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Caffeine acutely activates 5′adenosine monophosphate–activated protein kinase and increases insulin-independent glucose transport in rat skeletal muscles

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Abstract

Caffeine (1,3,7-trimethylxanthine) has been implicated in the regulation of glucose and lipid metabolism including actions such as insulin-independent glucose transport, glucose transporter 4 expression, and fatty acid utilization in skeletal muscle. These effects are similar to the exercise-induced and 5′adenosine monophosphate–activated protein kinase (AMPK)–mediated metabolic changes in skeletal muscle, suggesting that caffeine is involved in the regulation of muscle metabolism through AMPK activation. We explored whether caffeine acts on skeletal muscle to stimulate AMPK. Incubation of rat epitrochlearis and soleus muscles with Krebs buffer containing caffeine (≥3 mmol/L, ≥15 minutes) increased the phosphorylation of AMPKα Thr^{172}, an essential step for full kinase activation, and acetyl–coenzyme A carboxylase Ser^{79}, a downstream target of AMPK, in dose- and time-dependent manners. Analysis of isoform-specific AMPK activity revealed that both AMPKα1 and α2 activities increased significantly. This enzyme activation was associated with a reduction in phosphocreatine content and an increased rate of 3-*O*-methyl-D-glucose transport activity in the absence of insulin. These results suggest that caffeine has similar actions to exercise by acutely stimulating skeletal muscle AMPK activity and insulin-independent glucose transport with a reduction of the intracellular energy status.
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1. Introduction

Skeletal muscle is the major site of whole-body glucose transport and metabolism. Insulin and exercise (contractile activity) are the most potent and physiologically relevant stimuli of glucose transport, the rate-limiting step in glucose utilization under physiologic conditions [1,2]. Similar to insulin stimulation, exercise acutely increases the rate of glucose transport into contracting skeletal muscle by the translocation of glucose transporter (GLUT) 4 to the plasma membrane and transverse tubules. However, a growing body of data indicates that exercise and insulin use distinct signaling pathways in skeletal muscle; and 5′adenosine monophosphate–activated protein kinase (AMPK) has been identified as part of the mechanisms leading to exercise-stimulated glucose transport (reviewed in [3-5]).

Skeletal muscle AMPK is also implicated in a variety of antidiabetic properties of exercise, including GLUT4 expression [6,7], glycogen regulation [8,9], fatty acid oxidation [10,11], and enhanced insulin sensitivity [7,12,13]. In addition, skeletal muscle AMPK mediates part of glucose and lipid homeostasis by adipokines, including leptin and adiponectin, and the hypoglycemic effect of metformin (reviewed in [3-5]). Thus, through these metabolic effects in skeletal muscle, AMPK fosters a metabolic milieu that may reduce the risk for type 2 diabetes mellitus.

Caffeine (1,3,7-trimethylxanthine) has been implicated in the regulation of glucose and lipid metabolism in skeletal muscle. Caffeine stimulates muscle glucose transport in the absence of insulin in rodents [14-16], increases GLUT4 messenger RNA or protein expression in cultured myotubes [17,18], and enhances fatty acid metabolism in perfused rat skeletal muscles [19]. These effects are similar to the

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exercise-induced and AMPK-mediated metabolic changes in skeletal muscle, suggesting that caffeine regulates muscle metabolisms through AMPK activation.

AMPK is a heterotrimeric kinase consisting of a catalytic α subunit and 2 regulatory subunits, β and γ. Two distinct α isoforms (α1 and α2) exist in skeletal muscle [20]. A recent study by Jensen et al [16] demonstrated that caffeine acutely stimulates AMPKα1 activity, but not α2 activity, in incubated mouse and rat soleus muscles. However, they did not observe significant phosphorylation on AMPKα Thr^{172}, an essential step for full kinase activation [21]. Similarly, Wright et al [14] and Canto et al [15] also reported that caffeine has no effect on AMPKα Thr^{172} phosphorylation in incubated rat skeletal muscles; but they did not measure AMPK activity. In contrast, Raney and Turcotte [19] demonstrated that caffeine increases AMPKα2 activity in perfused rat hind limb muscles; but they did not measure AMPKα Thr^{172} phosphorylation.

The purpose of the present study was to reevaluate whether caffeine has the potential to act on skeletal muscle and stimulate AMPK. For this purpose, we used an isolated rat skeletal muscle preparation to eliminate the effects of systemic confounders such as circulatory, humoral, and neural factors and of intestinal absorption of caffeine. We determined the effect of caffeine on AMPKα Thr^{172} phosphorylation as well as on α1 and α2 isoform-specific AMPK activities in fast-twitch epitrochlearis and slow-twitch soleus muscles in vitro.

