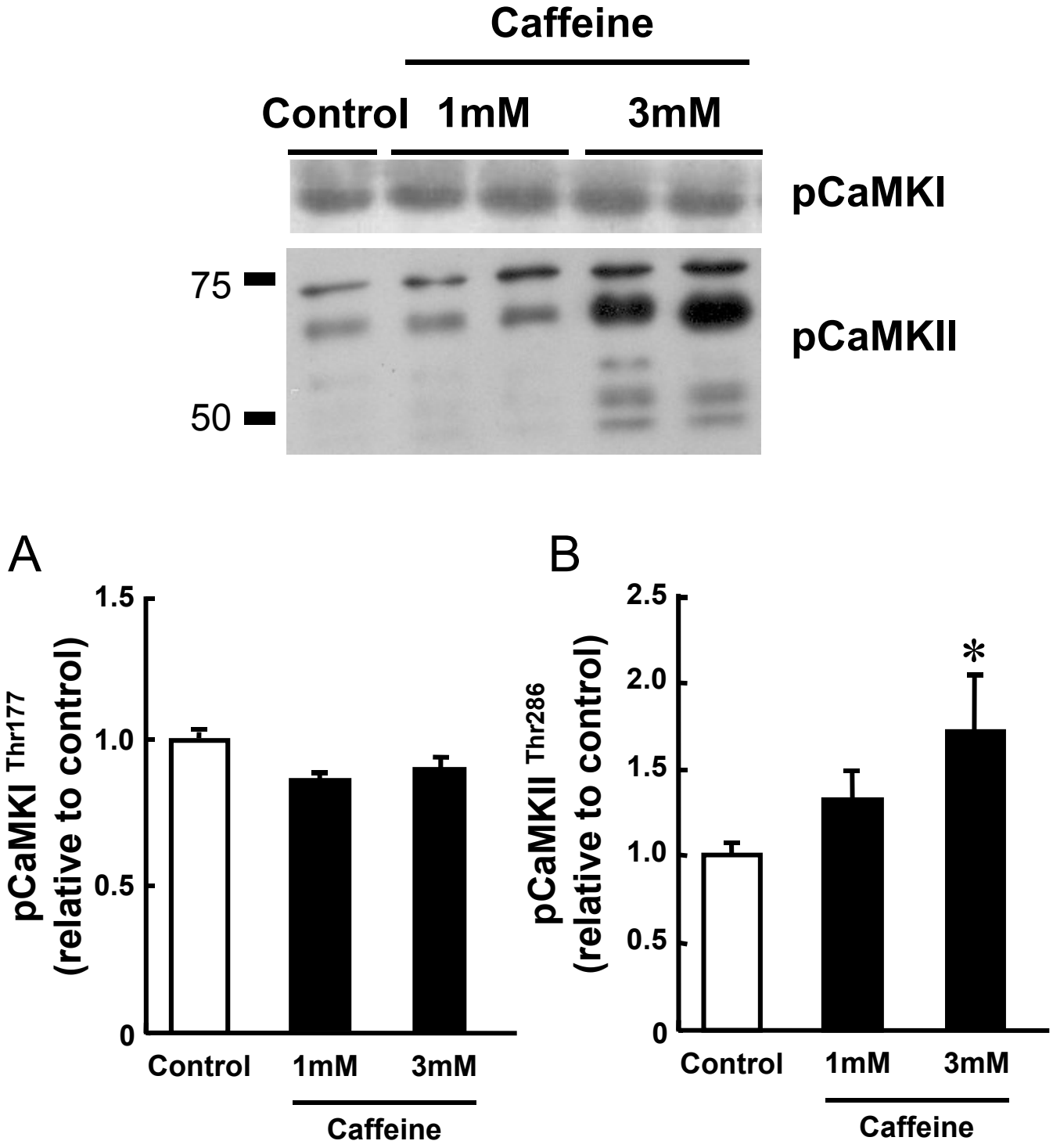
Caffeine
Control
1mM
3mM
pCaMKI
pCaMKII
75
50
A
pCaMKI Thr177
(relative to control)
1.5
1.0
0.5
0
Control
1mM
3mM
Caffeine
B
pCaMKII Thr286
(relative to control)
2.5
2.0
1.5
1.0
0.5
0
*
Control
1mM
3mM
Caffeine

