

Caffeine acutely activates 5'AMP-activated protein kinase and increases insulin-independent glucose transport in rat skeletal muscles

Tatsuro Egawa^a, Taku Hamada^b, Naoko Kameda^c, Kouhei Karaike^a, Xiao Ma^a, Shinya Masuda^a, Nobumasa Iwanaka^a, Tatsuya Hayashi^{a,*}

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

^b *Graduate School of Sport and Exercise Science, Osaka University of Health and Sport Sciences, Osaka, 590-0496, Japan*

^c *Laboratory of Applied Physiology, Graduate School of Human and Environmental studies, Kyoto University, Kyoto, 606-8501, Japan*

* 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

Financial disclosure statement

Tatsuya Hayashi was supported by a research grant from the Japan Society for the

25 Promotion of Science (20500576). Tatsuya Hayashi and Taku Hamada were
26 supported by a research grant from the All Japan Coffee Association.

27

28

29 **Conflicts of interest**

30 The authors state that there are no conflicts of interest.

31

32 **Abstract**

33

34 Caffeine (1, 3, 7-trimethylxanthine) has been implicated in the regulation of glucose
35 and lipid metabolism including actions such as insulin-independent glucose transport,
36 glucose transporter (GLUT) 4 expression, and fatty acid utilization in skeletal
37 muscle. These effects are similar to the exercise-induced and 5'AMP-activated
38 protein kinase (AMPK)-mediated metabolic changes in skeletal muscle, suggesting
39 that caffeine is involved in the regulation of muscle metabolism through AMPK
40 activation. We explored whether caffeine acts on skeletal muscle to stimulate
41 AMPK. Incubation of rat epitrochlearis and soleus muscles with Krebs buffer
42 containing caffeine (≥ 3 mmol/L, ≥ 15 min) increased the phosphorylation of
43 AMPK α Thr¹⁷², an essential step for full kinase activation, and acetyl CoA
44 carboxylase Ser⁷⁹, a downstream target of AMPK, in dose- and time-dependent
45 manners. Analysis of isoform-specific AMPK activity revealed that both AMPK α 1
46 and α 2 activities increased significantly. This enzyme activation was associated
47 with a reduction in phosphocreatine content and an increased rate of
48 3-O-methyl-D-glucose transport activity in the absence of insulin. These results
49 suggest that caffeine has similar actions to exercise, by acutely stimulating skeletal
50 muscle AMPK activity and insulin-independent glucose transport with a reduction of
51 the intracellular energy status.

52

53 **1. Introduction**

54

55 Skeletal muscle is the major site of whole-body glucose transport and
56 metabolism. Insulin and exercise (contractile activity) are the most potent and
57 physiologically relevant stimuli of glucose transport, the rate-limiting step in glucose
58 utilization under physiological conditions [1,2]. Similar to insulin stimulation,
59 exercise acutely increases the rate of glucose transport into contracting skeletal
60 muscle by the translocation of glucose transporter (GLUT) 4 to the plasma
61 membrane and transverse tubules. However, a growing body of data indicates that
62 exercise and insulin use distinct signaling pathways in skeletal muscle, and
63 5'AMP-activated protein kinase (AMPK) has been identified as part of the
64 mechanisms leading to exercise-stimulated glucose transport (reviewed in [3-5]).

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

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

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

79 AMPK is a heterotrimeric kinase, consisting of a catalytic α -subunit and two
80 regulatory subunits, β and γ . Two distinct α -isoforms ($\alpha 1$ and $\alpha 2$) exist in skeletal
81 muscle [20]. A recent study by Jensen et al [16] demonstrated that caffeine acutely
82 stimulates AMPK $\alpha 1$ activity, but not $\alpha 2$ activity, in incubated mouse and rat soleus
83 muscles. However, they did not observe significant phosphorylation on AMPK α
84 Thr¹⁷², an essential step for full kinase activation [21]. Similarly, Wright et al [14]
85 and Canto et al [15] also reported that caffeine has no effect on AMPK α Thr¹⁷²
86 phosphorylation in incubated rat skeletal muscles, but they did not measure AMPK
87 activity. In contrast, Raney et al [19] demonstrated that caffeine increases
88 AMPK $\alpha 2$ activity in perfused rat hindlimb muscles, but they did not measure
89 AMPK α Thr¹⁷² phosphorylation.

90 The purpose of the present study was to reevaluate whether caffeine has the
91 potential to act on skeletal muscle and stimulate AMPK. For this purpose, we used
92 an isolated rat skeletal muscle preparation to eliminate the effects of systemic
93 confounders such as circulatory, humoral and neural factors, and of intestinal
94 absorption of caffeine. We determined the effect of caffeine on AMPK α Thr¹⁷²
95 phosphorylation as well as on $\alpha 1$ and $\alpha 2$ isoform-specific AMPK activities in
96 fast-twitch epitrochlearis and slow-twitch soleus muscles in vitro.

97

98 **2. Materials and Methods**

99

100 *2.1. Animals*

101 Ninety-seven male Sprague-Dawley rats weighing 100 g were obtained from
102 Shimizu Breeding Laboratories (Kyoto, Japan). Animals were housed in an animal
103 room maintained at 22-24°C with a 12:12-h light-dark cycle and fed a standard
104 laboratory diet (Certified Diet MF; Oriental Koubo, Tokyo, Japan) and water ad
105 libitum. Rats were fasted overnight before the experiments and were randomly
106 assigned to the experimental groups. All protocols for animal use and euthanasia
107 were reviewed and approved by the Kyoto University Graduate School of Human
108 and Environmental Studies, Kyoto University Graduate School of Medicine, and
109 Kyoto University Radioisotope Research Center in Japan.

110

111 *2.2. Muscle incubation*

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

117 Muscles were treated as we described previously [24,25]. Rats were sacrificed
118 by cervical dislocation without anesthesia, and the muscles of each side were rapidly
119 removed. Both ends of each muscle were tied with sutures (silk 3-0; Natsume
120 Seisakusho, Tokyo, Japan) and the muscles were mounted on an incubation
121 apparatus with a tension set to 0.5 g. The buffers were continuously gassed with

122 95% O₂-5% CO₂ and maintained at 37°C. Muscles were preincubated in 7 mL of
123 Krebs-Ringer bicarbonate buffer (KRB) (117 mmol/L NaCl, 4.7 mmol/L KCl, 2.5
124 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, 24.6 mmol/L NaHCO₃)
125 containing 2 mmol/L pyruvate (KRBP) for 40 min. For the time- and
126 dose-dependent effects of caffeine, muscles were then randomly assigned to
127 incubation in 7 mL of fresh buffer in the presence of 3 mmol/L caffeine for up to 60
128 min, or in 7 mL of fresh buffer in the absence or presence of 1-15 mmol/L caffeine
129 for 15 min, respectively. Immediately after incubation, muscles were frozen in
130 liquid nitrogen, weighed, and stored at -80°C, and then subjected to Western blot
131 analysis for phosphorylated AMPK α , total AMPK α , phosphorylated acetyl CoA
132 carboxylase (ACC) and total ACC. The wet muscle weight of epitrochlearis and
133 soleus were 12.6 $\pm$ 3.0 (mean $\pm$ SD, n=152) mg and 35.4 $\pm$ 7.9 (mean $\pm$ SD, n=145)
134 mg, respectively. Some frozen muscles collected for the time-dependent effect
135 were subjected to measurements for adenosine triphosphate (ATP), phosphocreatine
136 (PCr) (see *ATP and PCr Assay*) and glycogen (see *Muscle glycogen content*) content.
137 For other experiments we incubated muscles in the absence or presence of 3 mmol/L
138 caffeine for 15 min (see RESULTS). Immediately after incubation, some muscles
139 were used for the measurement of glucose transport activity (see
140 *3-O-methyl-D-glucose transport*), and others were frozen in liquid nitrogen, stored at
141 -80°C, and analyzed for isoform-specific AMPK activity or Western blot analysis
142 for GLUT4.

143

144 2.3. Western blot analysis

145 Sample preparation and Western blot analysis for detection of phosphorylated

146 AMPK α , total AMPK α , phosphorylated ACC, and total ACC were performed as we
147 described previously [25]. Muscles were homogenized in ice-cold lysis buffer
148 (1:40 wt/vol) containing 20 mmol/L Tris·HCl (pH 7.4), 1% Triton X, 50 mmol/L
149 NaCl, 250 mmol/L sucrose, 50 mmol/L NaF, 5 mmol/L sodium pyrophosphate, 2
150 mmol/L dithiothreitol, 4 mg/L leupeptin, 50 mg/L trypsin inhibitor, 0.1 mmol/L
151 benzamidine, and 0.5 mmol/L phenylmethylsulfonyl fluoride and centrifuged at
152 16,000 *g* for 40 min at 4°C. Lysates were solubilized in Laemmli's sample buffer
153 containing mercaptoethanol and boiled.

154 Sample preparation and Western blot analysis for detection of GLUT4 were
155 performed as we described previously [13]. Muscles were homogenized in ice-cold
156 buffer containing 250mmol/L sucrose, 20 mmol/L
157 2-[4-(2-hydroxyethyl)-1-piperadiny] ethonsulfonic acid (HEPES) (pH 7.4), and 1
158 mmol/L EDTA, and centrifuged at 1200 *g* for 5 minutes. The supernatant was
159 centrifuged at 200, 000 *g* for 60 minutes at 4°C. The resulting pellet was
160 solubilized in Laemmli's sample buffer containing dithiothreitol.

