Caffeine modulates phosphorylation of insulin receptor substrate-1 and impairs insulin signal transduction in rat skeletal muscle

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Egawa T, Tsuda S, Ma X, Hamada T, Hayashi T. Caffeine modulates phosphorylation of insulin receptor substrate-1 and impairs insulin signal transduction in rat skeletal muscle. J Appl Physiol 111: 1629–1636, 2011. First published September 22, 2011; doi:10.1152/japplphysiol.00249.2011.—Caffeine decreases insulin sensitivity and insulin-stimulated glucose transport in skeletal muscle; however, the precise mechanism responsible for this deleterious effect is not understood fully. We investigated the effects of incubation with caffeine on insulin signaling in rat epitrochlearis muscle. Caffeine (≥1 mM, ≥15 min) suppressed insulin-stimulated insulin receptor substrate (IRS)-1 Tyr612 phosphorylation in a dose- and time-dependent manner. These responses were associated with inhibition of the insulin-stimulated phosphorylation of phosphatidylinositol 3-kinase (PI3K) Tyr458, Akt Ser73, and glycogen synthase kinase-3 β (GSK3-β) Ser9 and with inhibition of insulin-stimulated 3-O-methyl-D-glucose (3MG) transport but not with inhibition of the phosphorylation of insulin receptor-β Tyr1158/1163. Furthermore, caffeine enhanced phosphorylation of IRS-1 Ser307 and an IRS-1 Ser307 kinase, inhibitor-α B kinase (IKK)-α/β Ser177/180. Blockade of IKK/IRS-1 Ser307 by caffeic acid ameliorated the caffeine-induced downregulation of IRS-1 Tyr612 phosphorylation and 3MG transport. Caffeine also increased the phosphorylation of IRS-1 Ser789 and an IRS-1 Ser789 kinase, 5′-AMP-activated protein kinase (AMPK). However, inhibition of IRS-1 Ser789 and AMPK phosphorylation by dantrolene did not rescue the caffeine-induced downregulation of IRS-1 Tyr612 phosphorylation or 3MG transport. In addition, caffeine suppressed the phosphorylation of insulin-stimulated IRS-1 Ser636/639 and upstream kinases, including the mammalian target of rapamycin and p70S6 kinase. Intravenous injection of caffeine at a physiological dose (5 mg/kg) in rats inhibited the phosphorylation of insulin-stimulated IRS-1 Tyr612 and Akt Ser473 in epitrochlearis muscle. Our results indicate that caffeine inhibits insulin signaling partly through the IKK/IRS-1 Ser307 pathway, via a Ca2+- and AMPK-independent mechanism in skeletal muscle.

serine phosphorylation; Akt; 5′-AMP-activated protein kinase; p70 S6 kinase; inhibitor-α B kinase

INSULIN-STIMULATED GLUCOSE TRANSPORT by skeletal muscle plays an important role in the maintenance of whole body glucose homeostasis (10). This biological response requires the tyrosine phosphorylation of insulin receptor (IR) substrate (IRS)-1 and activation of its downstream effectors, such as phosphatidylinositol 3-kinase (PI3K) and Akt. Maintaining proper responses of the IRS-PI3K-Akt pathway is crucial for normal insulin-mediated glucose metabolism in skeletal muscle. Many other signaling pathways can cross-talk with insulin metabolic signaling via the IRS-PI3K-Akt pathway, leading to reduced skeletal muscle responses to insulin and thereby contributing to systemic insulin resistance (2).

Caffeine (1,3,7-trimethylxanthine) is a xanthine alkaloid that has been implicated in the regulation of glucose metabolism in skeletal muscle. A number of studies (1, 11, 12, 26, 43) have shown that acute caffeine stimulation (≥1 mM) increases glucose transport in the absence of insulin in incubated or perfused rodent skeletal muscles, and 5′-AMP-activated protein kinase (AMPK) has been considered part of the mechanism leading to the metabolic activation by caffeine (1, 11, 12, 26). On the other hand, caffeine seems to induce insulin resistance by inhibiting insulin signaling in skeletal muscle. Foukas et al. (14) reported that 10 mM caffeine directly inhibits PI3K activity in vitro and blocks the ability of insulin to stimulate Akt in incubated rat soleus muscle. More recently, Kolnes et al. (28) reported that 10 mM caffeine blocks insulin-stimulated Akt phosphorylation and glucose transport in incubated rat skeletal muscles.

