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.—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 caffeine on insulin signaling in rat epimysial 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-3β Ser19 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 Ser707 and an IRS-1 Ser707 kinase, inhibitor-κB kinase (IKK)-α/β Ser172/180. Blockade of IKK/IRS-1 Ser707 by caffeic acid ameliorated the caffeine-induced downregulation of IRS-1 Tyr612 phosphorylation and 3MG transport. Caffeine also increased the phosphorylation of IRS-1 Ser707 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 epimysial muscle. Our results indicate that caffeine inhibits insulin signaling partly through 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 undergo 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-

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proven by the Kyoto University Graduate School of Human and Environmental Studies and Kyoto University Radioisotope Research Center. 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.

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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). Muscles were then incubated with or without 1 μM insulin 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.
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 Tyr1158/1162/1163, IRS-1 Tyr612 (Invitrogen, Carlsbad, CA), IR, IRS-1] and then incubated with insulin alone (Fig. 1A). Caffeine (3 mM, 15 min) blocked basal and insulin-stimulated IRS-1 Ser789 phosphorylation (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).

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 (Fig. 1A). The time-course experiments revealed that insulin-stimulated IRS-1 Tyr612 phosphorylation decreased after preincubation with caffeine for over 15 min (Fig. 1B).

Caffeine blocked basal and insulin-stimulated PI3K p85 Tyr458 and Akt Ser473 phosphorylation and inhibited insulin-stimulated glucose transport. To determine the activation state of PI3K and Akt, we performed Western blot analysis with a phoshospecific anti-PI3K p85 antibody that recognizes phosphorylated Tyr458, an indicator of PI3K activation (29), and an anti-Akt antibody that recognizes phosphorylated Ser473. Insulin 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...
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 Ser\(^{307}\) and IKK-\(\alpha/\beta\) Ser\(^{176/180}\) 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 Ser\(^{307}\), a molecular indicator of insulin resistance (6, 18). Caffeine significantly increased IRS-1 Ser\(^{307}\) phosphorylation under both basal and insulin-stimulated conditions (Fig. 3A). IKK is a mediator of insulin resistance through IRS-1 Ser\(^{307}\) phosphorylation (9, 16), and other stress-related kinases, such as JNK, ERK, and p38 MAPK, have also been shown to promote Ser\(^{307}\) phosphorylation (4, 7–9, 22). Therefore, we next evaluated the effects of caffeine on the phosphorylation states of IKK-\(\alpha/\beta\) Ser\(^{176/180}\), JNK Thr\(^{183/185}\), ERK Thr\(^{202/204}\)/Tyr\(^{204}\), and p38 MAPK Thr\(^{180}/\)Tyr\(^{182}\). Caffeine clearly increased IKK-\(\alpha/\beta\) Ser\(^{176/180}\) 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 Ser\(^{307}\) (Fig. 4A) and IKK-\(\alpha/\beta\) Ser\(^{176/180}\) (Fig. 4B) by 5 mM caffeine acid (32) was associated with significant reductions in the inhibitory effects of caffeine on insulin-stimulated IRS-1 Tyr\(^{612}\) (Fig. 4C) and Akt Ser\(^{773}\) (Fig. 4D) phosphorylation as well as 3MG transport (Fig. 4E).

Caffeine-induced IRS-1 Ser\(^{789}\) and AMPK Thr\(^{172}\) phosphorylation were not paralleled by the inhibition of insulin signaling or insulin-stimulated glucose transport. Ser\(^{789}\) 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 Ser\(^{789}\), AMPK Thr\(^{172}\), and ACC Ser\(^{79}\), a well-characterized substrate of AMPK. The phosphorylation state of Ser\(^{789}\) was significantly enhanced by caffeine in both the presence and absence of insulin (Fig. 5A). Phosphorylation of AMPK Thr\(^{172}\) (Fig. 5B) and phosphorylation of ACC Ser\(^{79}\) (Fig. 5C) were increased by caffeine, as observed in our previous studies (11, 12), and displayed a pattern similar to that of IRS-1 Ser\(^{789}\) phosphorylation (Fig. 5A). Cancelling the stimulatory effect of caffeine on the phosphorylation of IRS-1 Ser\(^{789}\) (Fig. 6A) and AMPK Thr\(^{172}\) (Fig. 6B) by the Ca\(^{2+}\) release inhibitor dantrolene (10 \(\mu\)M) did not rescue the inhibitory effects of caffeine on insulin-stimulated IRS-1 Tyr\(^{612}\) (Fig. 6C) and Akt Ser\(^{773}\) (Fig. 6D) phosphorylation as well as 3MG transport (Fig. 6E).

Caffeine decreased insulin-stimulated IRS-1 Ser\(^{636/639}\), mTOR Ser\(^{2448}\), and p70S6K Thr\(^{389}\) phosphorylation. We performed Western blot analysis using an anti-IRS-1 antibody that recognizes phosphorylated Ser\(^{636/639}\), a residue involved in insulin resistance (40, 42). Caffeine did not change basal Ser\(^{636/639}\) phosphorylation; however, insulin-stimulated Ser\(^{636/639}\) 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 Ser\(^{636/639}\) (40, 42), we performed Western blot analysis using an anti-mTOR antibody that recognizes phosphorylated...
Ser\textsuperscript{2448} and an anti-p70S6K antibody that recognizes phosphorylated Thr\textsuperscript{389}. Consistent with the inhibitory effect of caffeine on insulin-stimulated IRS-1 Ser\textsuperscript{307/308} phosphorylation, the stimulatory effect of insulin on mTOR and p70S6K phosphorylation was blocked by caffeine (Fig. 7, B and C). Caffeine alone decreased basal mTOR phosphorylation but not p70S6K phosphorylation (Fig. 7, B and C).