161 The samples (10 μ g of protein) were separated on either 10% polyacrylamide gel
162 for AMPK and GLUT4 or 7.5% gel for ACC. Proteins were then transferred to
163 polyvinylidene difluoride membranes (PolyScreen; PerkinElmer, Wellesley, MA) at
164 100 V for 1 h. Membranes were blocked for 1h at room temperature in TBS-T
165 (TBS with 0.1% Tween 20) containing 5% nonfat dry milk and were then incubated
166 over night at 4°C with phosphospecific AMPK α Thr¹⁷² (#2531; Cell Signaling
167 Technology, Beverly, MA) diluted 1:1000, AMPK α (#2532; Cell Signaling
168 Technology, Beverly, MA) diluted 1:1000, phosphospecific ACC Ser⁷⁹ (#07-303;
169 Upstate Biotechnology, Lake Placid, NY) diluted 1:1000, ACC (#3662; Cell

170 Signaling Technology, Beverly, MA) diluted 1:1000, or GLUT4 (#4670-1704;
171 Biogenesis; South Coast, United Kingdom) diluted 1:2000. The membranes were
172 then washed, incubated for 1h at room temperature with anti-mouse IgG antibody
173 (GE Healthcare, Buckinghamshire, UK) diluted 1:2500, and developed with
174 enhanced chemiluminescence reagents according to the manufacturer's instructions
175 (Amersham Biosciences, Buckinghamshire, UK). The intensity of the signals was
176 quantified using Multi-Analyst software (Bio-Rad, Hercules, CA). The mean
177 intensity of control samples in each membrane was used as reference for controlling
178 gel-to-gel variation. Equal protein loading and transfer was confirmed by
179 Coomassie brilliant blue staining of the membranes.

180

181 *2.4. Isoform-specific AMPK activity assay*

182 We have raised AMPK polyclonal antibodies in rabbit against isoform-specific
183 peptides derived from the amino acid sequences of rat $\alpha 1$ (residues 339–358) or $\alpha 2$
184 (residues 490–514) [25]. AMPK activity assay was performed as we described
185 previously [25,26]. Muscles were homogenized as described in *Western blot*
186 *analysis*, and resultant supernatants (100 μ g of protein) were immunoprecipitated
187 with the $\alpha 1$ or $\alpha 2$ AMPK antibody and protein A-Sepharose beads (Amersham
188 Biosciences, Uppsala, Sweden). Immunoprecipitates were washed twice both in
189 lysis buffer and in wash buffer (240 mmol/L HEPES and 480 mmol/L NaCl).
190 Kinase reactions were performed in 40 HEPES (pH 7.0), 0.1 mmol/L SAMS peptide
191 [25,26], 0.2 mmol/L AMP, 80 mmol/L NaCl, 0.8 mmol/L dithiothreitol, 5 mmol/L
192 $MgCl_2$, 0.2 mmol/L ATP (2 μ Ci of $[\gamma\text{-}^{32}P]$ ATP) (PerkinElmer, Wellesley, MA), in a
193 final volume of 40 μ l for 20 min at 30°C. At the end of the reaction, a 15- μ l aliquot

194 was removed and spotted onto Whatman P81 paper (Whatman International,
195 Maidstone, UK). The papers were washed six times in 1% phosphoric acid and
196 once in acetone. ^{32}P incorporation was quantitated with a scintillation counter, and
197 kinase activity was expressed as fold increases relative to the control samples.

198

199 2.5. ATP and PCr assay

200 ATP and PCr content were measured fluorometrically in perchloric acid extracts
201 of epitrochlearis and soleus muscles according to the method of Lowry and
202 Passonneau [27]. In brief, each frozen muscle was homogenized in 0.2 mol/L
203 HClO_4 (3:25 w/v) in an ethanol-dry ice bath ($-20 \sim -30^\circ\text{C}$) and centrifuged at 16,000
204 g for 2 min at -9°C . The supernatant of the homogenate was neutralized with a
205 solution of 2 mol/L KOH, 0.4 mol/L KCl and 0.4 mol/L imidazole and then
206 centrifuged at 16,000 g for 2 min at -9°C , and then subjected to enzymatic analysis
207 [27]. ATP and PCr content were expressed as nanomoles per milligram wet weight
208 of muscle.

209

210 2.6. Muscle glycogen content

211 Glycogen content was assayed as we described previously [7,9]. Each frozen
212 muscle was digested in 1 mol/L NaOH at 85°C for 10 minutes, and the digestates
213 were neutralized with HCl. The glycogen in the digestates was hydrolyzed by
214 incubated in 2 mol/L HCL for 2 h at 85°C . The digestates were neutralized with
215 NaOH, and the concentration of hydrolyzed glucose residues was measured
216 enzymatically using Glucose CII Test (Wako, Osaka, Japan). Glycogen content
217 was expressed as nanomoles of glucose per milligram wet weight of muscle.

218

219 2.7. 3-O-methyl-D-glucose (3MG) transport

220 3MG transport assay was performed as we described previously [24,25].
221 Muscles were transferred to 2 mL of KRB containing 1 mmol/L [³H]3-MG (1.5 µCi/
222 mL) (American Radiolabeled Chemicals, St. Louis, MO) and 7 mmol/L
223 D-[1-¹⁴C]mannitol (0.3 µCi/ mL) (American Radiolabeled Chemicals, St. Louis, MO)
224 at 30°C and further incubated for 10 min. The muscles were then blotted onto filter
225 paper, trimmed, frozen in liquid nitrogen, and stored at -80°C. Each frozen muscle
226 was weighed and processed by incubating them in 300 µl of 1 mol/L NaOH at 80°C
227 for 10 min. Digestates were neutralized with 300 µl of 1 mol/L HCl, and
228 particulates were precipitated by centrifugation at 20,000 g for 2 min.
229 Radioactivity in aliquots of the digested protein was determined by liquid
230 scintillation counting for dual labels, and the extracellular and intracellular spaces
231 were calculated [28].

232

233 2.8. Statistical analysis

234 Results are presented as means ± SE. One-way ANOVA was used to estimate
235 the variance of the dose-response and time-course studies (Figs. 1 and 2, Table 1),
236 and statistical significance of difference between control and caffeine-treated groups
237 was evaluated by Dunnet's post hoc test. Student's t test was used to examine the
238 significant differences between control and caffeine-treated groups in AMPK
239 activity assay (Fig. 3), 3MG transport assay (Fig. 4), and analysis of GLUT4 content
240 (Fig. 5). Differences between groups were considered statistically significant at *P*
241 < .05.

242

243 3. Results

244

245 3.1. Caffeine increases the phosphorylation of muscle AMPK α Thr¹⁷² and ACC Ser⁷⁹ 246 in time- and dose-dependent manners

247 In both $\alpha 1$ and $\alpha 2$ catalytic subunits, the primary site responsible for AMPK
248 activation is the Thr¹⁷² residue [21]. To determine whether caffeine stimulation
249 activates AMPK, we measured the degree of phosphorylation of AMPK α Thr¹⁷² by
250 Western blot analysis using a phosphospecific antibody in muscle homogenates that
251 had been stimulated with caffeine at 3 mmol/L for various times. The time-course
252 study showed that phosphorylation of AMPK α Thr¹⁷² increased within 15 min of
253 caffeine stimulation in epitrochlearis and soleus muscles (Figs. 1A and C).
254 Phosphorylation of ACC Ser⁷⁹ displayed a pattern similar to that for AMPK
255 phosphorylation in both muscles (Figs. 1B and D). ACC is a downstream target of
256 AMPK in skeletal muscle, and phosphorylation of the Ser⁷⁹ site of ACC reflects total
257 AMPK activity [29,30]. We chose a caffeine concentration of 3 mmol/L to
258 reevaluate the results of preceding studies in which stimulation with 3-3.5 mmol/L
259 of caffeine for 15 min failed to demonstrate an increase in AMPK α Thr¹⁷²
260 phosphorylation in incubated rat epitrochlearis [14], rat soleus [15,16] and mouse
261 soleus [16] muscles. We also determined the effects of 15 min of stimulation with
262 various concentrations of caffeine. The dose-response study revealed that
263 phosphorylation of AMPK α Thr¹⁷² and ACC Ser⁷⁹ increased at caffeine
264 concentrations of 3 mmol/L or higher in both epitrochlearis (Figs. 2A and B) and
265 soleus (Figs. 2C and D) muscles. The total AMPK and ACC content of the
266 muscles did not change during the study (Figs. 1 and 2).

267

268 3.2. Caffeine increases both AMPK α 1 and AMPK α 2 activities in skeletal muscles

269 To identify which catalytic subunit is activated by caffeine, isoform-specific
270 AMPK activity was determined in anti- α 1 and anti- α 2 immunoprecipitates from
271 epitrochlearis and soleus muscles after treatment with caffeine (3 mmol/L, 15 min).
272 We chose this stimulation protocol to reevaluate the preceding studies by Jensen et
273 al [16], in which 3 mmol/L of caffeine stimulation for 15 min increased AMPK α 1
274 activity but not AMPK α 2 activity in incubated rat soleus muscle, and that of Raney
275 et al [19], in which stimulation with 3 mmol/L caffeine for 20 min increased
276 AMPK α 2 activity in perfused rat hindlimb muscles. In contrast to the results of
277 these two studies, in our study caffeine clearly increased AMPK α 1 activity by
278 2.3-fold and 1.8-fold, and AMPK α 2 activity by 2.6-fold and 3.0-fold in
279 epitrochlearis (Fig. 3A) and soleus muscle (Fig. 3B), respectively.

280

281 3.3. Caffeine decreases ATP, PCr and glycogen content in skeletal muscles

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

291 PCr content was significantly decreased at 15, 30 and 60 min of caffeine incubation.
292 Glycogen content was significantly lower at 60 min of incubation in both
293 epitrochlearis and soleus muscles.

294

295 *3.4. Caffeine acutely increases insulin-independent glucose transport activity in* 296 *skeletal muscles*

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

301

302 *3.5. Caffeine does not affect GLUT4 content in skeletal muscles*

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

309

310 **4. Discussion**

311

312 Our data show three novel findings relating to the metabolic effect of caffeine on
313 skeletal muscle. First, caffeine had the ability to increase AMPK α Thr¹⁷²
314 phosphorylation (Figs. 1 and 2) and both AMPK α 1 and α 2 activities (Fig. 3). The
315 enhanced phosphorylation of ACC, an endogenous substrate of AMPK, (Figs. 1 and
316 2) as well as increased 3MG transport activity (Fig. 4) is indicative of a substantial
317 increase in AMPK activity in vivo. Second, these effects were observed in both
318 fast-glycolytic epitrochlearis and slow-oxidative soleus muscles, suggesting that the
319 stimulatory effect of caffeine on AMPK is not specific to a particular muscle type.
320 Third, caffeine-stimulated AMPK activation was associated with a reduction in the
321 fuel status of skeletal muscle (Table 1), as with contraction-stimulated AMPK
322 activation.

