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

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Running head: EFFECTS OF CAFFEINE ON INSULIN SIGNALING

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ABSTRACT

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 Ser⁴⁷³ and glycogen synthase kinase (GSK) 3 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 Tyr^{1158/62/63}. Furthermore, caffeine enhanced phosphorylation of IRS-1 Ser³⁰⁷ and an IRS-1 Ser³⁰⁷ kinase, inhibitor- κ B kinase (IKK) α/β Ser^{176/180}. 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 Ser^{636/639} and upstream kinases including the mammalian target of rapamycin (mTOR) and p70S6 kinase (p70S6K). 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.

Keywords: serine phosphorylation, Akt, 5'AMP-activated protein kinase, p70 S6 kinase, inhibitor- κ B kinase

INTRODUCTION

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 the insulin receptor 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 crosstalk 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 have shown that acute caffeine stimulation (≥ 1 mM) increases glucose transport in the absence of insulin in incubated or perfused rodent skeletal muscles (1, 11, 12, 26, 43), 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) have 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) have 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 insulin receptor (IR) allows 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 in human and rodent muscles have suggested the increased serine phosphorylation of IRS-1 in the insulin-resistant state (33, 45).

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 approved 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 described previously (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 a 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, 24.6 mM NaHCO₃) containing 2 mM pyruvate (KRBP) for 40 min. For the dose- and time-dependent effects of caffeine, muscles were then randomly assigned to incubation in 7 mL of

fresh buffer in the absence or presence of 0.1-3 mM caffeine for 15 min, or in 7 mL of fresh buffer in the absence or presence of 3 mM caffeine for up to 60 min, respectively. In other experiments, muscles were incubated in the absence or presence of 3 mM caffeine for 15 min. Muscles were then incubated in 7 ml of fresh buffer with 1 μ M insulin in the absence or presence of caffeine for 15min. 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- κ B 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 immediately frozen in liquid nitrogen and subsequently analyzed for western blot.

Muscle treatment in vivo. Muscles were treated as we described previously (12). Caffeine was dissolved in saline and injected into the tail vein without anesthesia at 5 mg/kg body weight. The injection volume was 1 ml/kg body weight. 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 anaesthetized with intraperitoneal administration of pentobarbital sodium (50 mg/kg body weight), and then insulin (100 mU/kg body weight) or saline was injected intravenously. One min after injection epitrochlearis muscle was rapidly dissected and immediately frozen in liquid nitrogen.

Western blot analysis. Sample preparation and western blot analysis was performed

as we described previously (11, 24, 39). Muscles were homogenized in ice-cold lysis buffer (1:40 wt/vol) containing 20 mM Tris·HCl (pH 7.4), 1% Triton X, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 2 mM dithiothreitol, 4 mg/L leupeptin, 50 mg/L trypsin inhibitor, 0.1 mM benzamidine, 1 mM Na₃VO₄, and 0.5 mM phenylmethylsulfonyl fluoride and centrifuged at 16,000 g for 40 min at 4°C. Lysates were solubilized in Laemmli's sample buffer containing mercaptoethanol and boiled. The samples (10 µg of protein) were separated by SDS-PAGE using 7.5% polyacrylamide gel. Proteins were then transferred to polyvinylidene difluoride membranes (PolyScreen; PerkinElmer, Wellesley, MA) at 100 V for 1 h. Membranes were blocked for 1h at room temperature in TBS-T (TBS with 0.1% Tween 20) containing 5% nonfat dry milk and were then incubated overnight at 4°C with primary antibody [IR Tyr^{1158/1162/1163}, IRS-1 Tyr⁶¹² (Invitrogen, Carlsbad, CA), IR, IRS-1 Ser^{636/639}, mammalian target of rapamycin (mTOR) Ser²⁴⁴⁸, mTOR, p70 S6 kinase (p70S6K) Thr³⁸⁹, p70S6K, IKK α/β Ser^{176/180}, IKKα, IKKβ, c-Jun N-terminal kinase (JNK) Thr¹⁸³/Tyr¹⁸⁵, JNK, p44/42 MAPK (ERK) Thr²⁰²/Tyr²⁰⁴, p44/42 MAPK, p38 MAPK Thr¹⁸⁰/Tyr¹⁸², p38 MAPK, PI3K p85 Tyr⁴⁵⁸, Akt Ser⁴⁷³, Akt, glycogen synthase kinase (GSK) 3β Ser⁹, GSK3β, AMPK Thr¹⁷², AMPKα, acetyl CoA carboxylase (ACC) (Cell Signaling Technology, Danvers, MA), PI3K p85, ACC Ser⁷⁹ (Millipore, Billerica, MA)]. The membranes were then washed and incubated for 1h at room temperature with anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK). To immunoprecipitate the IRS-1, an aliquot of supernatant was prepared as described above (100 µg of protein) and incubated with anti-IRS-1 (Millipore) and protein A-Sepharose CL4B beads (GE Healthcare) overnight with end-over-end rotation at 4 °C. The immunoprecipitate was washed

three times in the lysis buffer, and then centrifuged at $4000\times g$ for 30 s at 4 °C. The supernatant was removed, and the beads were mixed with the 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 TBS-T containing 5% nonfat dry milk for 1h at room temperature, and then incubated with anti-IRS-1 or anti- IRS-1 Ser³⁰⁷ (Cell Signaling Technology). The membranes were then washed, incubated for 1h 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 reference for controlling gel-to-gel variation. Equal protein loading and transfer was confirmed by Coomassie brilliant blue staining of the membranes.

3-O-methyl-D-glucose (3MG) transport. 3MG transport assay was performed as we described previously (11, 20, 39). To measure 3MG transport after caffeine or insulin incubation, muscles were transferred to 2 mL of KRB containing 1 mM [³H]3-MG (1.5 μ Ci/mL) (American Radiolabeled Chemicals, St. Louis, MO) and 7 mM D-[1-¹⁴C] mannitol (0.3 μ Ci/mL) (American Radiolabeled Chemicals, St. Louis, MO) at 30°C and further incubated for 10 min. The muscles were then blotted onto filter paper, trimmed, frozen in liquid nitrogen, and stored at -80°C. Each frozen muscle was weighed and processed by incubating them in 300 μ l of 1 M NaOH at 80°C for 10 min. Digestates were neutralized with 300 μ l 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 \pm SE. Multiple means were compared by ANOVA followed by 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 muscle 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 study 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 study revealed that insulin-stimulated IRS-1 Tyr⁶¹² phosphorylation decreased after preincubation with caffeine for over 15 min (Fig. 1B).