Figure 5

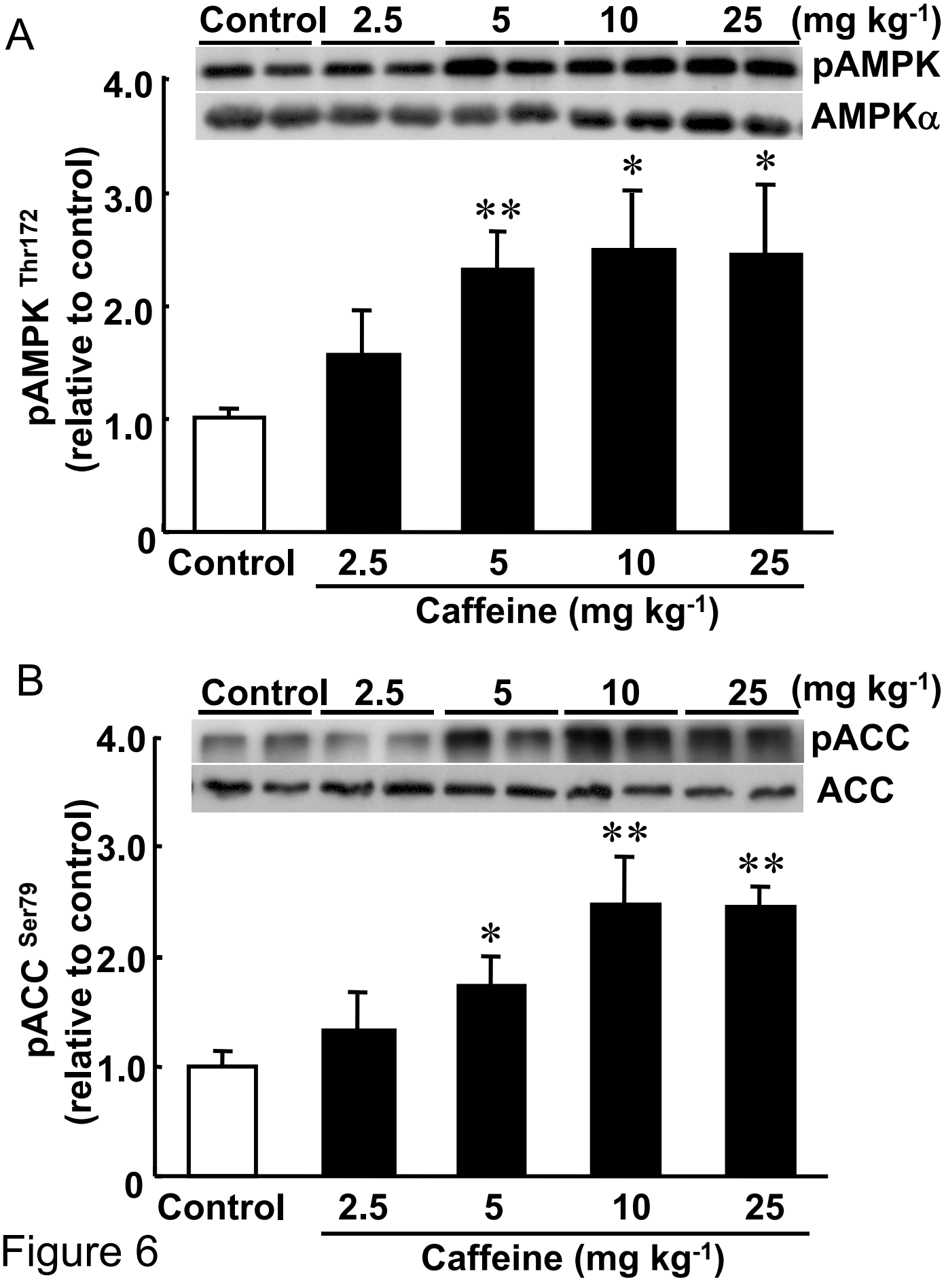
A
Control
2.5
5
10
25
(mg kg-1)
pAMPK
AMPKα
4.0
3.0
2.0
1.0
0
pAMPK Thr172
(relative to control)
**
*
*
Control
2.5
5
10
25
Caffeine (mg kg-1)
B
Control
2.5
5
10
25
(mg kg-1)
pACC
ACC
4.0
3.0
2.0
1.0
0
pACC Ser79
(relative to control)
*
**
**
Control
2.5
5
10
25
Caffeine (mg kg-1)