Tyrosine phosphorylation of IRS-1 by the IR allows the binding and activation of PI3K and the subsequent activation of Akt (2, 6, 18, 37). On the other hand, serine phosphorylation of IRS-1 generally negates its ability to undergone tyrosine phosphorylation, thereby leading to an impairment in the insulin signaling cascade (2, 6, 18, 37). Several studies (33, 45) in human and rodent muscles have suggested the increased serine phosphorylation of IRS-1 in the insulin-resistant state.

We hypothesized that the antagonistic effect of caffeine on insulin-stimulated Akt phosphorylation and glucose transport is caused by inhibiting IRS-1 tyrosine phosphorylation through increasing in IRS-1 serine phosphorylation. To test this hypothesis, we investigated the effects of caffeine on insulin signaling molecules (IR, IRS-1, PI3K, and Akt) and glucose transport using an isolated rat skeletal muscle model. We also examined whether a physiological blood concentration of caffeine affects insulin-stimulated Akt phosphorylation and IRS-1 tyrosine phosphorylation in rat skeletal muscle.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 100–120 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 ap-
Previously described (12). Caffeine was dissolved in saline and injected directly into the tail vein without anesthesia at 5 mg/kg body wt. The injection volume was 1 ml/kg body wt. Saline was injected as a control. The rat was temporarily restricted in a Ballman’s cage during injection. Sixty minutes after caffeine or saline injection, rats were anesthetized with intraperitoneal administration of pentobarbital sodium (50 mg/kg).

**Muscle treatment in vitro.** Muscles were treated as we have previously described (11, 20, 39). Rats were killed by cervical dislocation without anesthesia, and the epitrochlearis muscles of each side were rapidly removed. Both ends of each muscle were tied with sutures (silk 3-0, Nitcho Kogyo, Tokyo, Japan), and the muscles were mounted on an incubation apparatus with tension set to 0.5 g. The buffers were continuously gassed with 95% O2-5% CO2 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 CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, and 24.6 mM NaHCO3) containing 2 mM pyruvate for 40 min. The tissue lysate was subjected to Western blot analysis. Fold increases are expressed relative to the level of signal in control muscles. Representative immunoblots are shown. Values are means ± SE; n = 5–16 muscles/group. *P < 0.05 and ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. insulin alone.

**Muscle treatment in vivo.** Muscles were treated as we have previously described (11, 20, 39). Rats were killed by cervical dislocation without anesthesia, and the epitrochlearis muscles of each side were rapidly removed. Both ends of each muscle were tied with sutures (silk 3-0, Nitcho Kogyo, Tokyo, Japan), and the muscles were mounted on an incubation apparatus with tension set to 0.5 g. The buffers were continuously gassed with 95% O2-5% CO2 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 CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, and 24.6 mM NaHCO3) containing 2 mM pyruvate for 40 min. The tissue lysate was subjected to Western blot analysis. Fold increases are expressed relative to the level of signal in control muscles. Representative immunoblots are shown. Values are means ± SE; n = 5–16 muscles/group. *P < 0.05 and ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. insulin alone.

**Fig. 1. Dose- and time-dependent effects of caffeine on insulin receptor (IR) substrate (IRS)-1 Tyr(1122) phosphorylation (A) or p-Tyr(1158/1162/1163) phosphorylation of IRS-1 (B) in incubated rat epitrochlearis muscle.** A: isolated muscles were preincubated for 40 min and incubated in the presence of caffeine at the indicated concentrations for 15 min. Muscles were then incubated with or without 1 μM insulin in the presence of caffeine at the indicated concentrations for 15 min. B: isolated muscles were preincubated for 40 min and incubated with 3 mM caffeine for the indicated times. Muscles were then incubated with 1 μM insulin in the presence of caffeine at the indicated concentrations for 15 min. Control muscles (open bars) were incubated without caffeine or insulin for 30 min. The tissue lysate was subjected to Western blot analysis. Fold increases are expressed relative to the level of signal in control muscles. Representative immunoblots are shown. Values are means ± SE; n = 5–16 muscles/group. *P < 0.05 and ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. insulin alone.