2. Materials and methods

2.1. Animals

Ninety-seven 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°C to 24°C with a 12:12-hour 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 were reviewed and approved by the Kyoto University Graduate School of Human and Environmental Studies, Kyoto University Graduate School of Medicine, and Kyoto University Radioisotope Research Center in Japan.

2.2. Muscle incubation

Two muscles, epitrochlearis and soleus, were chosen because of their specific fiber-type composition. The epitrochlearis is composed predominantly of fast-twitch glycolytic fibers (60%-65% fast-twitch white, 20% fast-twitch red, 15% slow-twitch red) [22], and the soleus is composed primarily of slow-twitch oxidative fibers (0% fast-twitch white, 13% fast-twitch red, 87% slow-twitch red) [23].

Muscles were treated as we described previously [24,25]. Rats were killed by cervical dislocation without anesthesia, and the 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 (117 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, and 24.6 mmol/L $NaHCO_3$) containing 2 mmol/L pyruvate for 40 minutes. For the time- and dose-dependent effects of caffeine, muscles were then randomly assigned to incubation in 7 mL of fresh buffer in the presence of 3 mmol/L caffeine for up to 60 minutes, or in 7 mL of fresh buffer in the absence or presence of 1 to 15 mmol/L caffeine for 15 minutes, respectively. Immediately after incubation, muscles were frozen in liquid nitrogen, weighed, stored at −80°C, and then subjected to Western blot analysis for phosphorylated AMPKα, total AMPKα, phosphorylated acetyl–coenzyme A carboxylase (ACC), and total ACC. The wet muscle weight of epitrochlearis and soleus was 12.6 ± 3.0 (mean ± SD, n = 152) mg and 35.4 ± 7.9 (mean ± SD, n = 145) mg, respectively. Some frozen muscles collected for the time-dependent effect were subjected to measurements for adenosine triphosphate (ATP), phosphocreatine (PCr) ("ATP and PCr assay"), and glycogen ("Muscle glycogen content") content. For other experiments, we incubated muscles in the absence or presence of 3 mmol/L caffeine for 15 minutes ("Results"). Immediately after incubation, some muscles were used for the measurement of glucose transport activity ("3-*O*-methyl-D-glucose transport"); and others were frozen in liquid nitrogen, stored at −80°C, and analyzed for isoform-specific AMPK activity or Western blot analysis for GLUT4.

2.3. Western blot analysis

Sample preparation and Western blot analysis for detection of phosphorylated AMPKα, total AMPKα, phosphorylated ACC, and total ACC were performed as we described previously [25]. Muscles were homogenized in ice-cold lysis buffer (1:40 wt/vol) containing 20 mmol/L Tris-HCl (pH 7.4), 1% Triton X, 50 mmol/L NaCl, 250 mmol/L sucrose, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 2 mmol/L dithiothreitol, 4 mg/L leupeptin, 50 mg/L trypsin inhibitor, 0.1 mmol/L benzamidine, and 0.5 mmol/L phenylmethylsulfonyl fluoride and centrifuged at 16 000*g* for 40 minutes at 4°C. Lysates were solubilized in Laemmli sample buffer containing mercaptoethanol and boiled.

Sample preparation and Western blot analysis for detection of GLUT4 were performed as we described previously [13]. Muscles were homogenized in ice-cold buffer containing 250 mmol/L sucrose, 20 mmol/L 2-[4-(2-

hydroxyethyl)-1-piperadinyl] ethonsulforic acid (HEPES) (pH 7.4), and 1 mmol/L EDTA and centrifuged at 1200*g* for 5 minutes. The supernatant was centrifuged at 200 000*g* for 60 minutes at 4°C. The resulting pellet was solubilized in Laemmli sample buffer containing dithiothreitol.

The samples (10 μg of protein) were separated on either 10% polyacrylamide gel for AMPK and GLUT4 or 7.5% gel for ACC. Proteins were then transferred to polyvinylidene difluoride membranes (PolyScreen; PerkinElmer, Wellesley, MA) at 100 V for 1 hour. Membranes were blocked for 1 hour at room temperature in Tris-buffered saline with 0.1% Tween 20 containing 5% nonfat dry milk and were then incubated overnight at 4°C with phosphospecific AMPKα Thr^{172} (2531; Cell Signaling Technology, Beverly, MA) diluted 1:1000, AMPKα (2532, Cell Signaling Technology) diluted 1:1000, phosphospecific ACC Ser^{79} (07-303; Upstate Biotechnology, Lake Placid, NY) diluted 1:1000, ACC (3662, Cell Signaling Technology) diluted 1:1000, or GLUT4 (4670-1704; Biogenesis; South Coast, United Kingdom) diluted 1:2000. The membranes were then washed, incubated for 1 hour at room temperature with anti-mouse immunoglobulin G antibody (GE Healthcare, Buckinghamshire, United Kingdom) diluted 1:2500, and developed with enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham Biosciences, Buckinghamshire, United Kingdom). The intensity of the signals was quantified using Multi-Analyst software (Bio-Rad, Hercules, CA). The mean intensity of control samples in each membrane was used as reference for controlling gel-to-gel variation. Equal protein loading and transfer were confirmed by Coomassie brilliant blue staining of the membranes.