Caffeine did not change IR β Tyr^{1158/62/63} phosphorylation. We performed western blot analysis using an anti-IR β antibody that recognizes phosphorylated Tyr^{1158/1162/1163} to identify whether caffeine affects tyrosine phosphorylation of IR β . Tyrosine phosphorylation of IR β 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 IR β (Fig. 2A).

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 phosphospecific anti-PI3K p85 antibody that recognizes phosphorylated Tyr⁴⁵⁸, an indicator of PI3K activation (29), and 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-3 β 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 3 MG 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 3 MG transport activity was decreased significantly compared with muscles incubated with insulin alone (Fig. 2E).

Caffeine-induced IRS-1 Ser³⁰⁷ and IKK α/β 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 Ser³⁰⁷—a molecular indicator of insulin resistance (6, 18). Caffeine significantly increased IRS-1 Ser³⁰⁷ phosphorylation in 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 Ser^{176/180}, JNK Thr¹⁸³/Tyr¹⁸⁵, ERK Thr²⁰²/Tyr²⁰⁴ and p38 MAPK Thr¹⁸⁰/Tyr¹⁸². Caffeine clearly increased IKK α/β Ser^{176/180} phosphorylation in both basal and insulin-stimulated conditions (Fig. 3B), whereas phosphorylation of JNK, ERK, and p38 MAPK were not affected by caffeine (Fig. 3C–E). The cancelling of the caffeine-induced phosphorylation of IRS-1 Ser³⁰⁷ (Fig. 4A) and IKK α/β Ser^{176/180} (Fig. 4B) by 5 mM caffeic 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, and 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 was 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 Ser⁷⁹, 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 ACC Ser⁷⁹ (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). Cancelling the stimulatory effect of caffeine on the phosphorylation of IRS-1 Ser⁷⁸⁹ (Fig. 6A) and AMPK Thr¹⁷² (Fig. 6B) by the Ca²⁺ release inhibitor dantrolene (10 μ M) did not rescue the inhibitory effects of caffeine on insulin-stimulated IRS-1 Tyr⁶¹² (Fig. 6C)

and Akt Ser⁴⁷³ (Fig. 6D) phosphorylation and 3MG transport (Fig. 6E).

Caffeine decreased insulin-stimulated IRS-1 Ser^{636/639}, mTOR Ser²⁴⁴⁸, and p70S6K Thr³⁸⁹ 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 Ser²⁴⁴⁸ and anti-p70S6K antibody that recognizes phosphorylated Thr³⁸⁹. Consistent with the inhibitory effect of caffeine on insulin-stimulated IRS-1 Ser^{636/639} phosphorylation, the stimulatory effect of insulin on mTOR and p70S6K phosphorylation was blocked by caffeine (Figs. 7B and 7C). Caffeine alone decreased basal mTOR phosphorylation but not p70S6K phosphorylation (Figs. 7B and 7C).

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 Tyr⁶¹² and Akt Ser⁴⁷³ in muscles dissected 60 min after intravenous injection of 5 mg/kg caffeine was estimated. In humans, following an ingestion of caffeine in amounts corresponding to 2–3 cups of coffee, plasma caffeine levels reach 20–50 μ M (15). Habitual coffee consumers (7 cups/day)

show a peak plasma caffeine concentration of about 50 μM , with a mean 24 h plasma level of about 25 μM (31). We showed previously that administration of 5 mg/kg caffeine into the tail vein of the rats increases the blood concentration of caffeine to 50 μM 60 min after the injection (12). Caffeine significantly suppressed basal and insulin-stimulated phosphorylation of IRS-1 Tyr⁶¹² (Fig. 8A) and Akt Ser⁴⁷³ (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 IR β 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 IR β , and impairs PI3K and Akt activity and glucose transport in skeletal muscle.

Ser³⁰⁷ 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 subsequent activation of PI3K (4, 22). We found here that caffeine upregulated the phosphorylation state of IRS-1 Ser³⁰⁷ (Fig. 3A) and enhanced both the basal and insulin-stimulated phosphorylation of IKK (Fig. 3B) but not of JNK, ERK or p38 MAPK (Fig. 3C–E). In addition, blockade of the IKK/NF- κ B 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- κ B activation in response to inflammatory stimuli, and the IKK/NF- κ B 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 Ser³⁰⁷ phosphorylation.

We also found that caffeine increased both basal and insulin-stimulated IRS-1 Ser⁷⁸⁹ phosphorylation (Fig. 5A) with similar increases in AMPK and ACC phosphorylation (Figs. 5B and 5C). Ser⁷⁸⁹ of IRS-1 was shown to be phosphorylated

in vitro by AMPK and to promote insulin signaling in muscle C₂C₁₂ cells (25). By contrast, some studies have proposed an association between Ser⁷⁸⁹ phosphorylation and insulin resistance (23, 34, 41). For example, Tzatsos et al. (41) showed that energy depletion and oxidative stress stimulated AMPK and IRS-1 Ser⁷⁸⁹ phosphorylation and suppressed insulin-stimulated IRS-1-associated PI3K activity and Akt phosphorylation in L6 myoblasts. In the present study, we found that Ca²⁺ 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 Ser⁷⁸⁹ phosphorylation, but did not rescue IRS-1 tyrosine phosphorylation, Akt phosphorylation or insulin-stimulated glucose transport (Fig. 6). Similarly, Kolnes et al. (28) demonstrated recently that dantrolene does not rescue caffeine-mediated blockade of insulin-stimulated Akt phosphorylation and glucose transport in incubated rat skeletal muscles. These observations are not surprising because muscle contraction, a physiological stimulator of both Ca²⁺ release and AMPK activity, does not induce insulin resistance in skeletal muscle. Taken together, these data suggest that caffeine inhibits insulin signaling via Ca²⁺-independent and AMPK/IRS-1 Ser⁷⁸⁹-independent mechanisms in skeletal muscle.