**Fig. 2. Effect of caffeine on IRβ Tyr1158/1162/1163 phosphorylation (A), phosphatidylinositol 3-kinase p85 phosphorylation (B), Akt Ser473 phosphorylation (C), glycogen synthase kinase (GSK)-3β Ser9 phosphorylation (D), and 3-O-methyl-d-glucose (3MG) transport assay (E) in incubated rat epitrochlearis muscle.** Isolated muscles were preincubated for 40 min and incubated for 15 min in the absence or presence of 3 mM caffeine. Muscles were then incubated with or without 1 μM insulin in the absence or presence of 3 mM caffeine for 15 min. Control muscles were then incubated with or without 1 μM insulin in the absence or presence of 3 mM caffeine for 15 min. Control samples were identically preincubated and incubated without caffeine or insulin stimulation. In some experiments, caffeic acid (Sigma, St. Louis, MO) or dantrolene (Sigma) was added during the preincubation and incubation periods to inhibit inhibitor-κB kinase (IKK) (32) and AMPK (26), respectively. The maximal concentration of vehicle (DMSO) for dantrolene was 0.1%, which did not affect any assay. The muscles were then used for the measurement of glucose transport or 3-0, Nitcho Kogyo, Tokyo, Japan), and the muscles were mounted on an incubation apparatus with tension set to 0.5 g. The buffers were continuously gassed with 95% O2-5% CO2 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 CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, and 24.6 mM NaHCO3) containing 2 mM pyruvate for 40 min. The tissue lysate was subjected to Western blot analysis. Fold increases are expressed relative to the level of signal in control muscles. Representative immunoblots are shown. Values are means ± SE; n = 5–16 muscles/group. *P < 0.05 and ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. insulin alone.

**A**

**B**

**C**

**D**

**E**

**Fig. 2. Effect of caffeine on IRβ Tyr1158/1162/1163 phosphorylation (A), phosphatidylinositol 3-kinase p85 phosphorylation (B), Akt Ser473 phosphorylation (C), glycogen synthase kinase (GSK)-3β Ser9 phosphorylation (D), and 3-O-methyl-d-glucose (3MG) transport assay (E) in incubated rat epitrochlearis muscle.** Isolated muscles were preincubated for 40 min and incubated for 15 min in the absence or presence of 3 mM caffeine. Muscles were then incubated with or without 1 μM insulin in the absence or presence of 3 mM caffeine for 15 min. Control muscles were then incubated with or without 1 μM insulin in the absence or presence of 3 mM caffeine for 15 min. Control samples were identically preincubated and incubated without caffeine or insulin stimulation. In some experiments, caffeic acid (Sigma, St. Louis, MO) or dantrolene (Sigma) was added during the preincubation and incubation periods to inhibit inhibitor-κB kinase (IKK) (32) and AMPK (26), respectively. The maximal concentration of vehicle (DMSO) for dantrolene was 0.1%, which did not affect any assay. The muscles were then used for the measurement of glucose transport or 3-0, Nitcho Kogyo, Tokyo, Japan), and the muscles were mounted on an incubation apparatus with tension set to 0.5 g. The buffers were continuously gassed with 95% O2-5% CO2 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 CaCl2, 1.2 mM KH2PO4, 1.2 mM MgSO4, and 24.6 mM NaHCO3) containing 2 mM pyruvate for 40 min. The tissue lysate was subjected to Western blot analysis. Fold increases are expressed relative to the level of signal in control muscles. Representative immunoblots are shown. Values are means ± SE; n = 5–16 muscles/group. *P < 0.05 and ***P < 0.001 vs. control; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. insulin alone.

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Muscles were homogenized in ice-cold lysis buffer [1:40 (wt/vol)] containing 20 mM Tris-HCl (pH 7.4), 1% Triton X-100, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 2 mM DTT, 4 mg/l leupeptin, 50 mg/l trypsin inhibitor, 0.1 mM benzamidine, 1 mM Na3VO4, and 0.5 mM PMSF and centrifuged at 16,000 g.

Western blot analysis. Samples were prepared and Western blot analysis performed as we have previously described (11, 24, 39).