323 The energy deprivation in our study may explain the difference between our
324 results and those of Jensen et al [16] which demonstrated predominant activation of
325 AMPK α 1 by caffeine stimulation. AMPK α 2 has greater AMP dependence than
326 AMPK α 1 in respect of allosteric activation by AMP and covalent activation by
327 upstream kinases [21,31], indicating that AMPK α 2 is more sensitive to energy
328 depletion than is AMPK α 1. In support of this idea, we previously demonstrated
329 that AMPK α 1, but not AMPK α 2, is activated in rat epitrochlearis muscles treated
330 with H₂O₂ and hypoxanthine/xanthine oxidase in the absence of an increase in AMP
331 or a decrease in PCr content [25]. We have also shown that AMPK α 1 is activated
332 in low-intensity contracting muscle in which AMP concentration is not elevated,
333 whereas AMPK α 1 and α 2 are activated in high-intensity contracting muscle, in

334 which the AMP concentration is significantly higher than the resting value [32]. In
335 the present study, we found that 15 min of treatment with 3 mmol/L of caffeine
336 significantly decreased the PCr content in both the epitrochlearis and soleus muscles
337 (Table 1). On the other hand, Jensen et al [16] did not detect any changes in energy
338 status in mouse soleus muscles treated with 3 mmol/L caffeine. Therefore,
339 although the reasons for the difference in the results of the energy assays are
340 unknown, the robust AMPK α 2 activation in our study may be explained by a
341 decrease in energy status induced by caffeine stimulation.

342 The difference between our results and those of the study by Raney et al [19],
343 who showed that caffeine increases AMPK α 2 activation, may be explained by the
344 different muscle preparations used: caffeine incubation in our study and caffeine
345 perfusion in their study. Because AMPK α 1, but not AMPK α 2, is activated
346 immediately as a postmortem artifact during the isolation procedure [32], we
347 measured AMPK activity after a preincubation period (40 min) that was sufficient to
348 stabilize AMPK α 1 activity at the basal level. This method enabled us to examine
349 the effect of caffeine on both AMPK α 1 and AMPK α 2 activities. In contrast, Raney
350 et al measured AMPK activity in muscles isolated after caffeine perfusion. The
351 actual AMPK α 1 activity may be increased by caffeine but it may also be disturbed
352 by additional activation during isolation, because an increase in AMPK α 1 activity
353 would be detectable only when the activation by caffeine exceeds that of the
354 isolating stimuli.

355 Preceding studies have shown that stimulation with 3-3.5 mmol/L caffeine for
356 15 min enhances glucose transport without an apparent increase in AMPK α Thr¹⁷²
357 phosphorylation in incubated skeletal muscles [14-16]. However, Jensen et al [16]

358 found that some individual pairs of rat soleus muscles incubated with 3 mmol/L
359 caffeine for 15 min clearly displayed greater AMPK α Thr¹⁷² phosphorylation, and
360 they proposed that evaluation of AMPK activation by measuring AMPK α Thr¹⁷²
361 phosphorylation is prone to statistical type 2 error, which may lead to false
362 conclusions that caffeine does not activate AMPK. In fact, Jensen et al [16] have
363 shown that in mice with muscle-specific expression of a dominant-negative,
364 kinase-dead AMPK mutant (AMPK-KD), glucose transport is blocked in response to
365 caffeine stimulation (3 mmol/L, 15 min) in isolated soleus muscles. Thus, it is
366 likely that AMPK is a signaling intermediary leading to caffeine-stimulated glucose
367 transport in skeletal muscle. We believe that we eliminated type 2 errors because
368 of the rapid and gentle isolation procedure, which minimally stimulated AMPK, and
369 because of sufficient preincubation, which decreased AMPK activity to a constant
370 level. We note that all muscle samples incubated with 3 mmol/L caffeine for 15
371 min showed stronger Western blot signals for AMPK α Thr¹⁷² phosphorylation than
372 control samples (data not shown).

373 The finding that caffeine increased AMPK α Thr¹⁷² phosphorylation in
374 epitrochlearis and soleus muscles (Figs. 1 and 2) provides evidence that caffeine
375 induces covalent modification via upstream kinases. The LKB1 complex is the
376 main kinase that regulates AMPK α 2 activity in mouse skeletal muscle during tetanic
377 contraction in situ and in vitro [33]. The LKB1 complex is constitutively active
378 and is not activated directly by AMP binding of AMP to AMPK facilitates the
379 phosphorylation of AMPK by the LKB1 complex [34,35]. Thus, LKB1 is believed
380 to be a crucial AMPK kinase in the response to energy deprivation in skeletal muscle
381 during intense contraction. In the present study, AMPK activation was

382 accompanied by a decrease in PCr content (Table 1), raising the possibility that
383 LKB1 is involved in AMPK α 2 activation by caffeine. On the other hand, Jensen et
384 al [16] have shown that caffeine-induced AMPK α 1 activation and 2-deoxyglucose
385 transport in mouse skeletal muscle is blocked by the Ca²⁺/calmodulin kinase kinase
386 (CaMKK) inhibitor, STO-609. Jensen et al [36] have also shown that STO-609
387 inhibits activation of AMPK α 1 and AMPK α 2 as well as AMPK α Thr¹⁷²
388 phosphorylation in mouse skeletal muscles after a low-intensity tetanic contraction
389 in vitro. Thus, CaMKK might be the upstream kinase responsible for the
390 caffeine-induced AMPK α 1 activation observed in our study.

391 Epidemiological studies show that long-term consumption of beverages
392 containing caffeine such as coffee and green tea is associated with a reduced risk of
393 type 2 diabetes [37-39]. Some researchers believe that caffeine reduces the risk of
394 diabetes [37,40], although others do not [38,39]. Considering that caffeine and
395 exercise exert similar effects in stimulating AMPK, caffeine may be the active
396 ingredient responsible for the preventive effect of coffee and green tea on the
397 development of type 2 diabetes. In this context, further studies are needed to
398 clarify whether oral administration of caffeine at a physiological dose results in
399 AMPK activation and induces AMPK-related metabolic events, including glucose
400 transport, in skeletal muscle.

401 In summary, we demonstrated for the first time that caffeine increases AMPK α
402 Thr¹⁷² phosphorylation and both AMPK α 1 and α 2 activities in fast- and slow-twitch
403 skeletal muscles, and that this activation is accompanied by insulin-independent
404 glucose transport and a reduction of muscle energy status. We propose that, similar
405 to exercise, caffeine can activate muscle glucose metabolism by stimulating AMPK.

406

407 **Acknowledgments**

408

409 We thank Licht Miyamoto and Taro Toyoda for suggestions. We also thank
410 Department of Medicine and Clinical Science Kyoto University Graduate School of
411 Medicine and Radioisotope Research Center of Kyoto University for instrumental
412 support. We are grateful to Kaoru Yuge, Kaoru Ijiri and Yoko Koyama for
413 secretarial assistance. Tatsuya Hayashi was supported by a research grant from the
414 Japan Society for the Promotion of Science (20500576). Tatsuya Hayashi and Taku
415 Hamada were supported by a research grant from the All Japan Coffee Association.

416

417 **References**

418

- 419 1 Kubo K and Foley JE, Rate-limiting steps for insulin-mediated glucose uptake
420 into perfused rat hindlimb. *Am J Physiol*, 1986;250:E100-2.
- 421 2 Cline GW, Petersen KF, Krssak M, et al., Impaired glucose transport as a cause
422 of decreased insulin-stimulated muscle glycogen synthesis in type 2 diabetes. *N*
423 *Engl J Med*, 1999;341:240-6.
- 424 3 Kahn BB, Alquier T, Carling D, et al., AMP-activated protein kinase: ancient
425 energy gauge provides clues to modern understanding of metabolism. *Cell*
426 *Metab*, 2005;1:15-25.
- 427 4 Hardie DG, Hawley SA, and Scott JW, AMP-activated protein
428 kinase--development of the energy sensor concept. *J Physiol*, 2006;574:7-15.
- 429 5 Musi N and Goodyear LJ, Insulin resistance and improvements in signal
430 transduction. *Endocrine*, 2006;29:73-80.
- 431 6 Zheng D, MacLean PS, Pohnert SC, et al., Regulation of muscle GLUT-4
432 transcription by AMP-activated protein kinase. *J Appl Physiol*,
433 2001;91:1073-83.
- 434 7 Nakano M, Hamada T, Hayashi T, et al., α 2 Isoform-specific activation of
435 5'adenosine monophosphate-activated protein kinase by
436 5-aminoimidazole-4-carboxamide-1-beta-d-ribonucleoside at a physiological
437 level activates glucose transport and increases glucose transporter 4 in mouse
438 skeletal muscle. *Metabolism*, 2006;55:300-8.
- 439 8 Jørgensen SB, Nielsen JN, Birk JB, et al., The α 2-5'AMP-activated protein
440 kinase is a site 2 glycogen synthase kinase in skeletal muscle and is responsive

441 to glucose loading. Diabetes, 2004;53:3074-81.

442 9 Miyamoto L, Toyoda T, Hayashi T, et al., Effect of acute activation of

443 5'-AMP-activated protein kinase on glycogen regulation in isolated rat skeletal

444 muscle. J Appl Physiol, 2007;102:1007-13.

445 10 Winder WW and Hardie DG, Inactivation of acetyl-CoA carboxylase and

446 activation of AMP-activated protein kinase in muscle during exercise. Am J

447 Physiol, 1996;270:E299-304.

448 11 Vavvas D, Apazidis A, Saha AK, et al., Contraction-induced changes in

449 acetyl-CoA carboxylase and 5'-AMP-activated kinase in skeletal muscle. J Biol

450 Chem, 1997;272:13255-61.

451 12 Fiedler M, Zierath JR, Selen G, et al.,

452 5-aminoimidazole-4-carboxy-amide-1-beta-D-ribofuranoside treatment

453 ameliorates hyperglycaemia and hyperinsulinaemia but not dyslipidaemia in

454 KKA^y-CETP mice. Diabetologia, 2001;44:2180-6.

455 13 Tanaka S, Hayashi T, Toyoda T, et al., High-fat diet impairs the effects of a

456 single bout of endurance exercise on glucose transport and insulin sensitivity in

457 rat skeletal muscle. Metabolism, 2007;56:1719-28.