Figure 6

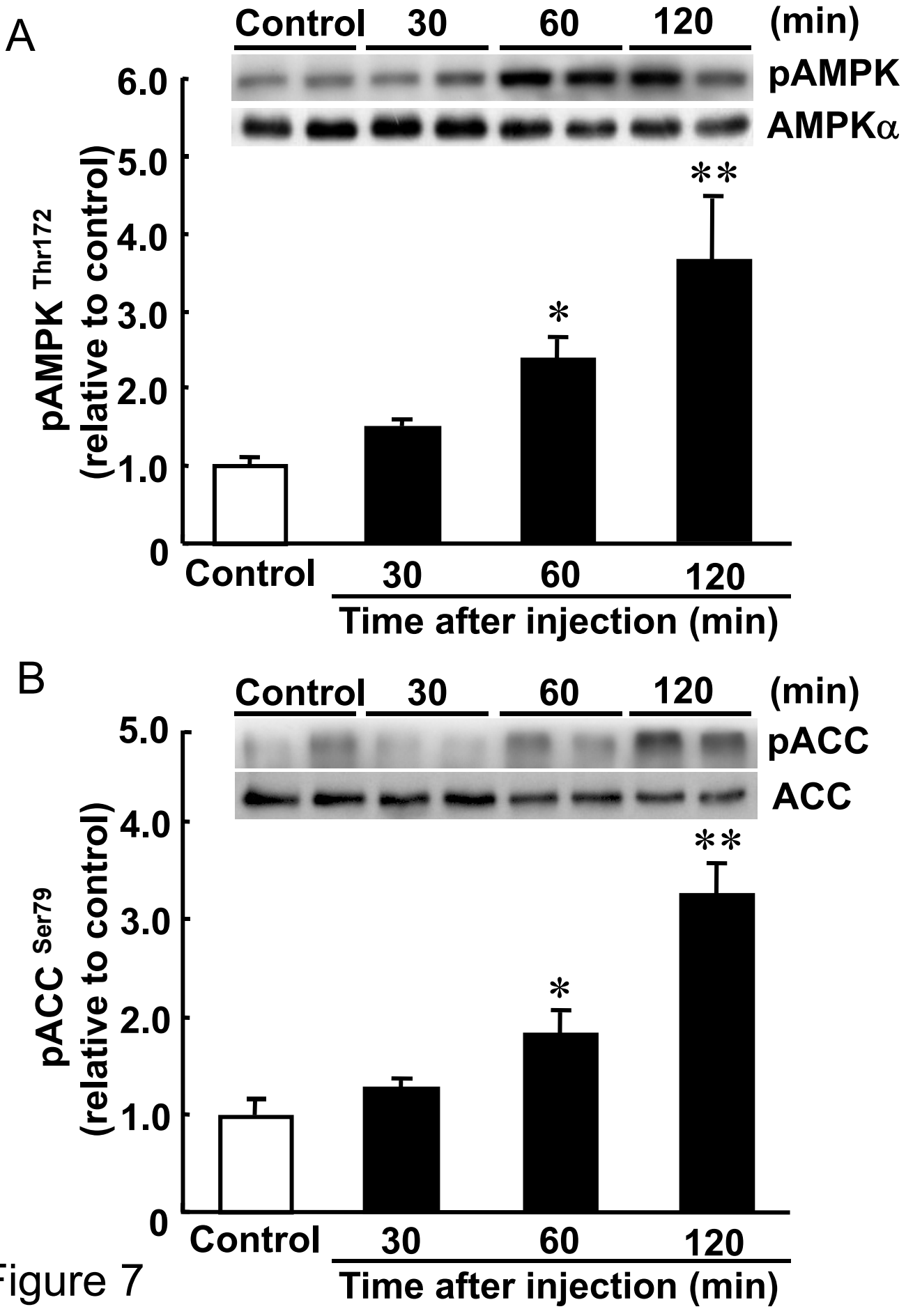
A
Control
30
60
120
(min)
pAMPK
AMPKα
6.0
5.0
4.0
3.0
2.0
1.0
0
pAMPK Thr172
(relative to control)
*
**
Control
30
60
120
Time after injection (min)
B
Control
30
60
120
(min)
pACC
ACC
5.0
4.0
3.0
2.0
1.0
0
pACC Ser79
(relative to control)
*
**
Control
30
60
120
Time after injection (min)