The Ser^{636/639} of IRS-1 is also a site involved in insulin resistance and mTOR/p70S6K could be responsible for this phosphorylation (40, 42). We have reported that the branched-chain amino acid leucine inhibits insulin-stimulated glucose transport in skeletal muscle and that this inhibition is associated with mTOR/p70S6K-induced Ser^{636/639} phosphorylation of IRS-1 (24). In the present study, contrary to our expectations, caffeine decreased insulin-stimulated IRS-1

Ser^{636/639} phosphorylation (Fig. 7A) and mTOR and p70S6K phosphorylation (Fig. 7B and 7C). Thus, the mTOR/p70S6K/IRS-1 Ser^{636/639} cascade might not be an important regulator of the caffeine-mediated reduction of insulin signaling. It is notable that insulin stimulation significantly increased Ser^{636/639} 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 Ser^{636/639} 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 have documented that acute caffeine administration decreases whole-body glucose tolerance and insulin sensitivity in humans (5, 17, 27, 30, 38). 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 insulin receptor tyrosine kinase activity, PI3K activity, or Akt phosphorylation in biopsy samples from caffeine-treated muscle. By 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-3 β Ser⁹ and 3MG transport without affecting IR β 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

The authors state that there are no conflicts of interest.

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LEGENDS TO FIGURES

Figure 1

The dose- and time-dependent effects of caffeine on IRS-1 Tyr⁶¹² phosphorylation (p-Tyr⁶¹²) in incubated rat epitrochlearis muscle. (A) Isolated muscle was preincubated for 40 min and incubated in the presence of caffeine at indicated concentrations for 15 min. Muscle was then incubated with or without 1 μ M insulin in the presence of caffeine at indicated concentrations for 15 min. (B) Isolated muscle was preincubated for 40 min and incubated with 3 mM caffeine for indicated times. Muscle was then incubated with 1 μ M insulin in the presence of caffeine at indicated concentrations for 15 min. Control muscle (open bar) was 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 the control muscles. Representative immunoblots are shown. Values are mean \pm SE; n = 5-16 per group. *P<0.05, ***P<0.001 vs. control. #P<0.05, ##P<0.01, ###P<0.001 vs. insulin alone.

Figure 2

The effect of caffeine on (A) IR β Tyr^{1158/1162/1163} phosphorylation (p-IR β), (B) PI3K p85 Tyr⁴⁵⁸ phosphorylation (p-PI3K p85), (C) Akt Ser⁴⁷³ phosphorylation (p-Akt), (D) GSK3 β Ser⁹ phosphorylation (p-GSK3 β), and (E) 3-O-methyl-D-glucose (3MG) transport activity in incubated rat epitrochlearis muscle. Isolated muscle was preincubated for 40 min and incubated for 15 min in the absence or presence of 3 mM caffeine. Muscle was then incubated with or without 1 μ M insulin in the

absence or presence of 3 mM caffeine for 15min. The muscle was subjected to western blot analysis (A-D) or 3MG transport assay (E). Fold increases are expressed relative to the level of signal in the control muscles. Representative immunoblots are shown. Values are mean \pm SE; n = 5-11 per group. *P<0.05, **P<0.01, ***P<0.001.

Figure 3

The effect of caffeine on (A) IRS-1 Ser³⁰⁷ phosphorylation (p-Ser³⁰⁷), (B) IKK Ser^{176/180} phosphorylation (p-IKK), (C) JNK Thr¹⁸³/Tyr¹⁸⁵ phosphorylation (p-JNK), (D) ERK Thr²⁰²/Tyr²⁰⁴ phosphorylation (p-ERK), and (E) p38 MAPK Thr¹⁸⁰/Tyr¹⁸² phosphorylation (p-p38 MAPK) in incubated rat epitrochlearis muscle. Isolated muscle was preincubated for 40 min and incubated for 15 min in the absence or presence of 3 mM caffeine. Muscle was then incubated with or without 1 μ M insulin in the absence or presence of 3 mM caffeine for 15min. The tissue lysate (B-E) and immunoprecipitate by anti IRS-1 antibody (IP:IRS-1) (A) was subjected to western blot analysis. Fold increases are expressed relative to the level of signal in the control muscles. Representative immunoblots are shown. Values are mean \pm SE; n = 5-10 per group. *P<0.05, **P<0.01, ***P<0.001.

Figure 4

The inhibitory effect of caffeine-induced (A) IRS-1 Ser³⁰⁷ phosphorylation (p-Ser³⁰⁷), and (B) IKK Ser^{176/180} phosphorylation (p-IKK) by caffeic acid on (C) IRS-1 Tyr⁶¹² phosphorylation (p-Tyr⁶¹²), (D) Akt phosphorylation (p-Akt), and (E) 3MG transport. Isolated muscle was preincubated for 40 min and incubated for 15 min in the

absence or presence of 3 mM caffeine. Muscle was then incubated with or without 1 μ M insulin in the absence or presence of 3 mM caffeine for 15min. When present, 5 mM caffeic was added throughout the preincubation and incubation periods. The tissue lysate (B-D) and immunoprecipitate by anti IRS-1 antibody (IP:IRS-1) (A) was subjected to western blot analysis. Muscle was also subjected to 3MG transport assay (E). Fold increases are expressed relative to the level of signal in the control muscles. Representative immunoblots are shown. Values are mean \pm SE; n = 6-8 per group. *P<0.05, ***P<0.001.

Figure 5

The effect of caffeine on (A) IRS-1 Ser⁷⁸⁹ phosphorylation (p-Ser⁷⁸⁹), (B) AMPK Thr¹⁷² phosphorylation (p-AMPK), and (C) ACC Ser⁷⁹ phosphorylation (p-ACC) in incubated rat epitrochlearis muscle. Isolated muscle was preincubated for 40 min and incubated for 15 min in the absence or presence of 3 mM caffeine. Muscle was then incubated with or without 1 μ M insulin in the absence or presence of 3 mM caffeine for 15min. The tissue lysate was subjected to western blot analysis. Fold increases are expressed relative to the level of signal in the control muscles. Representative immunoblots are shown. Values are mean \pm SE; n = 4-9 per group. *P<0.05, ***P<0.001.

Figure 6

The inhibitory effect of caffeine-induced (A) IRS-1 Ser⁷⁸⁹ phosphorylation (p-Ser⁷⁸⁹), and (B) AMPK Thr¹⁷² phosphorylation (p-AMPK) by dantrolene on (C) IRS-1 Tyr⁶¹² phosphorylation (p-Tyr⁶¹²), (D) Akt phosphorylation (p-Akt), and (E) 3MG transport.