Fig. 3. Effect of caffeine on IRS-1 Ser307 phosphorylation (A), inhibitor-κB kinase (IKK) Ser176/180 phosphorylation (B), JNK Thr183/Tyr185 phosphorylation (C), ERK Thr202/Tyr204 phosphorylation (D), and p38 MAPK Thr180/ Tyr182 phosphorylation (E) in incubated rat epitrochlearis muscle. Isolated muscles were preincubated for 40 min and incubated for 15 min in the absence or presence of 3 mM caffeine. Muscles were then incubated with or without 1 μM insulin in the absence or presence of 3 mM caffeine for 15 min. The tissue lysate (B–E) and immunoprecipitate by anti-IRS-1 antibody (IP:IRS-1; A) were subjected to Western blot analysis. Fold increases are expressed relative to the level of signal in control muscles. Representative immunoblots are shown. Values are means ± SE; n = 5–10 muscles/group. *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 4. Inhibitory effect of caffeine-induced IRS-1 Ser307 phosphorylation (A) and IKK Ser176/180 phosphorylation (B) by caffeic acid on IRS-1 Tyr612 phosphorylation (C), Akt phosphorylation (D) and 3MG transport (E). Isolated muscles were preincubated for 40 min and incubated for 15 min in the absence or presence of 3 mM caffeine. Muscles were then incubated with or without 1 μM insulin in the absence or presence of 3 mM caffeine for 15 min. When present, 5 mM caffeic acid was added throughout the preincubation and incubation periods. The tissue lysate (B–D) and immunoprecipitate by anti-IRS-1 antibody (A) were subjected to Western blot analysis. Muscles were also subjected to a 3MG transport assay (E). Fold increases are expressed relative to the level of signal in control muscles. Representative immunoblots are shown. Values are means ± SE; n = 6–8 muscles/group. *P < 0.05; ***P < 0.001.
for 40 min at 4°C. Lysates were solubilized in Laemmli’s sample buffer containing mercaptoethanol and boiled. Samples (10 μg protein) were separated by SDS-PAGE using a 7.5% polyacrylamide gel. Proteins were then transferred to polyvinylidene difluoride membranes (PolyScreen, Perkin Elmer, Wellesley, MA) at 100 V for 1 h. Membranes were blocked for 1 h at room temperature in Tris-buffered saline with 0.1% Tween 20 (TBST) containing 5% nonfat dry milk and then incubated overnight at 4°C with primary antibodies [IR Tyr458, p85 Tyr458, Akt Ser473, Akt, glycogen synthase kinase (GSK)-3 Ser636/639, mammalian target of rapamycin (mTOR) Ser2448, mTOR, p70 S6 kinase (p70S6K) Thr389, p70S6K, IKK-1 h at room temperature and then incubated with anti-IRS-1 or the corresponding antibody (Cell Signaling Technology, Danvers, MA), PI3K p85, Akt Ser77, Akt, glycogen synthase kinase (GSK)-3β Ser9, GSK-3β, AMPK Thr172, AMPK-α, acetyl CoA carboxylase (ACC) (Cell Signaling Technology, Danvers, MA), PI3K p85, and ACC Ser79 (Millipore, Billerica, MA)]. Membranes were then washed and incubated for 1 h at room temperature with anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK). To immunoprecipitate IRS-1, an aliquot of the supernatant was prepared as described above (100 μg protein) and incubated with anti-IRS-1 (Millipore) and protein A-Sepharose 4B beads (GE Healthcare) overnight with end over end rotation at 4°C. The immunoprecipitate was washed three times in lysis buffer and then centrifuged at 4,000 g for 30 s at 4°C. The supernatant was removed, and the beads were mixed with sample buffer and boiled. The denatured proteins were separated on a 7.5% polyacrylamide gel and then transferred as described above. The membrane was blocked with TBST containing 5% nonfat dry milk for 1 h at room temperature and then incubated with anti-IRS-1 or anti-IRS-1 Ser612 (Cell Signaling Technology). Membranes were then washed and incubated for 1 h at room temperature with rabbit TrueBlot IgG (eBioscience, San Diego, CA). Protein signals were detected with enhanced chemiluminescence reagents according to the manufacturer’s instructions (GE Healthcare) and an ImageCapture G3 (Liponics, Tokyo, Japan). The intensity of the signals was quantified using ImageJ (3). The mean intensity of control samples in each membrane was used as a reference for controlling gel-to-gel variation. Equal protein loading and transfer were confirmed by Coomassie brilliant blue staining of the membranes.