2.4. 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) [25]. The AMPK activity assay was performed as we described previously [25,26]. 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 mmol/L HEPES and 480 mmol/L NaCl). Kinase reactions were performed in 40 HEPES (pH 7.0), 0.1 mmol/L SAMS peptide [25,26], 0.2 mmol/L AMP, 80 mmol/L NaCl, 0.8 mmol/L dithiothreitol, 5 mmol/L $MgCl_2$, and 0.2 mmol/L ATP (2 μCi of [γ-^{32}P] ATP) (PerkinElmer, Wellesley, MA) in a final volume of 40 μL for 20 minutes at 30°C. At the end of the reaction, a 15-μL aliquot was removed and spotted onto Whatman P81 paper (Whatman International, Maidstone, United Kingdom). The papers were washed 6 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.

2.5. ATP and PCr assay

The ATP and PCr contents were measured fluorometrically in perchloric acid extracts of epitrochlearis and soleus muscles according to the method of Lowry and Passonneau [27]. In brief, each frozen muscle was homogenized in 0.2 mol/L $HClO_4$ (3:25 wt/vol) in an ethanol–dry-ice bath (−20∼−30°C) and centrifuged at 16 000*g* for 2 minutes at −9°C. The supernatant of the homogenate was neutralized with a solution of 2 mol/L KOH, 0.4 mol/L KCl, and 0.4 mol/L imidazole; centrifuged at 16 000*g* for 2 minutes at −9°C; and then subjected to enzymatic analysis [27]. The ATP and PCr contents were expressed as nanomoles per milligram wet weight of muscle.

2.6. Muscle glycogen content

Glycogen content was assayed as we described previously [7,9]. Each frozen muscle was digested in 1 mol/L NaOH at 85°C for 10 minutes, and the digestates were neutralized with HCl. The glycogen in the digestates was hydrolyzed by incubation in 2 mol/L HCl for 2 hours 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 content was expressed as nanomoles of glucose per milligram wet weight of muscle.

2.7. 3-*O*-methyl-D-glucose transport

The 3-*O*-methyl-D-glucose (3MG) transport assay was performed as we described previously [24,25]. Muscles were transferred to 2 mL of Krebs-Ringer bicarbonate buffer containing 1 mmol/L [^{3}H]3MG (1.5 μCi/mL) (American Radiolabeled Chemicals, St Louis, MO) and 7 mmol/L D-[1-^{14}C]mannitol (0.3 μCi/mL) (American Radiolabeled Chemicals) at 30°C and further incubated for 10 minutes. 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 mol/L NaOH at 80°C for 10 minutes. Digestates were neutralized with 300 μL of 1 mol/L HCl, and particulates were precipitated by centrifugation at 20 000*g* for 2 minutes. Radioactivity in aliquots of the digested protein was determined by liquid scintillation counting for dual labels, and the extracellular and intracellular spaces were calculated [28].

2.8. Statistical analysis

Results are presented as means ± SE. One-way analysis of variance was used to estimate the variance of the dose-response and time-course studies (Figs. 1 and 2, Table 1), and statistical significance of difference between control and caffeine-treated groups was evaluated by Dunnet post hoc test. Student *t* test was used to examine the significant