458 14 Wright DC, Hucker KA, Holloszy JO, et al., Ca²⁺ and AMPK both mediate

459 stimulation of glucose transport by muscle contractions. Diabetes,

460 2004;53:330-5.

461 15 Cantó C, Chibalin AV, Barnes BR, et al., Neuregulins mediate calcium-induced

462 glucose transport during muscle contraction. J Biol Chem, 2006;281:21690-7.

463 16 Jensen TE, Rose AJ, Hellsten Y, et al., Caffeine-induced Ca²⁺ release increases

464 AMPK-dependent glucose uptake in rodent soleus muscle. Am J Physiol

- 465 Endocrinol Metab, 2007;293:E286-92.
- 466 17 Ojuka EO, Jones TE, Nolte LA, et al., Regulation of GLUT4 biogenesis in
467 muscle: evidence for involvement of AMPK and Ca^{2+} . Am J Physiol Endocrinol
468 Metab, 2002;282:E1008-13.
- 469 18 Mukwevho E, Kohn TA, Lang D, et al., Caffeine induces hyperacetylation of
470 histones at the MEF2 site on the *Glut4* promoter and increases MEF2A binding
471 to the site via a CaMK-dependent mechanism. Am J Physiol Endocrinol Metab,
472 2008;294:E582-8.
- 473 19 Raney MA and Turcotte LP, Evidence for the involvement of CaMKII and
474 AMPK in Ca^{2+} -dependent signaling pathways regulating FA uptake and
475 oxidation in contracting rodent muscle. J Appl Physiol, 2008;104:1366-73.
- 476 20 Stapleton D, Mitchelhill KI, Gao G, et al., Mammalian AMP-activated protein
477 kinase subfamily. J Biol Chem, 1996;271:611-4.
- 478 21 Stein SC, Woods A, Jones NA, et al., The regulation of AMP-activated protein
479 kinase by phosphorylation. Biochem J, 2000;345 Pt 3:437-43.
- 480 22 Nesher R, Karl IE, Kaiser KE, et al., Epitrochlearis muscle. I. Mechanical
481 performance, energetics, and fiber composition. Am J Physiol,
482 1980;239:E454-60.
- 483 23 Armstrong RB and Phelps RO, Muscle fiber type composition of the rat
484 hindlimb. Am J Anat, 1984;171:259-72.
- 485 24 Hayashi T, Hirshman MF, Kurth EJ, et al., Evidence for 5' AMP-activated
486 protein kinase mediation of the effect of muscle contraction on glucose transport.
487 Diabetes, 1998;47:1369-73.
- 488 25 Toyoda T, Hayashi T, Miyamoto L, et al., Possible involvement of the $\alpha 1$

489 isoform of 5'AMP-activated protein kinase in oxidative stress-stimulated glucose
 490 transport in skeletal muscle. Am J Physiol Endocrinol Metab,
 491 2004;287:E166-73.

492 26 Hayashi T, Hirshman MF, Fujii N, et al., Metabolic stress and altered glucose
 493 transport: activation of AMP-activated protein kinase as a unifying coupling
 494 mechanism. Diabetes, 2000;49:527-31.

495 27 Lowry OH and Passonneau JV. A Flexible System of Enzymatic Analysis. New
 496 York: Academic Press. 1972. p. 151-156

497 28 Young DA, Uhl JJ, Cartee GD, et al., Activation of glucose transport in muscle
 498 by prolonged exposure to insulin. Effects of glucose and insulin concentrations.
 499 J Biol Chem, 1986;261:16049-53.

500 29 Davies SP, Sim AT, and Hardie DG, Location and function of three sites
 501 phosphorylated on rat acetyl-CoA carboxylase by the AMP-activated protein
 502 kinase. Eur J Biochem, 1990;187:183-90.

503 30 Park H, Kaushik VK, Constant S, et al., Coordinate regulation of malonyl-CoA
 504 decarboxylase, *sn*-glycerol-3-phosphate acyltransferase, and acetyl-CoA
 505 carboxylase by AMP-activated protein kinase in rat tissues in response to
 506 exercise. J Biol Chem, 2002;277:32571-7.

507 31 Salt I, Celler JW, Hawley SA, et al., AMP-activated protein kinase: greater
 508 AMP dependence, and preferential nuclear localization, of complexes containing
 509 the $\alpha 2$ isoform. Biochem J, 1998;334 (Pt 1):177-87.

510 32 Toyoda T, Tanaka S, Ebihara K, et al., Low-intensity contraction activates the
 511 $\alpha 1$ -isoform of 5'-AMP-activated protein kinase in rat skeletal muscle. Am J
 512 Physiol Endocrinol Metab, 2006;290:E583-90.

- 513 33 Sakamoto K, McCarthy A, Smith D, et al., Deficiency of LKB1 in skeletal
514 muscle prevents AMPK activation and glucose uptake during contraction. *Embo*
515 *J*, 2005;24:1810-20.
- 516 34 Hawley SA, Boudeau J, Reid JL, et al., Complexes between the LKB1 tumor
517 suppressor, STRAD α/β and MO25 α/β are upstream kinases in the
518 AMP-activated protein kinase cascade. *J Biol*, 2003;2:28.1-28.16.
- 519 35 Sakamoto K, Göransson O, Hardie DG, et al., Activity of LKB1 and
520 AMPK-related kinases in skeletal muscle: effects of contraction, phenformin,
521 and AICAR. *Am J Physiol Endocrinol Metab*, 2004;287:E310-7.
- 522 36 Jensen TE, Rose AJ, Jorgensen SB, et al., Possible CaMKK-dependent
523 regulation of AMPK phosphorylation and glucose uptake at the onset of mild
524 tetanic skeletal muscle contraction. *Am J Physiol Endocrinol Metab*,
525 2007;292:E1308-17.
- 526 37 Iso H, Date C, Wakai K, et al., The relationship between green tea and total
527 caffeine intake and risk for self-reported type 2 diabetes among Japanese adults.
528 *Ann Intern Med*, 2006;144:554-62.
- 529 38 van Dam RM, Willett WC, Manson JE, et al., Coffee, caffeine, and risk of type
530 2 diabetes: a prospective cohort study in younger and middle-aged U.S. women.
531 *Diabetes Care*, 2006;29:398-403.
- 532 39 Pereira MA, Parker ED, and Folsom AR, Coffee consumption and risk of type 2
533 diabetes mellitus: an 11-year prospective study of 28 812 postmenopausal
534 women. *Arch Intern Med*, 2006;166:1311-6.
- 535 40 Williams CJ, Fargnoli JL, Hwang JJ, et al., Coffee consumption is associated
536 with higher plasma adiponectin concentrations in women with or without type 2

537 diabetes: a prospective cohort study. Diabetes Care, 2008;31:504-7.

538

539

540

541 **Legends**

542

543 Fig. 1.

544 Caffeine stimulation increases phosphorylation of AMPK α Thr¹⁷² and ACC Ser⁷⁹ in
545 a time-dependent manner in rat skeletal muscles. Isolated epitrochlearis (A and B)
546 and soleus (C and D) muscles were incubated in the presence of 3 mmol/L caffeine
547 for indicated times. Muscle lysates were then analyzed for phosphorylation of
548 AMPK α Thr¹⁷² (pAMPK; A and C) and ACC Ser⁷⁹ (pACC; B and D) by Western
549 blot analysis. Fold increases are expressed relative to the level of muscles in the
550 control (0 min incubation) group. Representative immunoblots are shown. Values
551 are mean $\pm$ SE. The number of muscles in each group is as follows: epitrochlearis;
552 control (4), 15 min (4), 30 min (6), and 60 min (4), soleus; control (4), 15 min (6),
553 30 min (6), and 60 min (4). * p <.05, ** p <.01 vs. control.

554

555 Fig. 2.

556 Caffeine stimulation increases phosphorylation of AMPK α Thr¹⁷² and ACC Ser⁷⁹ in
557 a dose-dependent manner in rat skeletal muscles. Isolated epitrochlearis (A and B)
558 and soleus (C and D) muscles were incubated in the absence (Control) or presence of
559 caffeine at indicated concentration for 15 min. Muscle lysates were then analyzed
560 for phosphorylation of AMPK α Thr¹⁷² (pAMPK; A and C) and ACC Ser⁷⁹ (pACC; B
561 and D) by Western blot analysis. Fold increases are expressed relative to the level
562 of muscles in the control group. Representative immunoblots are shown. Values
563 are mean $\pm$ SE. The number of muscles in each group is as follows: epitrochlearis;
564 control (6), 1 mmol/L (6), 3 mmol/L (6), 6 mmol/L (8), 9 mmol/L (6) and 15

565 mmol/L (6), soleus; control (10), 1 mmol/L (6), 3 mmol/L (14), 6 mmol/L (6), 9
566 mmol/L (6) and 15 mmol/L (6). * $p < .05$, ** $p < .01$ vs. control.

567

568 Fig. 3.

569 Caffeine stimulation activates both AMPK α 1 and AMPK α 2 activity in rat skeletal
570 muscles. Isolated epitrochlearis (A) and soleus (B) muscles were incubated in the
571 absence (Control) or presence of 3 mmol/L caffeine for 15 min. Isoform-specific
572 AMPK activity was determined in anti-AMPK α 1 and -AMPK α 2
573 immunoprecipitates. Fold increases are expressed relative to the activity of
574 muscles in the control group. Values are mean $\pm$ SE. The number of muscles in
575 each group is as follows: epitrochlearis; control (8), caffeine (8), soleus; control (6),
576 caffeine (6). ** $p < .01$ vs. control.

577

578 Fig. 4.

579 Caffeine stimulation increases 3-O-methyl-D-glucose (3MG) transport in rat skeletal
580 muscles. Isolated epitrochlearis (A) and soleus (B) muscles were incubated in the
581 absence (Control) or presence of 3 mmol/L caffeine for 15 min, and then 3MG
582 transport activity was determined. Values are mean $\pm$ SE. The number of muscles
583 in each group is as follows: epitrochlearis; control (6), caffeine (5), soleus; control
584 (6), caffeine (6). ** $p < .01$, vs. control.