3-0-methyl-0-glucose transport. The 3-0-methyl-D-glucose (3MG) transport assay was performed as we previously described (11, 20, 39). To measure 3MG transport after caffeine or insulin incubation, muscles were transferred to 2 ml of KRB containing 1 mM [1H3]3MG (1.5 μCi/ml, American Radiolabeled Chemicals, St. Louis, MO) and 7 mM 0-l[14C]mannotol (0.3 μCi/ml, American Radiolabeled Chemicals) at 30°C and further incubated for 10 min. Muscles were then blotted onto filter paper, trimmed, frozen in liquid nitrogen, and stored at −80°C. Each frozen muscle was weighed and processed for 3-O-methyl-0-glucose transport. Afterward, the digests were neutralized with 300 μl of 1 M NaOH at 80°C for 10 min. The 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 (44).

Statistical analysis. Results are presented as means ± SE. Multiple means were compared by ANOVA followed by a post hoc comparison with Dunnet’s or Tukey’s test as appropriate. Differences between groups were considered statistically significant at P < 0.05.

RESULTS

Caffeine decreased basal and insulin-stimulated IRS-1 Tyr612 phosphorylation in a dose- and time-dependent manner. To examine the state of tyrosine phosphorylation of IRS-1, we measured the degree of phosphorylation of IRS-1 Tyr612 [a binding site for the p85 subunit of PI3K and important for the full activation of PI3K in response to insulin (13)] in muscles incubated with caffeine at various concentrations (0, 0.01, 0.1, 1, and 3 mM) and for various times (0, 5, 15, 30, and 60 min). This dose-response experiment showed that preincubation with caffeine at 1 and 3 mM lowered insulin-stimulated IRS-1 Tyr612 phosphorylation significantly more than in muscle incubated with insulin alone (Fig. 1A). Caffeine (3 mM) also decreased basal IRS-1 Tyr612 phosphorylation. The time-course experiments revealed that insulin-stimulated IRS-1 Tyr612 phosphorylation decreased after preincubation with caffeine for over 15 min (Fig. 1B).

Caffeine did not change IRβ Tyr1158/1162/1163 phosphorylation. We performed Western blot analysis using an anti-IRβ antibody that recognizes phosphorylated Tyr1158/1162/1163 to identify whether caffeine affects tyrosine phosphorylation of IRβ. Tyrosine phosphorylation of IRβ was clearly increased by insulin stimulation whether or not the muscle was incubated with caffeine (Fig. 2A). Caffeine did not affect the basal tyrosine phosphorylation state of IRβ (Fig. 2A).

Caffeine blocked basal and insulin-stimulated PI3K p85 Tyr548 phosphorylation and Akt Ser77 phosphorylation and inhibited insulin-stimulated glucose transport. To determine the activation state of PI3K and Akt, we performed Western blot analysis with a phosophospecific anti-PI3K p85 antibody that recognizes phosphorylated Tyr548, an indicator of PI3K activation (29), and an anti-Akt antibody that recognizes phosphorylated Thr172. Caffeine significantly increased PI3K and Akt phosphorylation, but caffeine (3 mM, 15 min) blocked basal and insulin-stimulated PI3K phosphorylation (Fig. 2B) and Akt phosphorylation (Fig. 2C). GSK-3β Ser9, a downstream target of Akt, also displayed

Fig. 5. Effect of caffeine on IRS-1 Ser612 phosphorylation (A), 5’-AMP-activated protein kinase (AMPK) Thr172 phosphorylation (B), and acetyl CoA carboxylase (ACC) Ser79 phosphorylation (C) in incubated rat epimysial muscle. Isolated muscles were preincubated for 40 min and incubated for 15 min in the absence or presence of 3 mM caffeine. Muscles were then incubated with or without 1 μM insulin in the absence or presence of 3 mM caffeine for 15 min. The tissue lysate was subjected to Western blot analysis. Fold increases are expressed relative to the level of signal in control muscles. Representative immunoblots are shown. Values are means ± SE; n = 4–9 muscles/group. *P < 0.05; ***P < 0.001.
a pattern similar to that for Akt phosphorylation (Fig. 2D). Next, we examined whether caffeine would affect basal and insulin-stimulated glucose transport in skeletal muscle. Caffeine increased 3MG transport activity by 2.2-fold compared with basal levels in the absence of insulin (Fig. 2E), as observed in our previous studies (11, 12). Insulin increased the activity by 5.3-fold, but in muscles incubated with caffeine, the rate of insulin-stimulated 3MG transport activity was significantly decreased compared with muscles incubated with insulin alone (Fig. 2E).