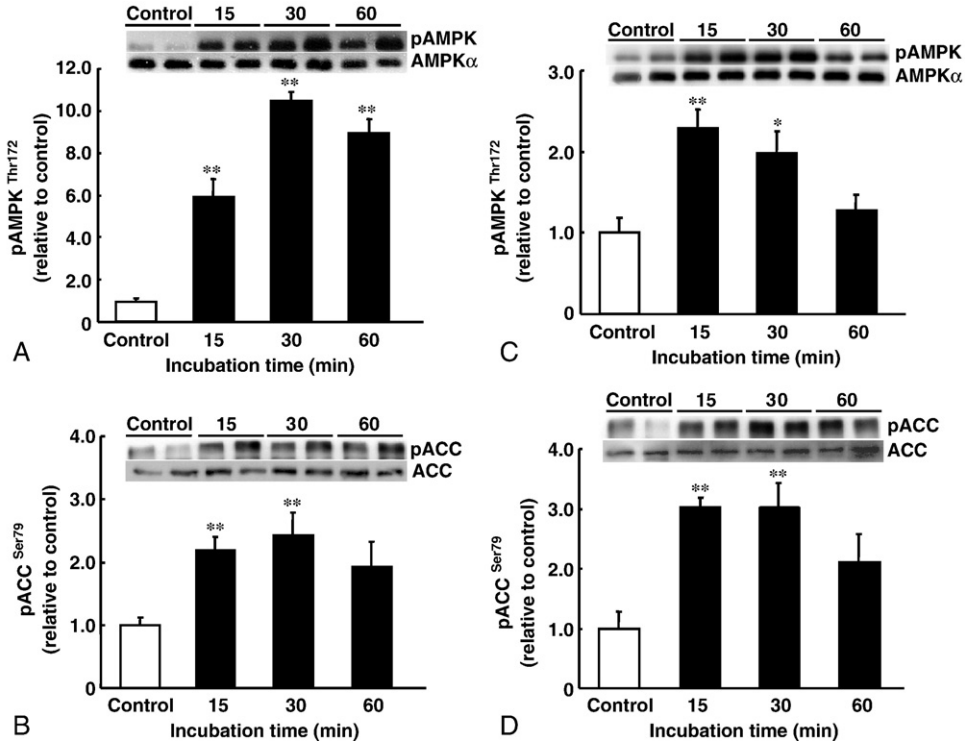

Fig. 1. Caffeine stimulation increases phosphorylation of AMPKα Thr^{172} and ACC Ser^{79} in a time-dependent manner in rat skeletal muscles. Isolated epitrochlearis (A and B) and soleus (C and D) muscles were incubated in the presence of 3 mmol/L caffeine for indicated times. Muscle lysates were then analyzed for phosphorylation of AMPKα Thr^{172} (pAMPK; A and C) and ACC Ser^{79} (pACC; B and D) by Western blot analysis. Fold increases are expressed relative to the level of muscles in the control (0-minute incubation) group. Representative immunoblots are shown. Values are mean ± SE. The number of muscles in each group is as follows: epitrochlearis—control (4), 15 minutes (4), 30 minutes (6), and 60 minutes (4); soleus—control (4), 15 minutes (6), 30 minutes (6), and 60 minutes (4). $*P < .05$ and $**P < .01$ vs control.