Isolated muscle was preincubated for 40 min and incubated for 15 min in the absence or presence of 3 mM caffeine. Muscle was then incubated with or without 1 μ M insulin in the absence or presence of 3 mM caffeine for 15min. When present, 5 μ M dantrolene was added throughout the preincubation and incubation periods. The muscle was subjected to western blot analysis (A-D) or 3MG transport assay (E). Fold increases are expressed relative to the level of signal in the control muscles. Representative immunoblots are shown. Values are mean \pm SE; n = 6-8 per group. *P<0.05, ***P<0.001.

Figure 7

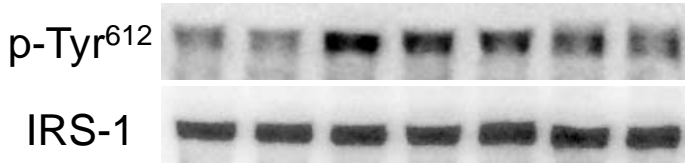
The effect of caffeine on (A) IRS-1 Ser^{636/639} phosphorylation (p-Ser^{636/639}), (B) mTOR Ser²⁴⁴⁸ phosphorylation (p-mTOR), and (C) p70S6K Thr³⁸⁹ phosphorylation (p-p70S6K) in incubated rat epitrochlearis muscle. Isolated muscle was preincubated for 40 min and incubated for 15 min in the absence or presence of caffeine. Muscle was then incubated with or without 1 μ M insulin in the absence or presence of 3 mM caffeine for 15min. The tissue lysate was subjected to western blot analysis. Fold increases are expressed relative to the level of signal in the control muscles. Representative immunoblots are shown. Values are mean \pm SE; n = 8-13 per group. **P<0.01, ***P<0.001.

Figure 8

The effect of caffeine on (A) IRS-1 Tyr⁶¹² phosphorylation (p-Tyr⁶¹²) and (B) Akt phosphorylation (p-Akt) in rat epitrochlearis muscle in vivo. Sixty min after intravenous injection of caffeine (5 mg/kg) or saline, insulin (100 mU/kg body

weight) or saline was injected intravenously. One min after insulin or saline injection epitrochlearis muscle was dissected, and tissue lysate was subjected to western blot analysis. Fold increases are expressed relative to the level of signal in the control muscles. Representative immunoblots are shown. Values are mean \pm SE; n = 6-15 per group. **P<0.01, ***P<0.001.

A



B

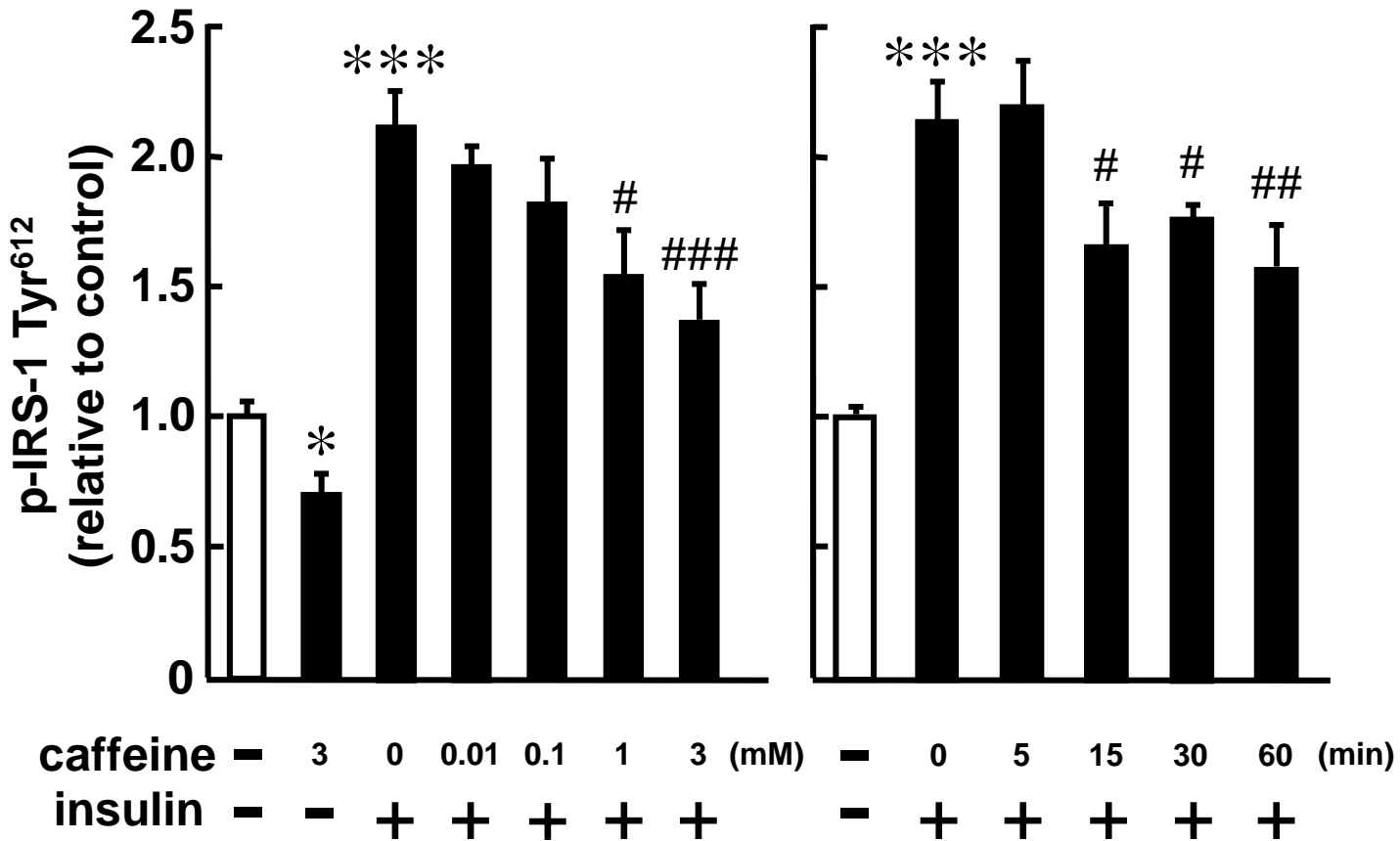
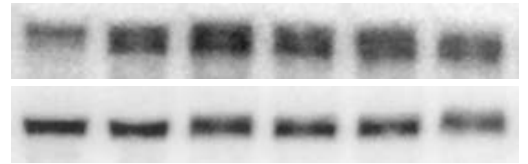