Caffeine-induced IRS-1 Ser307 and IKK-α/β Ser176/180 phosphorylation were associated with inhibition of insulin signaling and insulin-stimulated glucose transport. To examine whether caffeine affects the serine phosphorylation of IRS-1, we performed Western blot analysis using an anti-IRS-1 antibody that recognizes phosphorylated Ser307, a molecular indicator of insulin resistance (6, 18). Caffeine significantly increased IRS-1 Ser307 phosphorylation under both basal and insulin-stimulated conditions (Fig. 3A). IKK is a mediator of insulin resistance through IRS-1 Ser307 phosphorylation (9, 16), and other stress-related kinases, such as JNK, ERK, and p38 MAPK, have also been shown to promote Ser307 phosphorylation (4, 7–9, 22). Therefore, we next evaluated the effects of caffeine on the phosphorylation states of IKK-α/β Ser176/180, JNK Thr183/Tyr185, ERK Thr202/Tyr204, and p38 MAPK Thr180/Tyr182. Caffeine clearly increased IKK-α/β Ser176/180 phosphorylation under both basal and insulin-stimulated conditions (Fig. 3B), whereas phosphorylation of JNK, ERK, and p38 MAPK were not affected by caffeine (Fig. 3, C–E). The cancelling of the caffeine-induced phosphorylation of IRS-1 Ser307 (Fig. 4A) and IKK-α/β Ser176/180 (Fig. 4B) by 5 mM caffie acid (32) was associated with significant reductions in the inhibitory effects of caffeine on insulin-stimulated IRS-1 Tyr612 (Fig. 4C) and Akt Ser473 (Fig. 4D) phosphorylation as well as 3MG transport (Fig. 4E).

Caffeine-induced IRS-1 Ser789 and AMPK Thr172 phosphorylation were not paralleled by the inhibition of insulin signaling or insulin-stimulated glucose transport. Ser789 has been shown to negatively regulate insulin signal transduction, and this site seems to be a target for AMPK (41). Thus, we examined the effect of caffeine on the phosphorylation state of IRS-1 Ser789, AMPK Thr172, and ACC Ser279, a well-characterized substrate of AMPK. The phosphorylation state of Ser789 was significantly enhanced by caffeine in both the presence and absence of insulin (Fig. 5A). Phosphorylation of AMPK Thr172 (Fig. 5B) and phosphorylation of ACC Ser279 (Fig. 5C) were increased by caffeine, as observed in our previous studies (11, 12), and displayed a pattern similar to that of IRS-1 Ser789 phosphorylation (Fig. 5A). Cancellation of the stimulatory effect of caffeine on the phosphorylation of IRS-1 Ser789 (Fig. 6A) and AMPK Thr172 (Fig. 6B) by the Ca2+/calmodulin (CaM) release inhibitor dantrolene (10 μM) did not rescue the inhibitory effects of caffeine on insulin-stimulated IRS-1 Tyr612 (Fig. 6C) and Akt Ser473 (Fig. 6D) phosphorylation as well as 3MG transport (Fig. 6E).