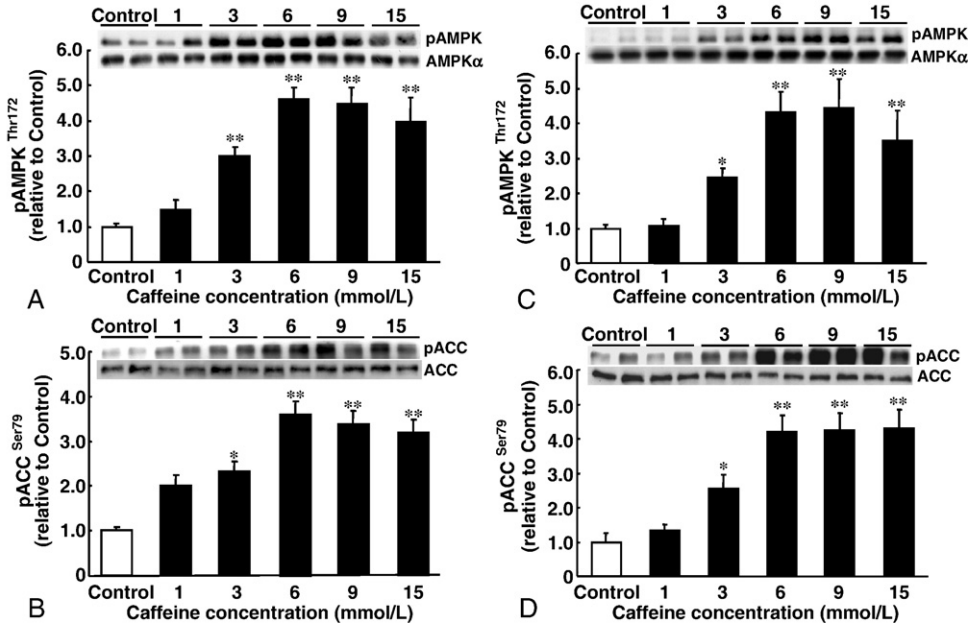

Fig. 2. Caffeine stimulation increases phosphorylation of AMPKα Thr^{172} and ACC Ser^{79} in a dose-dependent manner in rat skeletal muscles. Isolated epitrochlearis (A and B) and soleus (C and D) muscles were incubated in the absence (control) or presence of caffeine at indicated concentration for 15 minutes. Muscle lysates were then analyzed for phosphorylation of AMPKα Thr^{172} (pAMPK; A and C) and ACC Ser^{79} (pACC; B and D) by Western blot analysis. Fold increases are expressed relative to the level of muscles in the control group. Representative immunoblots are shown. Values are mean ± SE. The number of muscles in each group is as follows: epitrochlearis—control (6), 1 mmol/L (6), 3 mmol/L (6), 6 mmol/L (8), 9 mmol/L (6), and 15 mmol/L (6); soleus—control (10), 1 mmol/L (6), 3 mmol/L (14), 6 mmol/L (6), 9 mmol/L (6), and 15 mmol/L (6). $*P < .05$ and $**P < .01$ vs control.

Table 1
The ATP, PCr, and glycogen content in incubated skeletal muscles

		Incubation time			
		Control	15 min	30 min	60 min
EPI	ATP	5.8 ± 0.4 (10)	5.1 ± 0.5 (8)	5.3 ± 0.5 (6)	5.0 ± 0.3 (8)
	PCr	19.5 ± 0.6 (10)	15.0 ± 1.2† (8)	11.5 ± 1.0† (6)	10.3 ± 1.2† (8)
	Glycogen	26.5 ± 2.0 (4)	22.4 ± 0.4 (4)	18.7 ± 2.6 (4)	15.0 ± 2.6† (4)
SOL	ATP	3.4 ± 0.3 (10)	2.7 ± 0.3 (5)	2.3 ± 0.2* (6)	2.0 ± 0.2† (8)
	PCr	10.1 ± 1.4 (9)	5.5 ± 1.2* (5)	4.5 ± 0.5† (6)	4.3 ± 0.6† (8)
	Glycogen	13.8 ± 1.2 (4)	10.8 ± 0.3 (4)	10.7 ± 1.4 (4)	7.8 ± 0.2† (4)

The ATP, PCr and glycogen contents (in nanomoles per milligram wet weight of muscle) were measured in rat epitrochlearis and soleus muscles after incubation in the presence of 3 mmol/L caffeine for indicated times. Values are mean ± SE for the number of muscles given in parentheses. EPI indicates epitrochlearis; SOL, soleus. *$P < .05$ and †$P < .01$ vs control (0-minute incubation) group.

differences between control and caffeine-treated groups in AMPK activity assay (Fig. 3), 3MG transport assay (Fig. 4), and analysis of GLUT4 content (Fig. 5). Differences between groups were considered statistically significant at $P < .05$.

3. Results

3.1. Caffeine increases the phosphorylation of muscle AMPKα Thr^{172} and ACC Ser^{79} in time- and dose-dependent manners

In both α1 and α2 catalytic subunits, the primary site responsible for AMPK activation is the Thr^{172} residue [21]. To determine whether caffeine stimulation activates AMPK, we measured the degree of phosphorylation of AMPKα Thr^{172} by Western blot analysis using a phosphospecific antibody in muscle homogenates that had been stimulated with caffeine at 3 mmol/L for various times. The time-course study showed that phosphorylation of AMPKα Thr^{172} increased within 15 minutes of caffeine stimulation in epitrochlearis and soleus muscles (Fig. 1A, C). Phosphorylation of ACC Ser^{79} displayed a pattern similar to that of AMPK phosphorylation in both muscles (Fig. 1B, D). ACC is a downstream target of AMPK in skeletal muscle, and

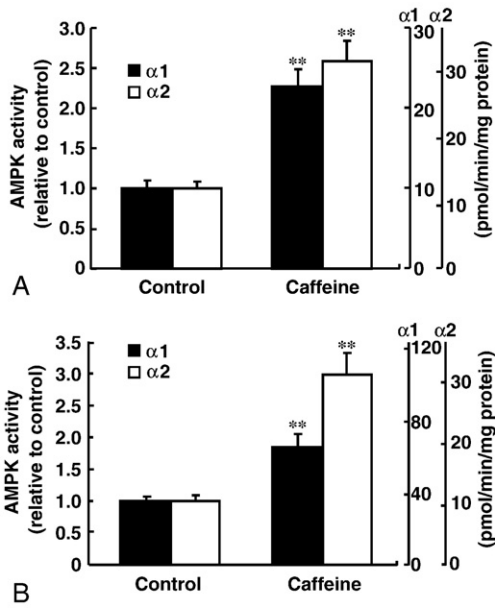

Fig. 3. Caffeine stimulation activates both AMPKα1 and AMPKα2 activity in rat skeletal muscles. Isolated epitrochlearis (A) and soleus (B) muscles were incubated in the absence (control) or presence of 3 mmol/L caffeine for 15 minutes. Isoform-specific AMPK activity was determined in anti-AMPKα1 and anti-AMPKα2 immunoprecipitates. Fold increases are expressed relative to the activity of muscles in the control group. Values are mean ± SE. The number of muscles in each group is as follows: epitrochlearis—control (8) and caffeine (8); soleus—control (6) and caffeine (6). **$P < .01$ vs control.