Figure. 1

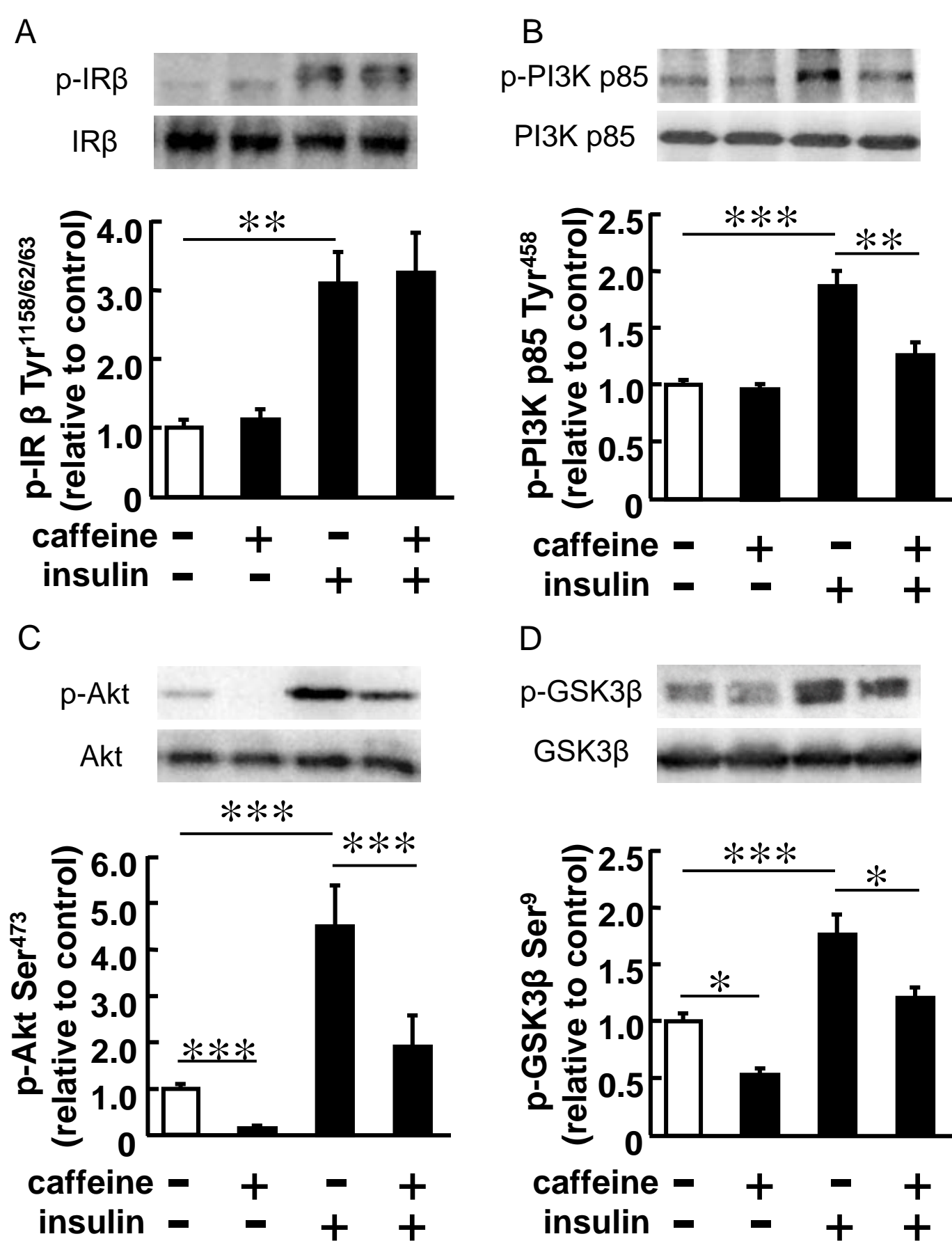
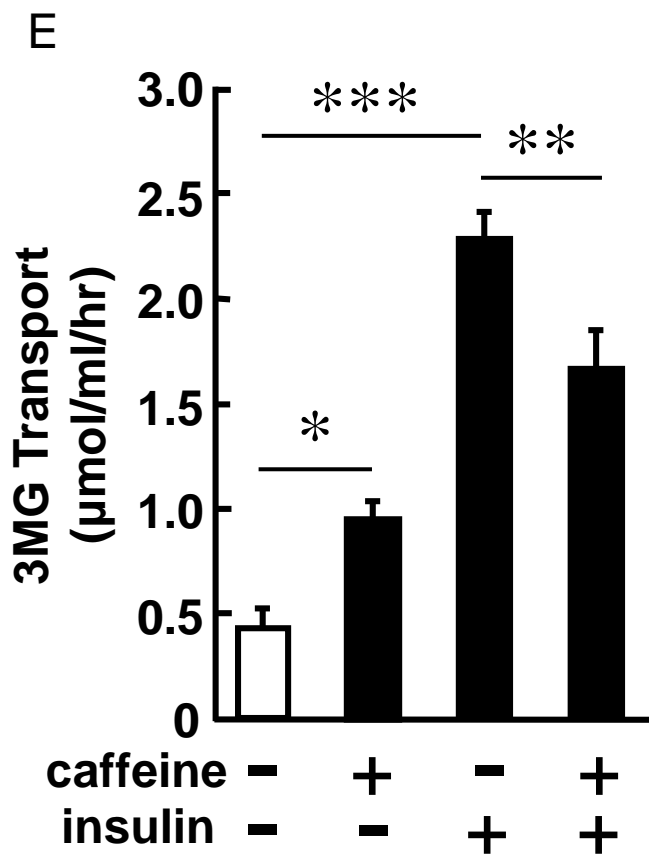