Caffeine decreased insulin-stimulated IRS-1 Ser636/639, mTOR Ser2448, and p70S6K Thr389 phosphorylation. We performed Western blot analysis using an anti-IRS-1 antibody that recognizes phosphorylated Ser636/639, a residue involved in insulin resistance (40, 42). Caffeine did not change basal Ser636/639 phosphorylation; however, insulin-stimulated Ser636/639 phosphorylation was significantly suppressed in the muscle incubated with caffeine (Fig. 7A). Next, to determine whether caffeine might modulate mTOR/p70S6K transduction, which is accompanied by a corresponding increase in IRS-1 phosphorylation of Ser636/639 (40, 42), we performed Western blot analysis using an anti-mTOR antibody that recognizes phosphorylated
Ser^{3448} and an anti-p70S6K antibody that recognizes phosphorylated Thr^{389}. Consistent with the inhibitory effect of caffeine on insulin-stimulated IRS-1 Ser^{307} phosphorylation, the stimulatory effect of insulin on mTOR and p70S6K phosphorylation was blocked by caffeine (Fig. 7, A and B). Caffeine alone decreased basal mTOR phosphorylation but not p70S6K phosphorylation (Fig. 7, B and C). The present study is the first report to show that caffeine inhibits basal and insulin-stimulated IRS-1 Tyr^{612} phosphorylation (Fig. 1) and PI3K phosphorylation (Fig. 2B) but not IRβ Tyr^{612} phosphorylation (Fig. 2A) in skeletal muscle. These inhibitory effects were also confirmed by in vivo caffeine treatment (Fig. 8). Our results suggest that caffeine affects the functions of IRS-1 either directly and/or through molecules other than IRβ and impairs PI3K and Akt activity and glucose transport in skeletal muscle.

Ser^{307} of IRS-1 is located close to the phosphotyrosine-binding domain of IRS-1 (36), and its phosphorylation has been demonstrated to inhibit insulin-stimulated tyrosine phosphorylation of IRS-1 and the subsequent activation of PI3K (4, 22). In the present study, we found that caffeine upregulated the phosphorylation state of IRS-1 Ser^{307} (Fig. 3A) and enhanced both the basal and insulin-stimulated phosphorylation of IKK (Fig. 3B) but not of JNK, ERK, or p38 MAPK (Fig. 3, C–E). In addition, blockade of the IKK/NF-κB pathway by caffeic acid ameliorated the caffeine-induced inhibition of insulin signaling and insulin-stimulated glucose transport (Fig. 4). IKK is the master regulator of NF-κB activation in response to inflammatory stimuli, and the IKK/NF-κB pathway is a core mechanism that conveys insulin resistance in peripheral tissues (35, 37). Our results suggest that the inhibitory effect of caffeine on insulin signaling occurs through IKK-induced IRS-1 Ser^{307} phosphorylation.

We also found that caffeine increased both basal and insulin-stimulated IRS-1 Ser^{789} phosphorylation (Fig. 5A) with similar increases in AMPK and ACC phosphorylation. Ser^{789} of IRS-1 has been shown to be phosphorylated in vitro by AMPK and to promote insulin signaling in muscle C_{2}C_{12} cells (25). In contrast, some studies (23, 34, 41) have proposed an association between Ser^{789} phosphorylation and insulin resistance. For example, Tzatsos et al. (41) showed that energy depletion and oxidative stress stimulated AMPK and IRS-1 Ser^{789} phosphorylation and suppressed insulin-stimulated IRS-1-associated PI3K activity and Akt phosphorylation in L6 myoblasts. In the present study, we found that the Ca^{2+} release blocker dantrolene, which has been reported to inhibit caffeine-induced AMPK activation in mouse skeletal muscle (26), suppressed both caffeine-induced AMPK phosphorylation and IRS-1 Ser^{789} phosphorylation but did not rescue IRS-1 Tyr^{612} phosphorylation, Akt phosphorylation, or insulin-stimulated glucose transport (Fig. 6). Similarily, Kolnes et al.

**DISCUSSION**

The present study is the first report to show that caffeine inhibits basal and insulin-stimulated IRS-1 Tyr^{612} phosphorylation (Fig. 1) and PI3K phosphorylation (Fig. 2B) but not IRβ Tyr^{612} phosphorylation (Fig. 2A) in skeletal muscle. These inhibitory effects were also confirmed by in vivo caffeine treatment (Fig. 8). Our results suggest that caffeine affects the functions of IRS-1 either directly and/or through molecules other than IRβ and impairs PI3K and Akt activity and glucose transport in skeletal muscle.

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phosphorylation independently of Ca\(^{2+}\) release or AMPK activation in skeletal muscle.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: T.E., T. Hamada, and T. Hayashi conception and design of research; T.E., S.T., and X.M. performed experiments; T.E. analyzed data; T.E., S.T., X.M., T. Hamada, and T. Hayashi interpreted results of experiments; T.E. prepared figures; T.E. drafted manuscript; T.E. and T. Hayashi edited and revised manuscript; T. Hayashi approved final version of manuscript.

REFERENCES
CAFFEINE ON INSULIN SIGNALING