Figure 7

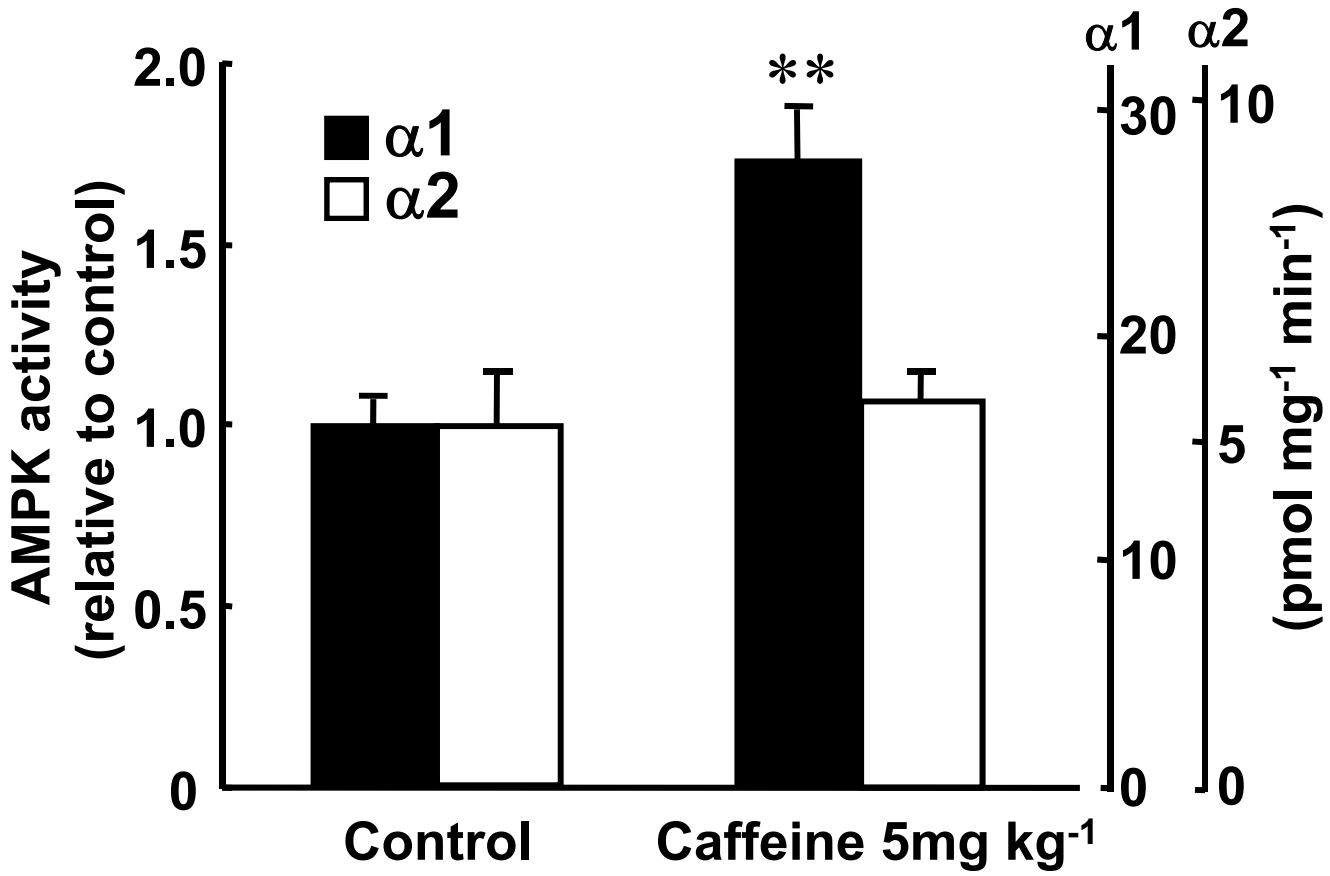
AMPK activity
(relative to control)
2.0
1.5
1.0
0.5
0
α1
α2
**
α1
α2
30
20
10
0
10
5
0
(pmol mg-1 min-1)
Control
Caffeine 5mg kg-1