585

586 Fig. 5.

587 Caffeine stimulation does not affect GLUT4 content in skeletal muscles. Isolated
588 epitrochlearis (A) and soleus (B) muscles were incubated in the absence (Control) or

589 presence of 3 mmol/L caffeine for 15 min. Muscle lysates were then analyzed for
590 GLUT4 content by Western blot analysis. Fold increases are expressed relative to
591 the level of muscles in the control group. Representative immunoblots are shown.
592 Values are mean $\pm$ SE. n=4 per group.

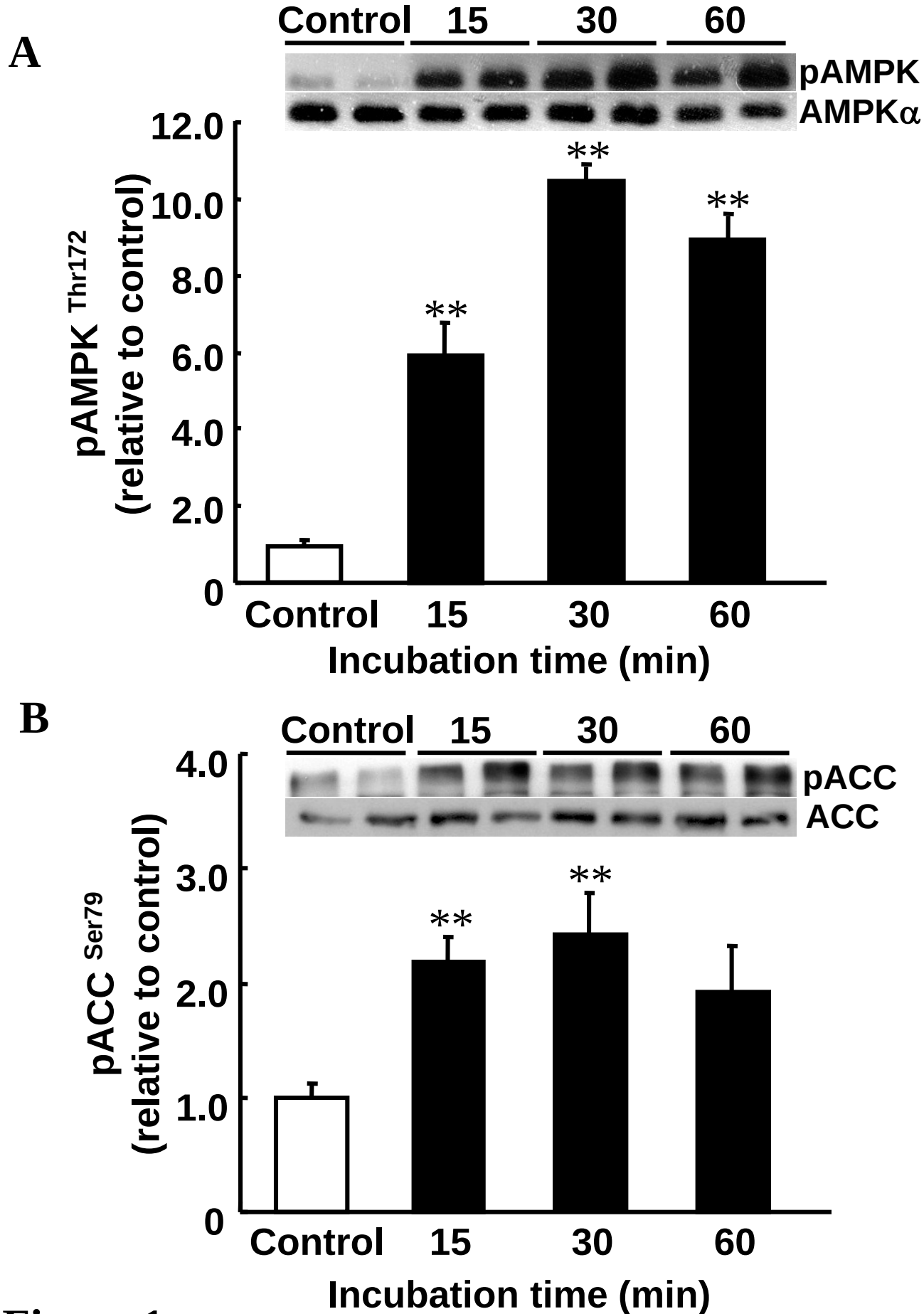
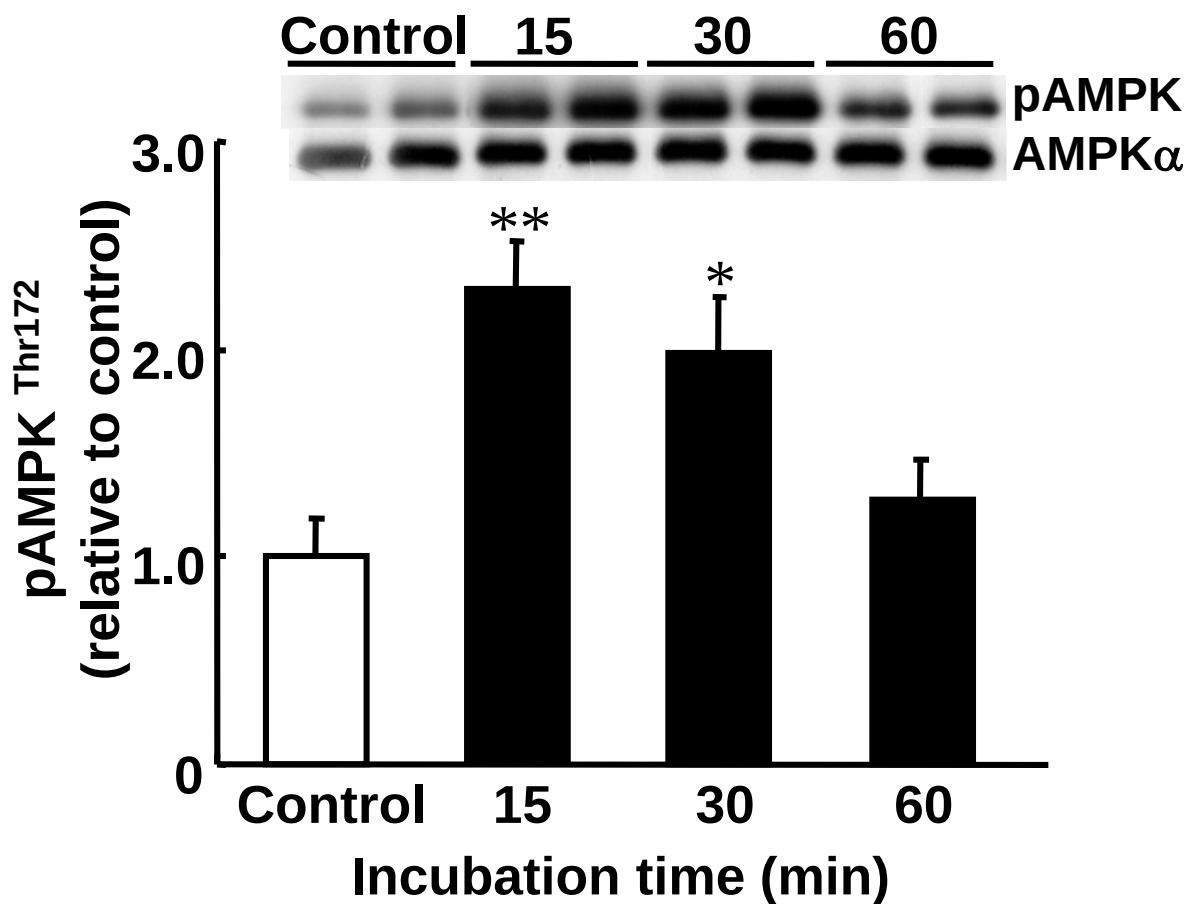
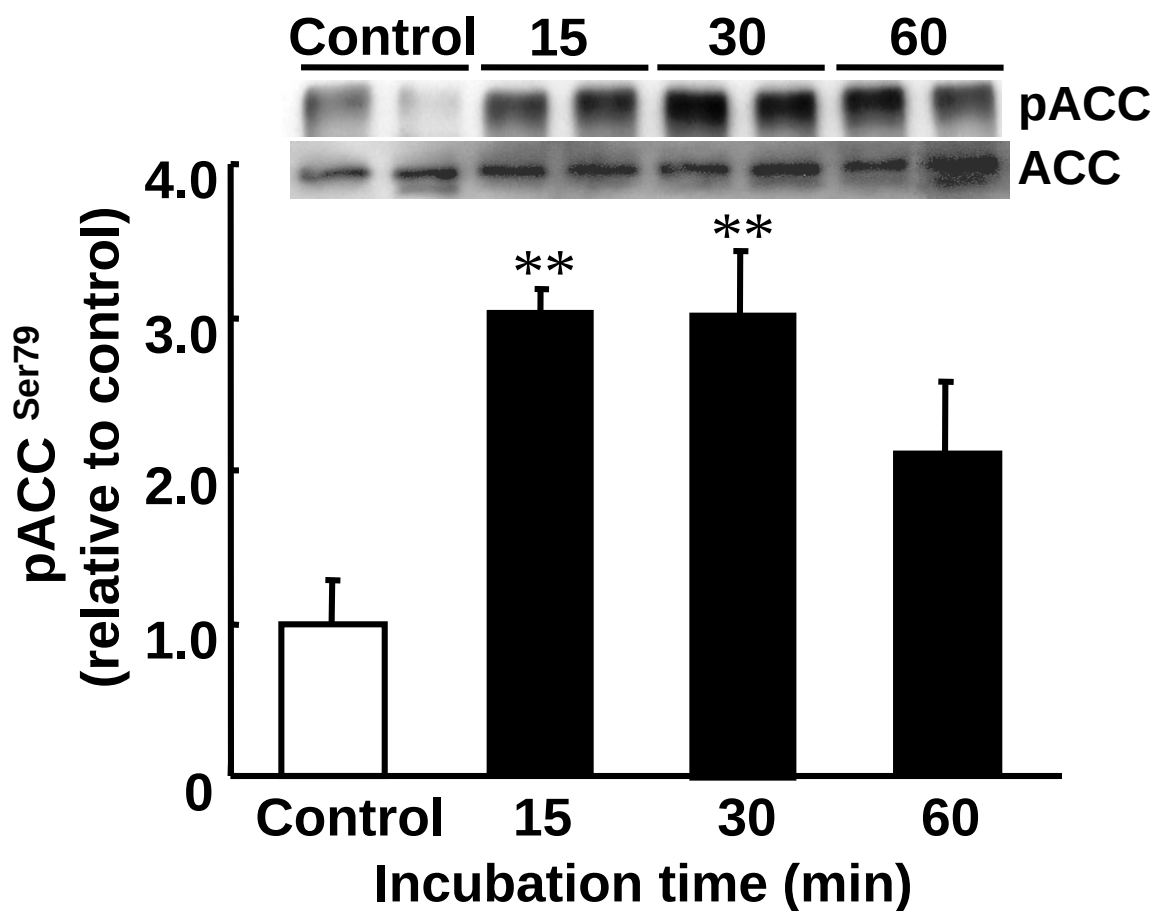
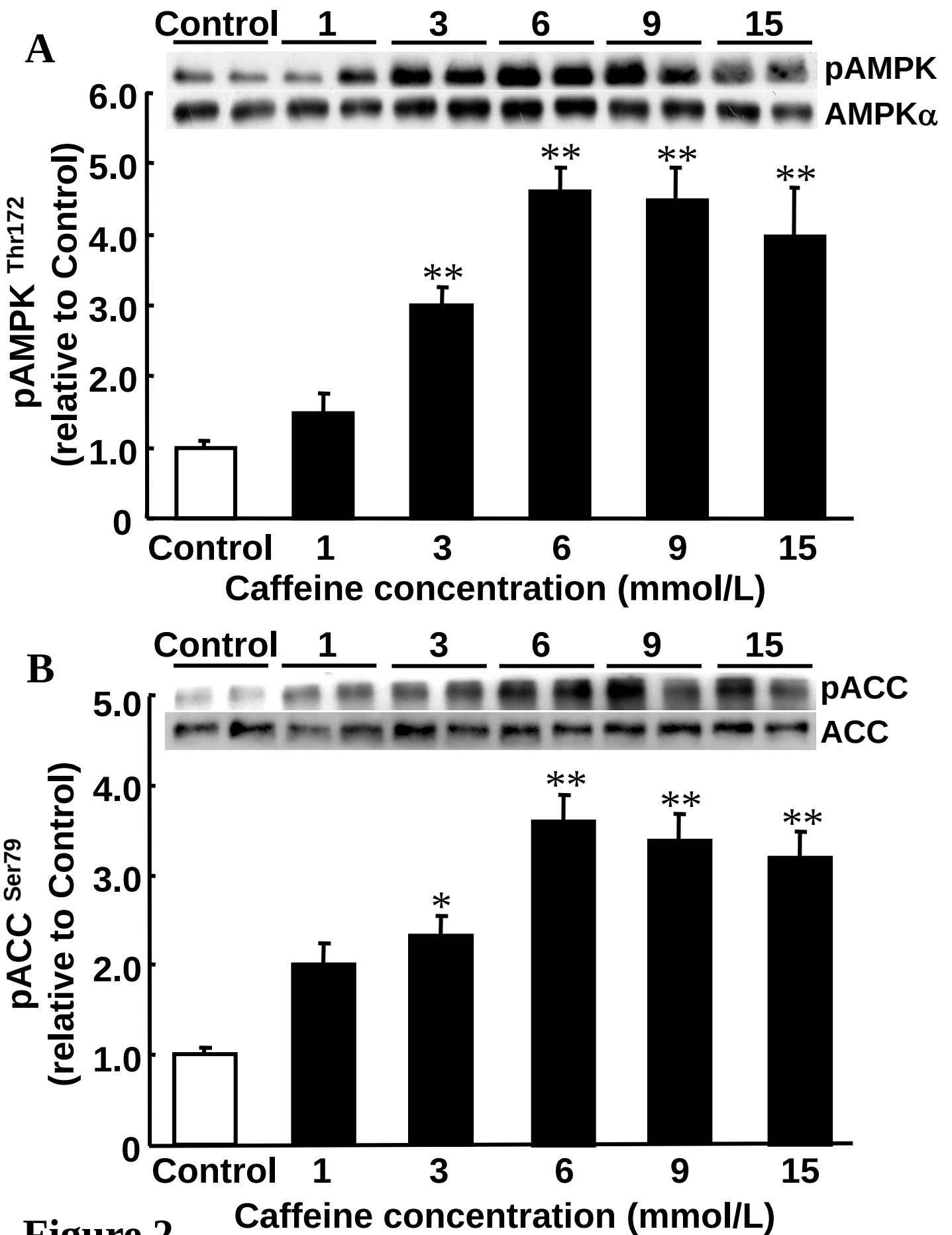
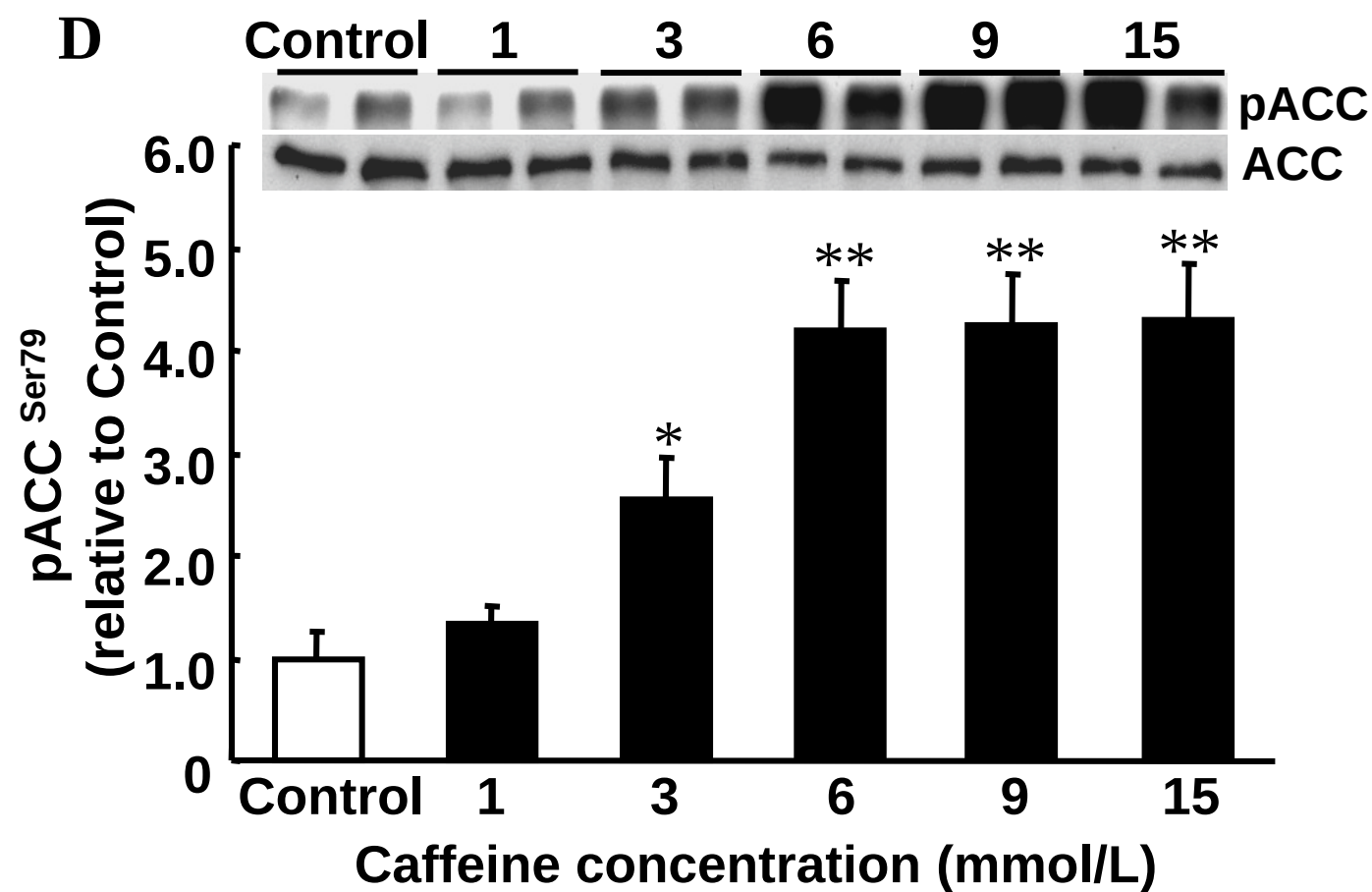
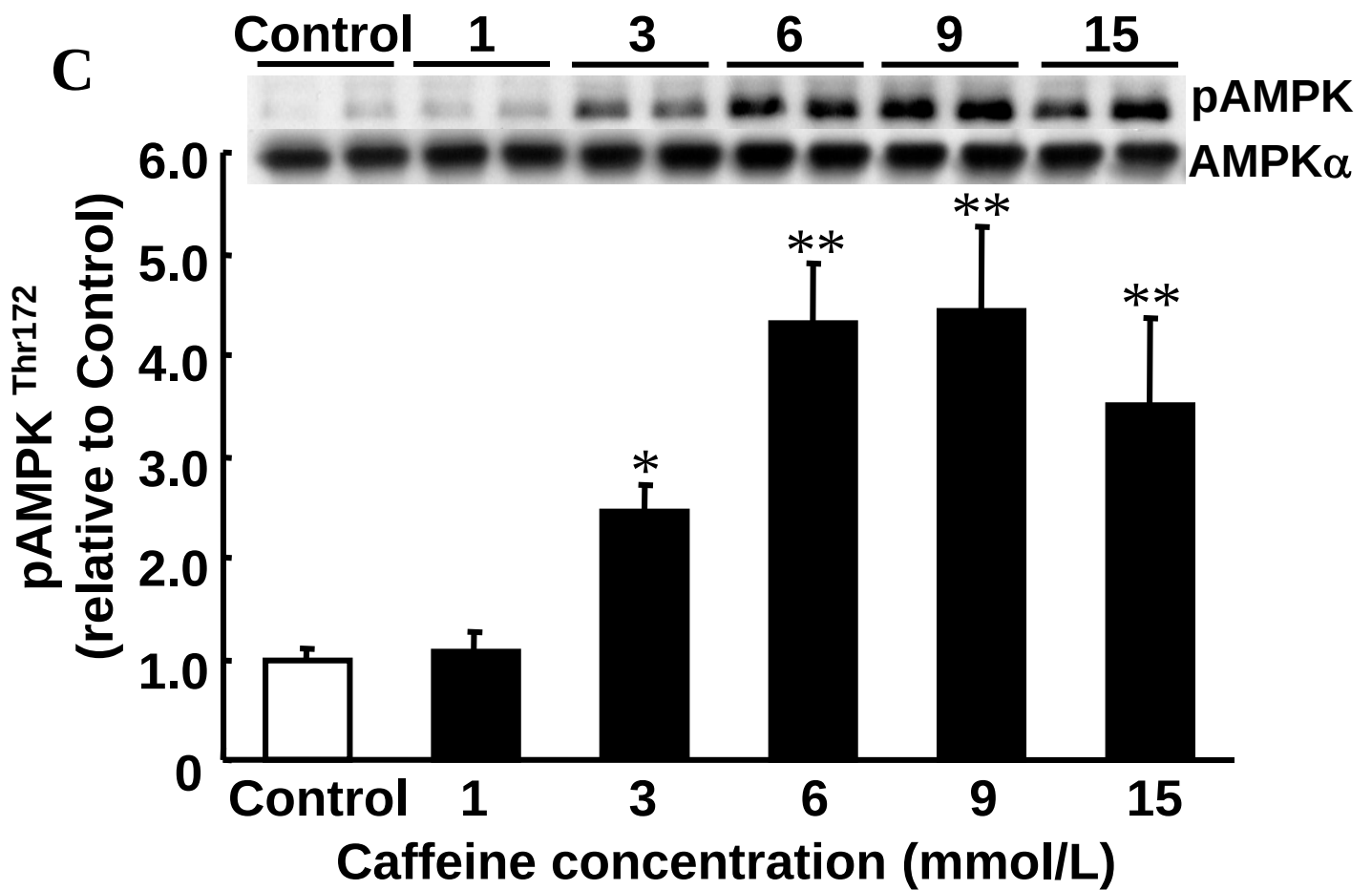
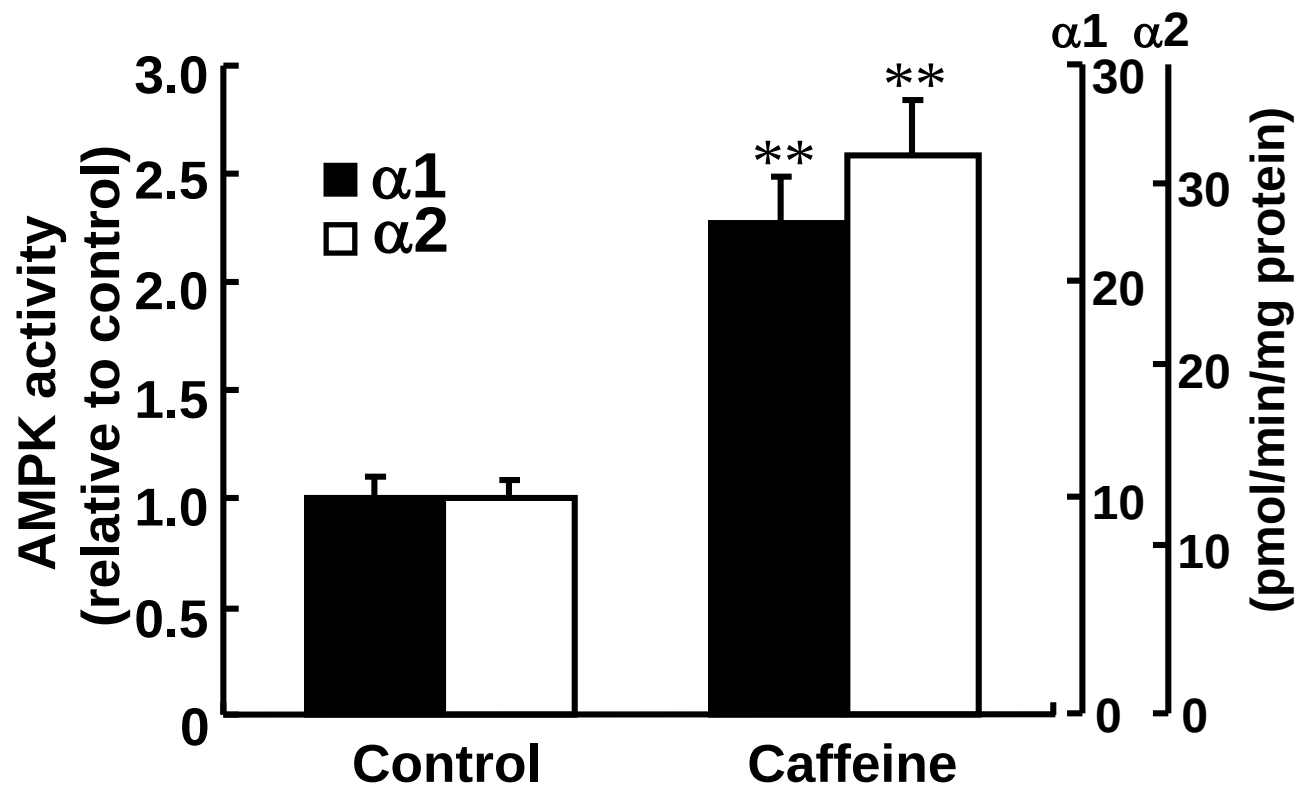
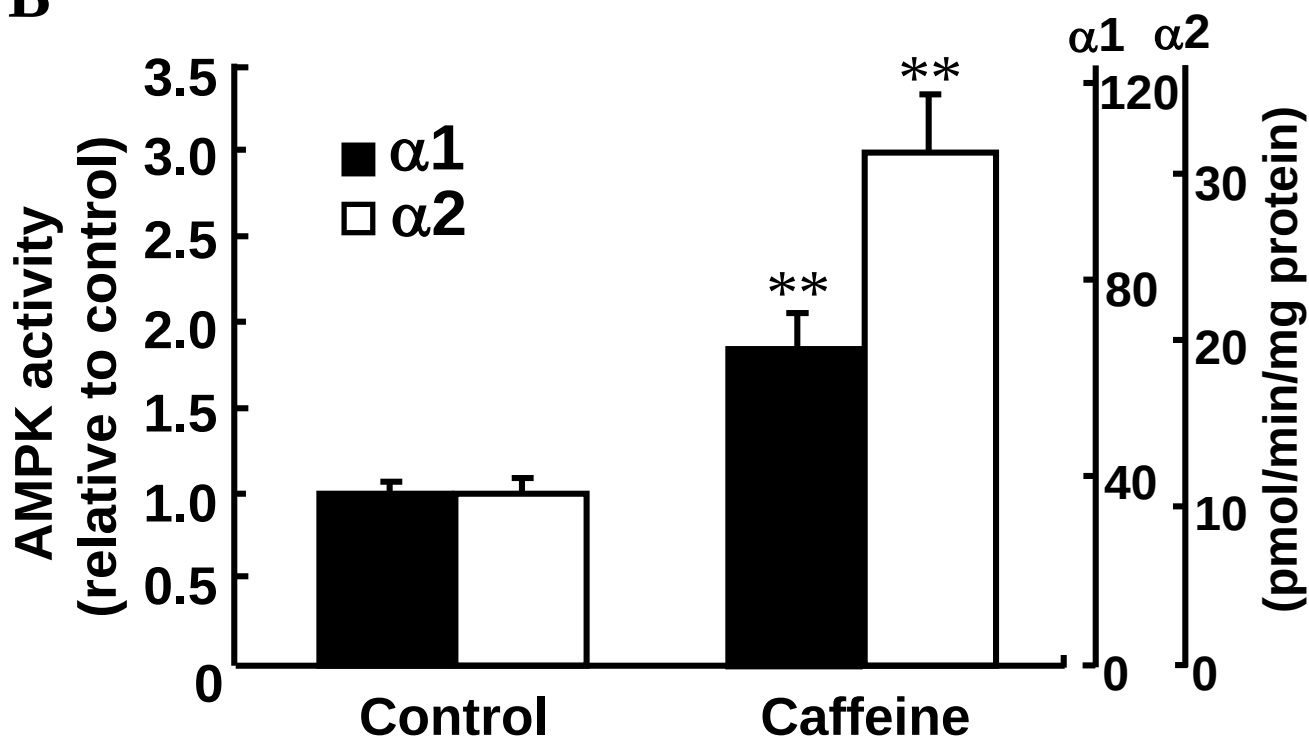