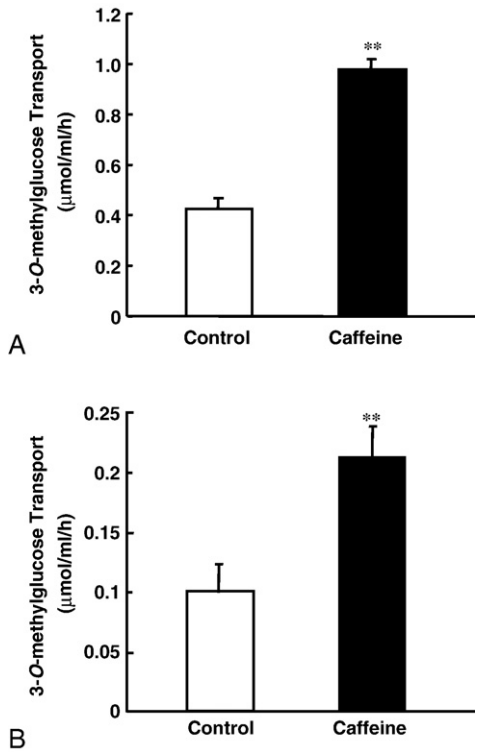

Fig. 4. Caffeine stimulation increases 3MG transport in rat skeletal muscles. Isolated epitrochlearis (A) and soleus (B) muscles were incubated in the absence (control) or presence of 3 mmol/L caffeine for 15 minutes, and then 3MG transport activity was determined. Values are mean ± SE. The number of muscles in each group is as follows: epitrochlearis—control (6) and caffeine (5); soleus—control (6) and caffeine (6). **$P < .01$ vs control.

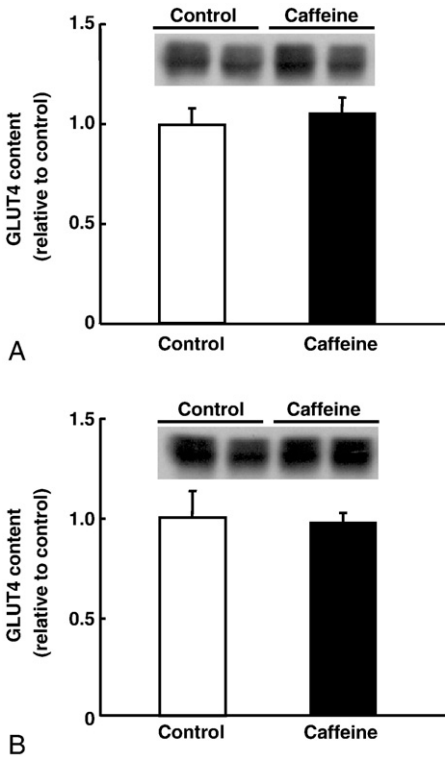

Fig. 5. Caffeine stimulation does not affect GLUT4 content in skeletal muscles. Isolated epitrochlearis (A) and soleus (B) muscles were incubated in the absence (control) or presence of 3 mmol/L caffeine for 15 minutes. Muscle lysates were then analyzed for GLUT4 content by Western blot analysis. Fold increases are expressed relative to the level of muscles in the control group. Representative immunoblots are shown. Values are mean ± SE. n = 4 per group.

phosphorylation of the Ser^{79} site of ACC reflects total AMPK activity [29,30]. We chose a caffeine concentration of 3 mmol/L to reevaluate the results of preceding studies in which stimulation with 3 to 3.5 mmol/L of caffeine for 15 minutes failed to demonstrate an increase in AMPKα Thr^{172} phosphorylation in incubated rat epitrochlearis [14], rat soleus [15,16], and mouse soleus [16] muscles. We also determined the effects of 15 minutes of stimulation with various concentrations of caffeine. The dose-response study revealed that phosphorylation of AMPKα Thr^{172} and ACC Ser^{79} increased at caffeine concentrations of 3 mmol/L or higher in both epitrochlearis (Fig. 2A, B) and soleus (Fig. 2C, D) muscles. The total AMPK and ACC content of the muscles did not change during the study (Figs. 1 and 2).