Figure. 2



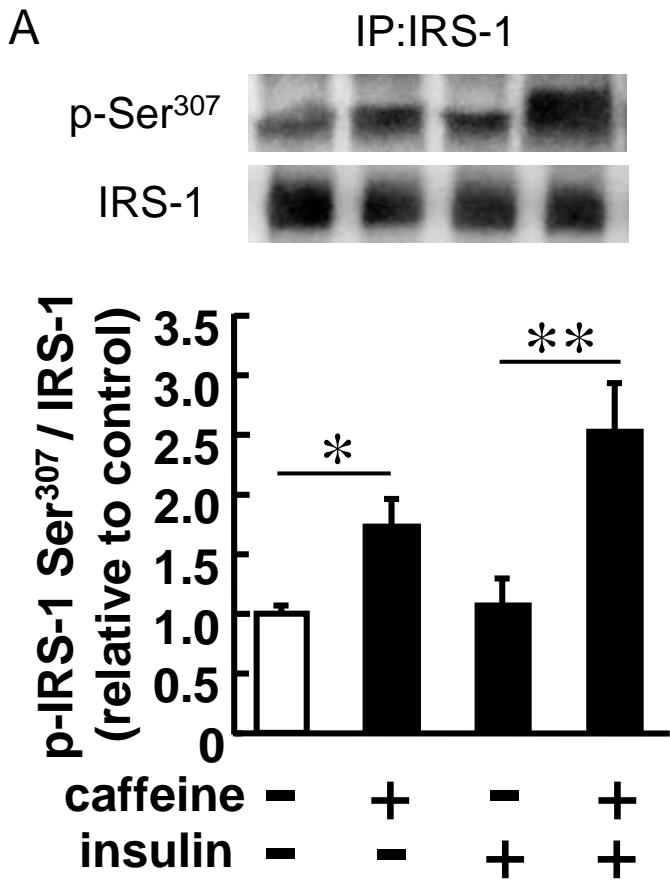
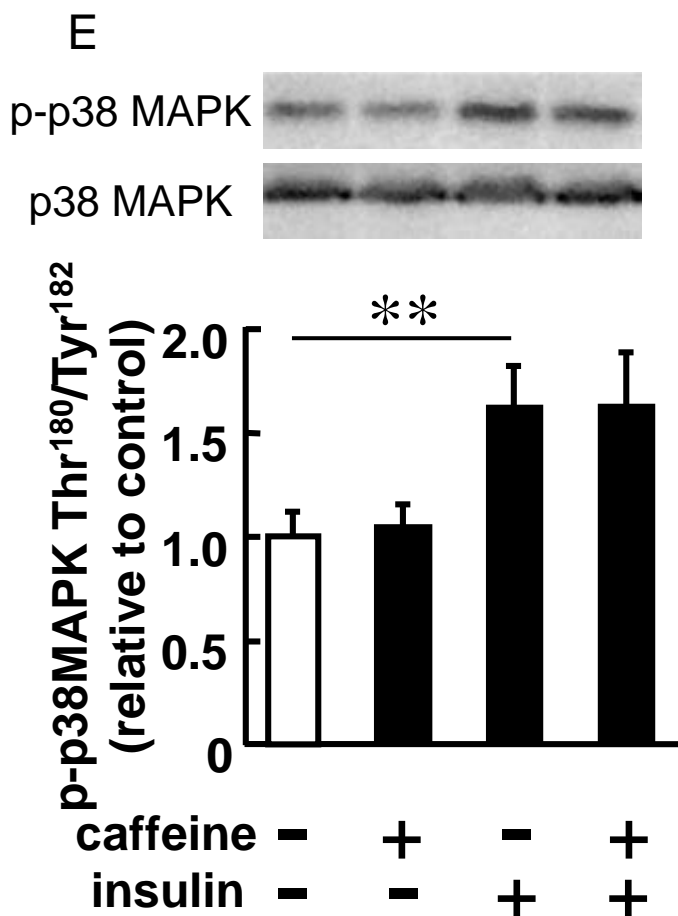
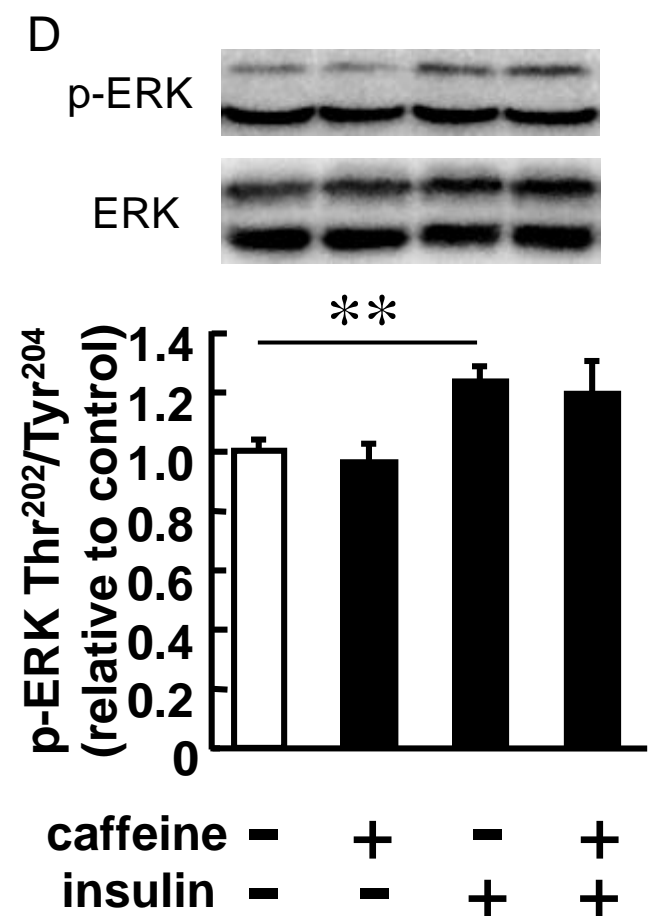
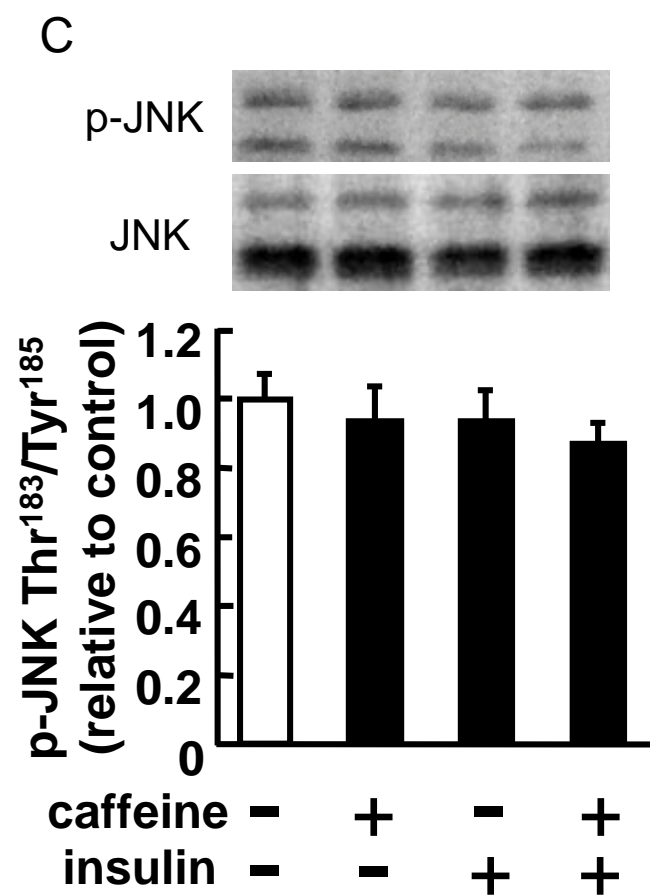
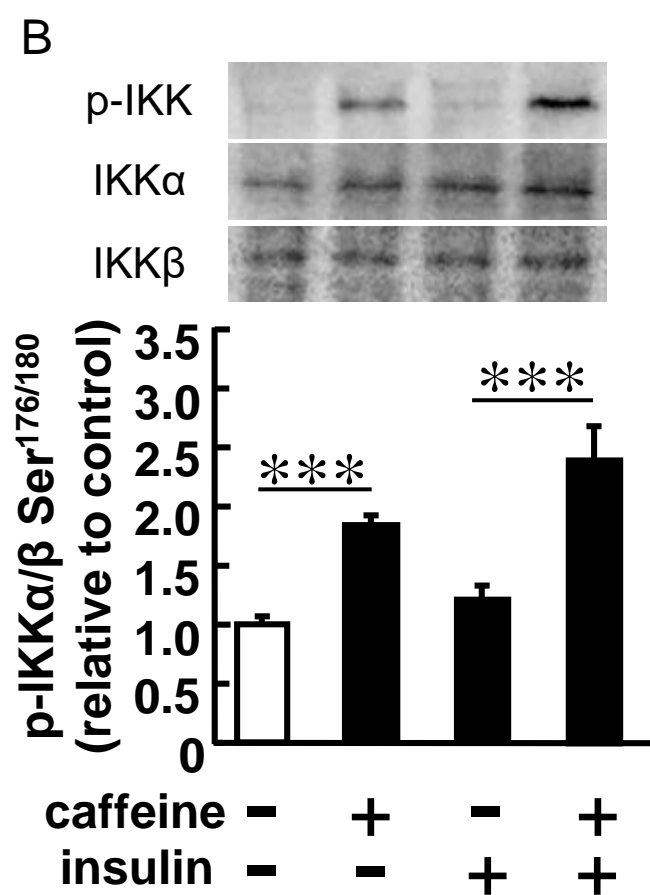