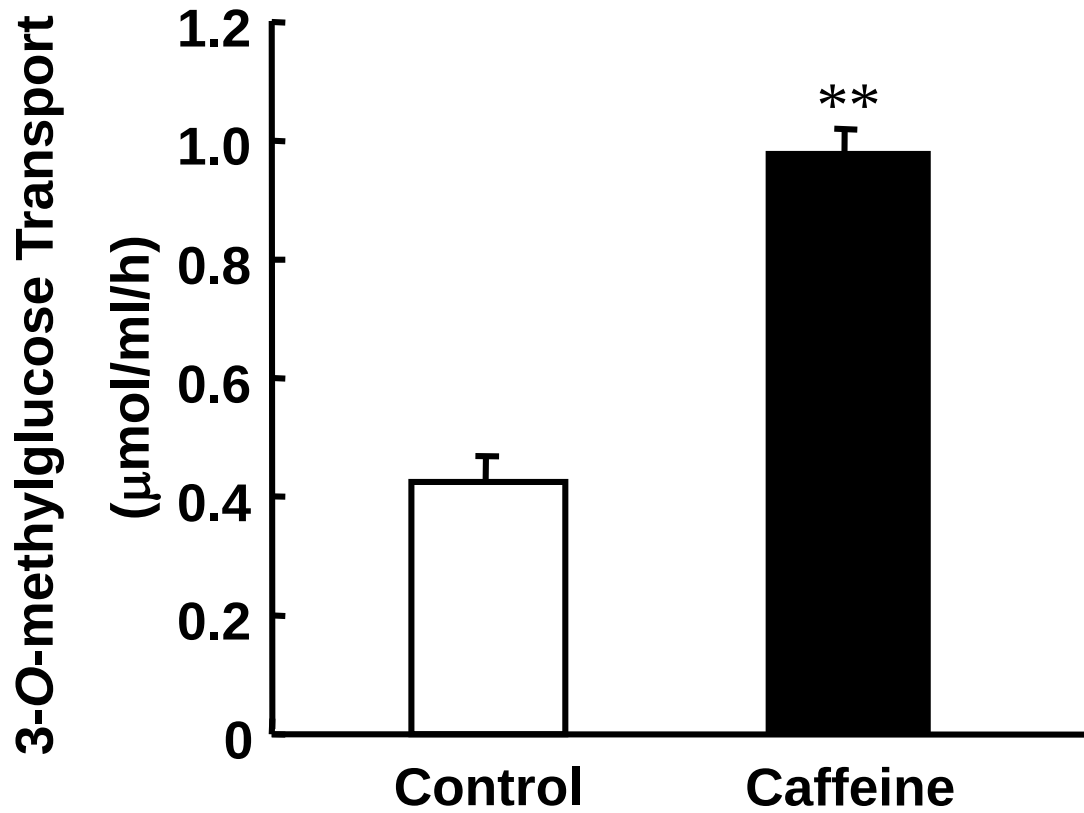
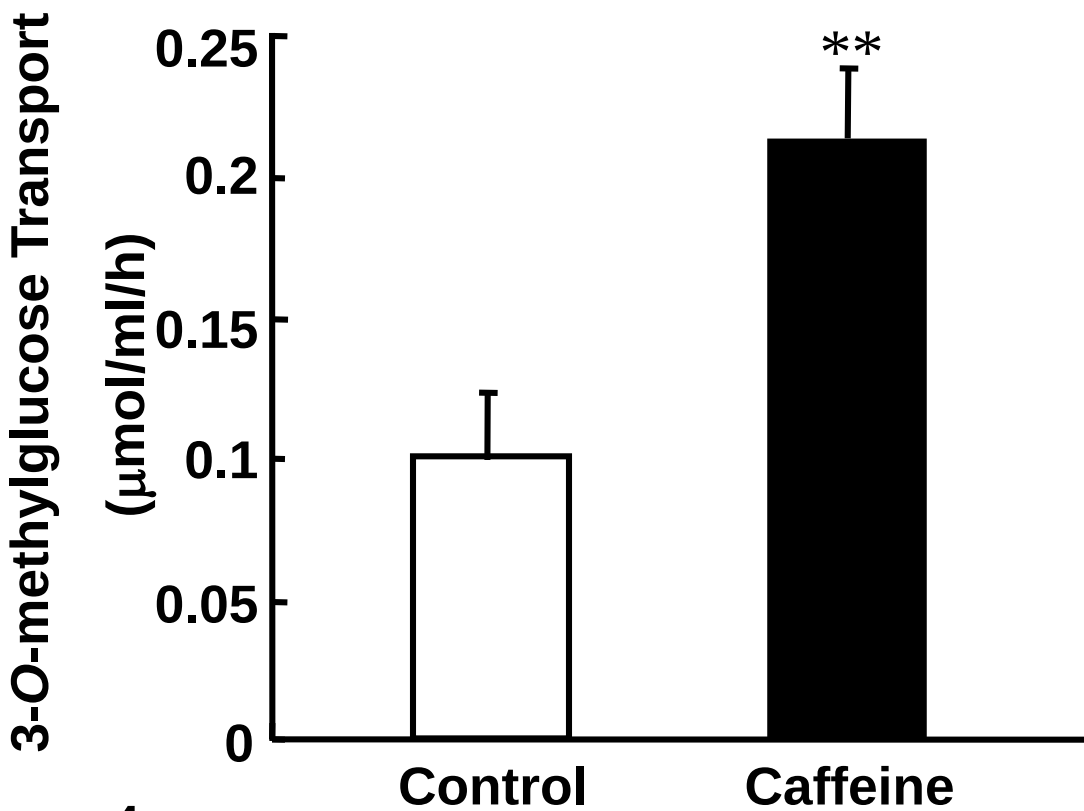
Figure 1