3.2. Caffeine increases both AMPKα1 and AMPKα2 activities in skeletal muscles

To identify which catalytic subunit is activated by caffeine, isoform-specific AMPK activity was determined in anti-α1 and anti-α2 immunoprecipitates from epitrochlearis and soleus muscles after treatment with caffeine (3 mmol/L, 15 minutes). We chose this stimulation protocol to reevaluate the preceding studies by Jensen et al [16], in which 3 mmol/L of caffeine stimulation for 15 minutes increased AMPKα1 activity but not AMPKα2 activity in incubated rat soleus muscle, and by Raney and Turcotte [19], in which stimulation with 3 mmol/L caffeine for 20 minutes increased AMPKα2 activity in perfused rat hind limb muscles. In contrast to the results of these 2 studies, in our study, caffeine clearly increased AMPKα1 activity by 2.3- and 1.8-fold and AMPKα2 activity by 2.6- and 3.0-fold in epitrochlearis (Fig. 3A) and soleus muscle (Fig. 3B), respectively.

3.3. Caffeine decreases ATP, PCr, and glycogen content in skeletal muscles

AMPK is activated in response to energy-depleting stresses such as muscle contraction, hypoxia, and inhibition of oxidative phosphorylation [26]. To determine whether caffeine increases AMPK activity in parallel with energy deprivation, we measured the time course of changes in the ATP, PCr, and glycogen content in muscles incubated in the presence of 3 mmol/L of caffeine for up to 60 minutes (Table 1). In epitrochlearis muscle, the ATP content did not differ at any time during incubation, whereas the PCr content at 15, 30, and 60 minutes of stimulation was significantly lower than that of the control. In soleus muscle, the ATP content at 30 and 60 minutes of stimulation was significantly lower than that of the control; and the PCr content was significantly decreased at 15, 30, and 60 minutes of caffeine incubation. Glycogen content was significantly lower at 60 minutes of incubation in both epitrochlearis and soleus muscles.

3.4. Caffeine acutely increases insulin-independent glucose transport activity in skeletal muscles

We next investigated whether the activation of AMPK in skeletal muscle by caffeine affects insulin-independent glucose transport activity. Incubation with 3 mmol/L caffeine for 15 minutes increased the rate of 3MG transport by 2.5-fold above the basal level in epitrochlearis (Fig. 4A) and by 2.2-fold in soleus (Fig. 4B).

3.5. Caffeine does not affect GLUT4 content in skeletal muscles

We investigated whether caffeine stimulation affects the GLUT4 content in skeletal muscles (Fig. 5). Incubation with 3 mmol/L caffeine for 15 minutes did not change the total amount of GLUT4 protein in the epitrochlearis (control: 1.00 ± 0.10, caffeine: 1.05 ± 0.11 arbitrary units relative to the control; n = 4 per group; $P = .75$) or soleus muscles (control: 1.00 ± 0.17, caffeine: 0.98 ± 0.03 arbitrary units relative to the control; n = 4 per group; $P = .92$).

4. Discussion

Our data show 3 novel findings relating to the metabolic effect of caffeine on skeletal muscle. First, caffeine had the ability to increase AMPKα Thr^{172} phosphorylation (Figs. 1 and 2) and both AMPKα1 and α2 activities (Fig. 3). The enhanced phosphorylation of ACC, an endogenous substrate of AMPK (Figs. 1 and 2), and the increased 3MG transport activity (Fig. 4) are indicative of a substantial increase in AMPK activity in vivo. Second, these effects were observed in both fast-glycolytic epitrochlearis and slow-oxidative soleus muscles, suggesting that the stimulatory effect of caffeine on AMPK is not specific to a particular muscle type. Third, caffeine-stimulated AMPK activation was associated with a reduction in the fuel status of skeletal muscle (Table 1), as with contraction-stimulated AMPK activation.

The energy deprivation in our study may explain the difference between our results and those of Jensen et al [16] that demonstrated predominant activation of AMPKα1 by caffeine stimulation. AMPKα2 has greater AMP dependence than AMPKα1 with respect to allosteric activation by AMP and covalent activation by upstream kinases [21,31], indicating that AMPKα2 is more sensitive to energy depletion than is AMPKα1. In support of this idea, we previously demonstrated that AMPKα1, but not AMPKα2, is activated in rat epitrochlearis muscles treated with H_2O_2 and hypoxanthine/xanthine oxidase in the absence of an increase in AMP or a decrease in PCr content [25]. We have also shown that AMPKα1 is activated in low-intensity contracting muscle, in which AMP concentration is not elevated, whereas AMPKα1 and α2 are activated in high-intensity contracting muscle, in which the AMP concentration is significantly higher than the resting value [32]. In the present study, we found that 15 minutes of treatment with 3 mmol/L of caffeine significantly decreased the PCr content in both the epitrochlearis and soleus muscles (Table 1). On the other hand, Jensen et al [16] did not detect any changes in energy status in mouse soleus muscles treated with 3 mmol/L caffeine. Therefore, although the reasons for the difference in the results of the energy assays are unknown, the robust AMPKα2 activation in our study may be explained by a decrease in energy status induced by caffeine stimulation.