Figure. 3



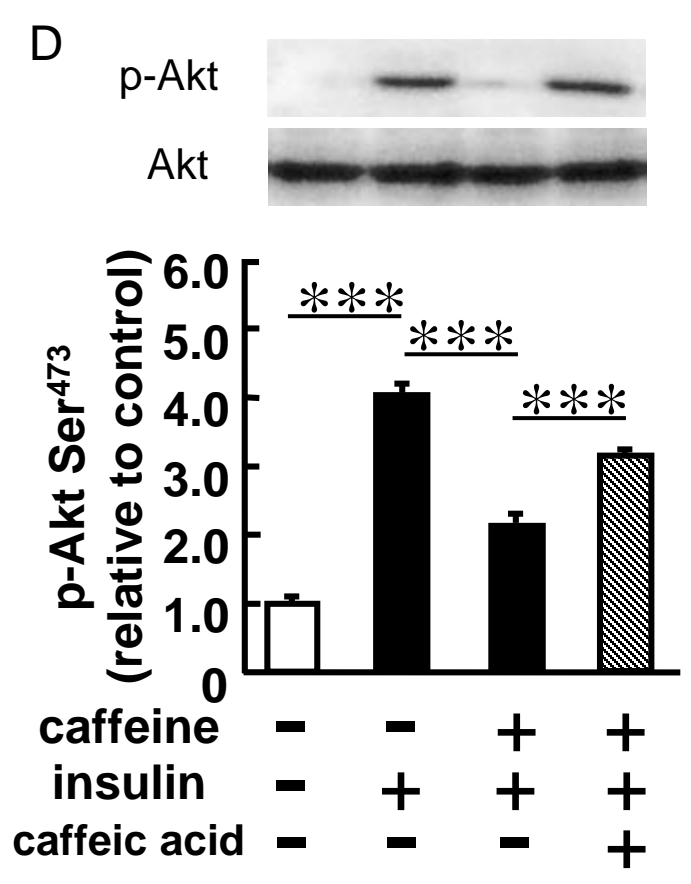
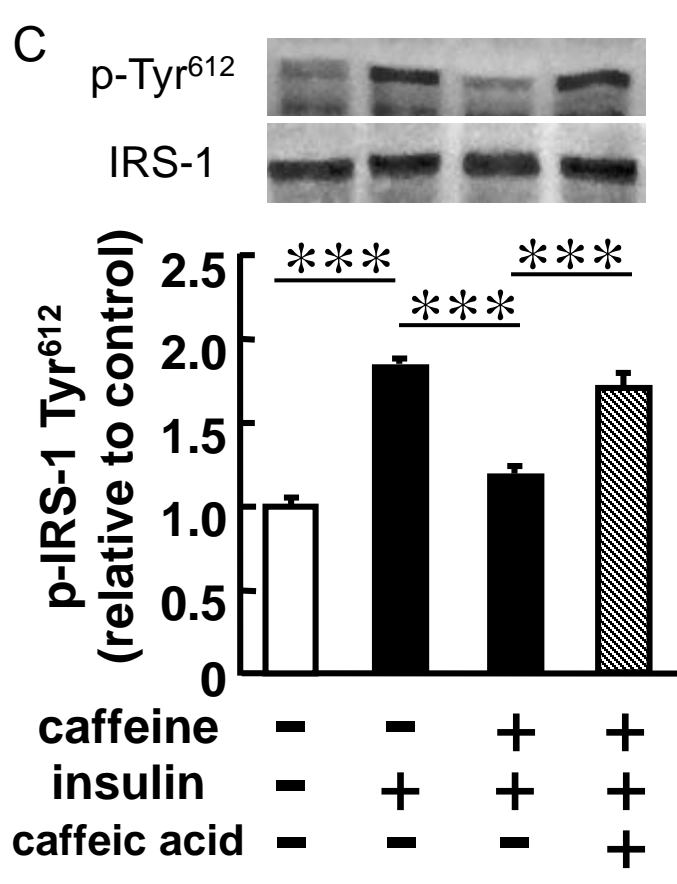