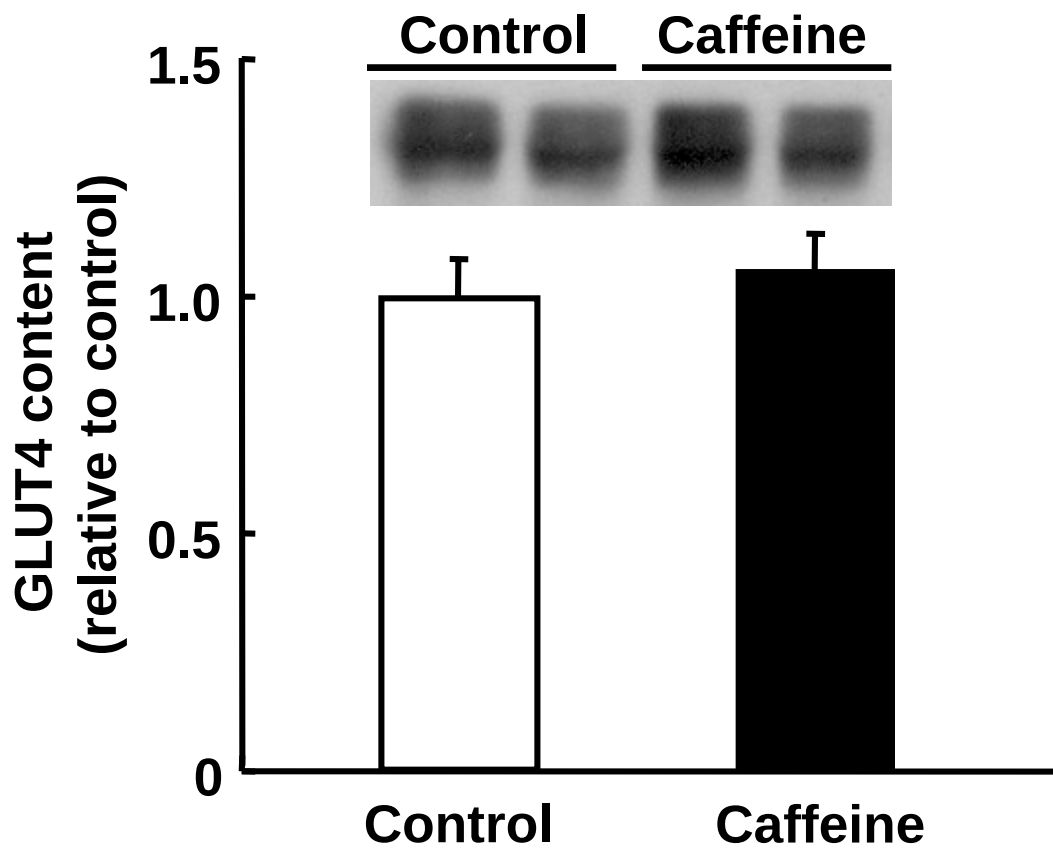
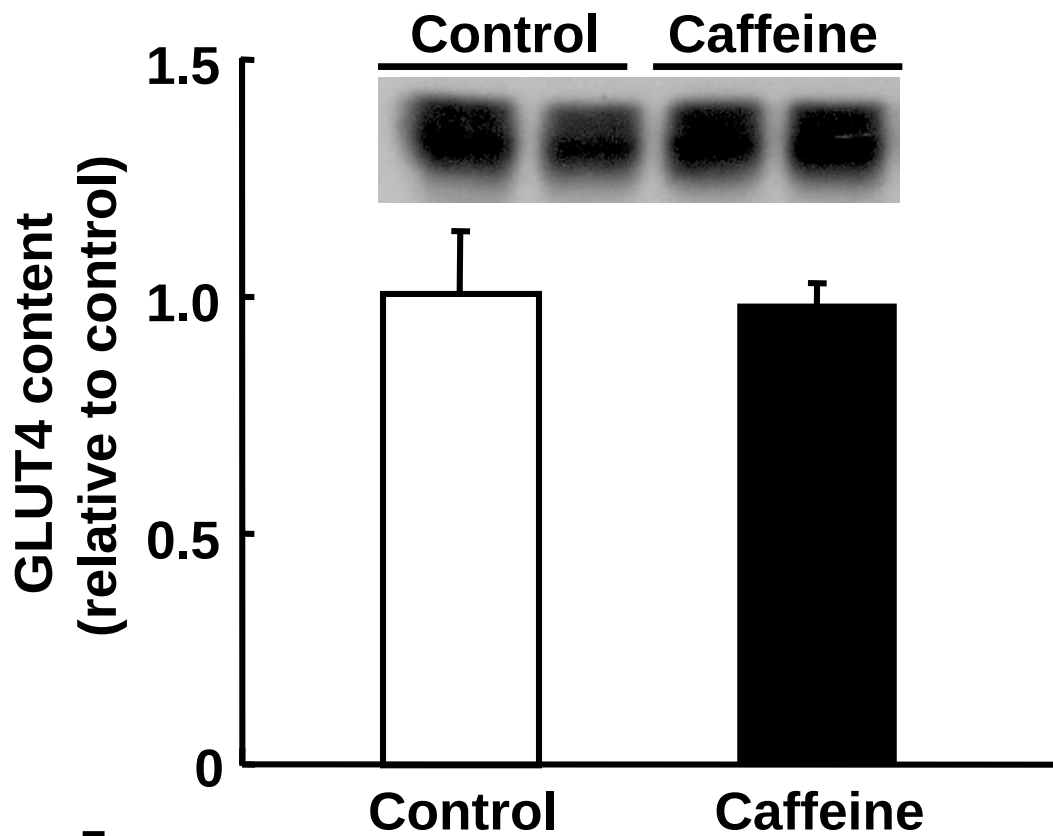
C**D**





A**B****Figure 3**

A**B****Figure 4**

A**B****Figure 5**