In vivo caffeine treatment inhibited insulin signaling in skeletal muscle. To determine whether a physiological blood concentration of caffeine affects insulin signaling, the phosphorylation of IRS-1 Tyr\textsuperscript{612} and Akt Ser\textsuperscript{473} in muscles dissected 60 min after an intravenous injection of 5 mg/kg caffeine was estimated. In humans, after an ingestion of coffee consumers (7 cups/day) show a peak plasma caffeine concentration of 50 M, with a mean 24-h plasma level of 16 M (15). Habitual coffee consumers (7 cups/day) show a peak plasma caffeine concentration of ~50 M, with a mean 24-h plasma level of ~25 μM (31). We (12) have previously shown that the administration of 5 mg/kg caffeine into the tail vein of rats increases the blood concentration of caffeine to 50 μM 60 min after the injection. Caffeine significantly suppressed basal and insulin-stimulated phosphorylation of IRS-1 Tyr\textsuperscript{612} (Fig. 8A) and Akt Ser\textsuperscript{473} (Fig. 8B).

**DISCUSSION**

The present study is the first report to show that caffeine inhibits basal and insulin-stimulated IRS-1 tyrosine phosphorylation (Fig. 1) and PI3K phosphorylation (Fig. 2B) but not IRβ tyrosine 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\textsuperscript{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\textsuperscript{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\textsuperscript{307} phosphorylation.

We also found that caffeine increased both basal and insulin-stimulated IRS-1 Ser\textsuperscript{780} phosphorylation (Fig. 5A) with similar increases in AMPK and ACC phosphorylation (Fig. 5, B and C). Ser\textsuperscript{780} of IRS-1 has been shown to be phosphorylated in vitro by AMPK and to promote insulin signaling in muscle C2C12 cells (25). In contrast, some studies (23, 34, 41) have proposed an association between Ser\textsuperscript{780} phosphorylation and insulin resistance. For example, Tzatzos et al. (41) showed that energy depletion and oxidative stress stimulated AMPK and IRS-1 Ser\textsuperscript{780} 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\textsuperscript{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\textsuperscript{780} phosphorylation but did not rescue IRS-1 tyrosine phosphorylation, Akt phosphorylation, or insulin-stimulated glucose transport (Fig. 6). Similarly, Kolnes et al.
glucose transport in skeletal muscle and that this inhibition is branched-chain amino acid leucine inhibits insulin-stimulated phosphorylation (40, 42). We (24) have previously reported that the resistance, and mTOR/p70S6K could be responsible for this phosphorylation (34). In the present study, contrary to our expectations, caffeine decreased insulin-stimulated IRS-1 Ser307 phosphorylation (Fig. 7A) and mTOR and p70S6K phosphorylation (7, B and C). Thus, the mTOR/p70S6K/IRS-1 Ser307 phosphorylation cascade might not be an important regulator of the caffeine-mediated reduction of insulin signaling. It is notable that insulin stimulation significantly increased Ser307 phosphorylation of IRS-1 (Fig. 7A), as reported in previous studies (21, 42) and one of our studies (24). Although the actual role of the p70S6K/IRS-1 Ser307 upregulation by insulin has not been clarified, it has been assumed that a feedback mechanism is evoked simultaneously to regulate the activation of insulin signaling including the PI3K/Akt pathway (19, 42).

A number of reports (5, 17, 27, 30, 38) have documented that acute caffeine administration decreases whole body glucose tolerance and insulin sensitivity in humans. In particular, acute caffeine ingestion reduces insulin-stimulated glucose disposal during a hyperinsulinemic clamp (5, 17, 30), indicating that caffeine-mediated insulin resistance occurs in skeletal muscle (10). Thong et al. (38) showed that caffeine ingestion impairs skeletal muscle glucose uptake in human skeletal muscle during a hyperinsulinemic clamp. However, Thong et al. (38) did not detect any alterations in IR tyrosine kinase activity, PI3K activity, or Akt phosphorylation in biopsy samples from caffeine-treated muscle. In contrast, our results show that a physiological concentration of caffeine in live rats blocks IRS-1 tyrosine phosphorylation and Akt phosphorylation in skeletal muscle (Fig. 8). Although we have no rational explanation for this difference, we believe that the present study gives new insights into how caffeine modulates insulin actions, including glucose transport in vivo.

In summary, we demonstrated that caffeine decreases insulin-stimulated phosphorylation of IRS-1 Tyr612, PI3K Tyr458, Akt Ser473, and GSK-3β Ser9 as well as 3MG transport without affecting IRβ tyrosine phosphorylation in rat skeletal muscle. We also found that caffeine promotes the phosphorylation of IRS-1 at Ser307 with corresponding increases in IKK phosphorylation and that suppressing the IKK/IRS-1 Ser307 cascade by caffeic acid ameliorates caffeine-induced insulin resistance. Caffeine also increased IRS-1 Ser789 and AMPK phosphorylation, but inhibition of IRS-1 Ser789 and AMPK phosphorylation by dantrolene did not rescue the caffeine-induced insulin resistance. We propose that caffeine acutely inhibits insulin signaling at least in part via the IKK-induced IRS-1 Ser307 phosphorylation independently of Ca2+ 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.

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