Figure 8

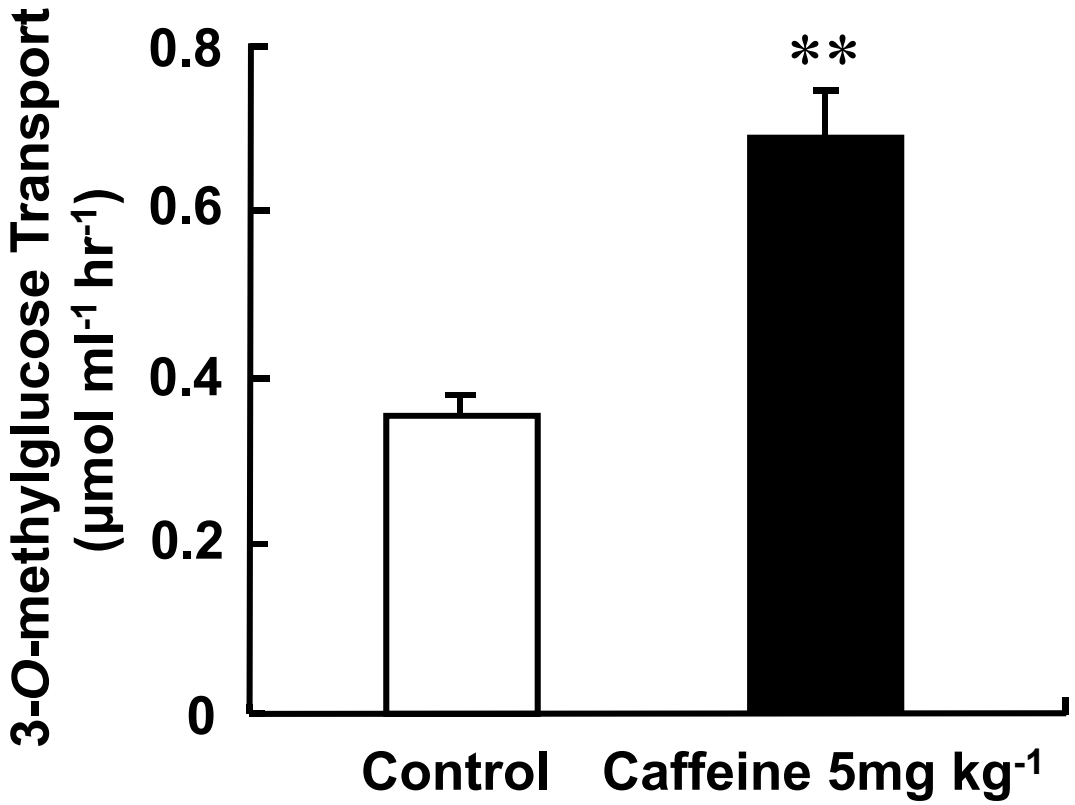
3-O-methylglucose Transport
(µmol ml-1 hr-1)
0.8
0.6
0.4
0.2
0
**
Control
Caffeine 5mg kg-1

Figure 9