The difference between our results and those of the study by Raney and Turcotte [19], who showed that caffeine increases AMPKα2 activation, may be explained by the different muscle preparations used: caffeine incubation in our study and caffeine perfusion in their study. Because AMPKα1, but not AMPKα2, is activated immediately as a postmortem artifact during the isolation procedure [32], we measured AMPK activity after a preincubation period (40 minutes) that was sufficient to stabilize AMPKα1 activity at the basal level. This method enabled us to examine the effect of caffeine on both AMPKα1 and AMPKα2 activities. In contrast, Raney and Turcotte measured AMPK activity in muscles isolated after caffeine perfusion. The actual AMPKα1 activity may be increased by caffeine, but it may also be disturbed by additional activation during isolation because an increase in AMPKα1 activity would be detectable only when the activation by caffeine exceeds that of the isolating stimuli.

Preceding studies have shown that stimulation with 3 to 3.5 mmol/L caffeine for 15 minutes enhances glucose transport without an apparent increase in AMPKα Thr^{172} phosphorylation in incubated skeletal muscles [14-16]. However, Jensen et al [16] found that some individual pairs of rat soleus muscles incubated with 3 mmol/L caffeine for 15 minutes clearly displayed greater AMPKα Thr^{172} phosphorylation; and they proposed that evaluation of AMPK activation by measuring AMPKα Thr^{172} phosphorylation is prone to statistical type 2 error, which may lead to false conclusions that caffeine does not activate AMPK. In fact, Jensen et al [16] have shown that in mice with muscle-specific expression of a dominant-negative, kinase-dead AMPK mutant (AMPK-KD), glucose transport is blocked in response to caffeine stimulation (3 mmol/L, 15 minutes) in isolated soleus muscles. Thus, it is likely that AMPK is a signaling intermediary leading to caffeine-stimulated glucose transport in skeletal muscle. We believe that we eliminated type 2 errors because of the rapid and gentle isolation procedure, which minimally stimulated AMPK, and because of sufficient preincubation, which decreased AMPK activity to a constant level. We note that all muscle samples incubated with 3 mmol/L caffeine for 15 minutes showed stronger Western blot signals for AMPKα Thr^{172} phosphorylation than control samples (data not shown).

The finding that caffeine increased AMPKα Thr^{172} phosphorylation in epitrochlearis and soleus muscles (Figs. 1 and 2) provides evidence that caffeine induces covalent modification via upstream kinases. The LKB1 complex is the main kinase that regulates AMPKα2 activity in mouse skeletal muscle during tetanic contraction in situ and in vitro [33]. 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 [34,35]. 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, AMPK activation was accompanied by a decrease in PCr content (Table 1), raising the possibility that LKB1 is involved in AMPKα2 activation by caffeine. On the other hand, Jensen et al [16] have shown that caffeine-induced AMPKα1 activation and 2-deoxyglucose transport in mouse skeletal muscle are blocked by the Ca^{2+}/calmodulin kinase kinase inhibitor STO-609. Jensen et al [36] have also shown that STO-609 inhibits activation of AMPKα1 and AMPKα2 as well as AMPKα Thr^{172} phosphorylation in mouse skeletal muscles after a low-intensity tetanic contraction in vitro. Thus, Ca^{2+}/calmodulin kinase kinase might be the upstream kinase responsible for the caffeine-induced AMPKα1 activation observed in our study.

Epidemiologic studies show that long-term consumption of beverages containing caffeine such as coffee and green tea

is associated with a reduced risk of type 2 diabetes mellitus [37-39]. Some researchers believe that caffeine reduces the risk of diabetes [37,40], although others do not [38,39]. Considering that caffeine and exercise exert similar effects in stimulating AMPK, caffeine may be the active ingredient responsible for the preventive effect of coffee and green tea on the development of type 2 diabetes mellitus. In this context, further studies are needed to clarify whether oral administration of caffeine at a physiologic dose results in AMPK activation and induces AMPK-related metabolic events, including glucose transport, in skeletal muscle.

In summary, we demonstrated for the first time that caffeine increases AMPKα Thr^{172} phosphorylation and both AMPKα1 and α2 activities in fast- and slow-twitch skeletal muscles, and that this activation is accompanied by insulin-independent glucose transport and a reduction of muscle energy status. We propose that, similar to exercise, caffeine can activate muscle glucose metabolism by stimulating AMPK.

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