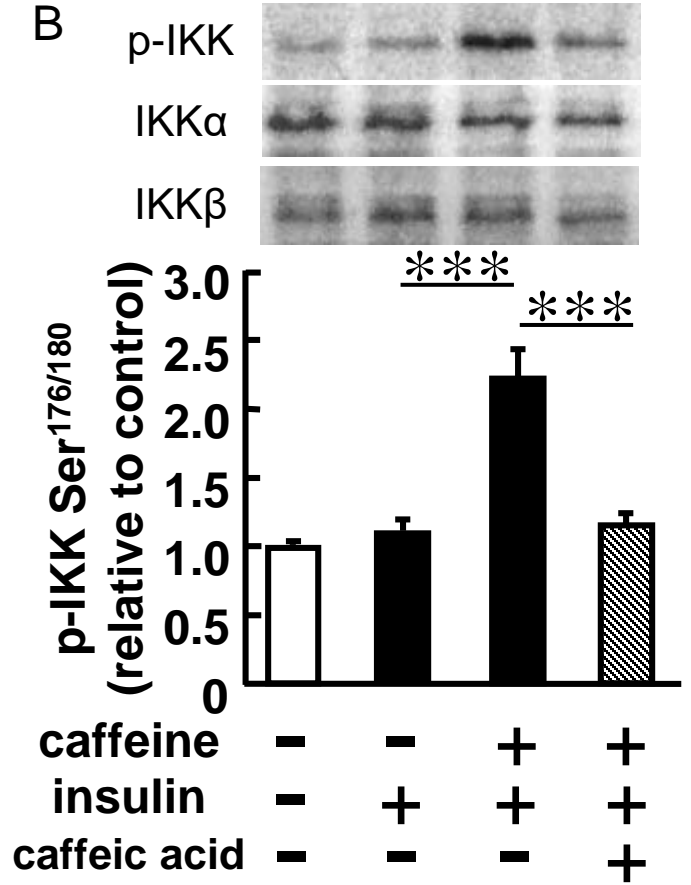
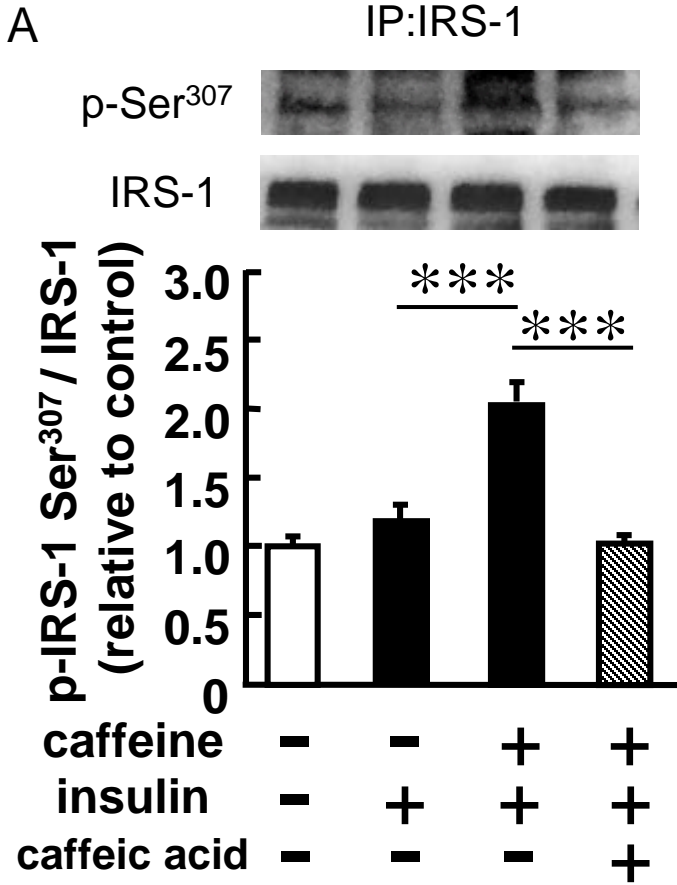
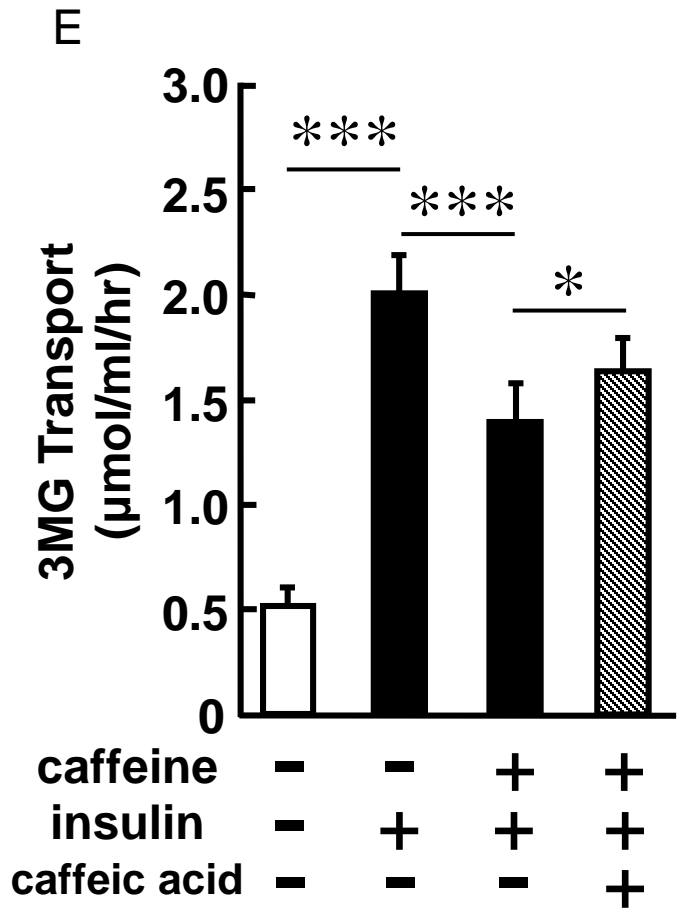
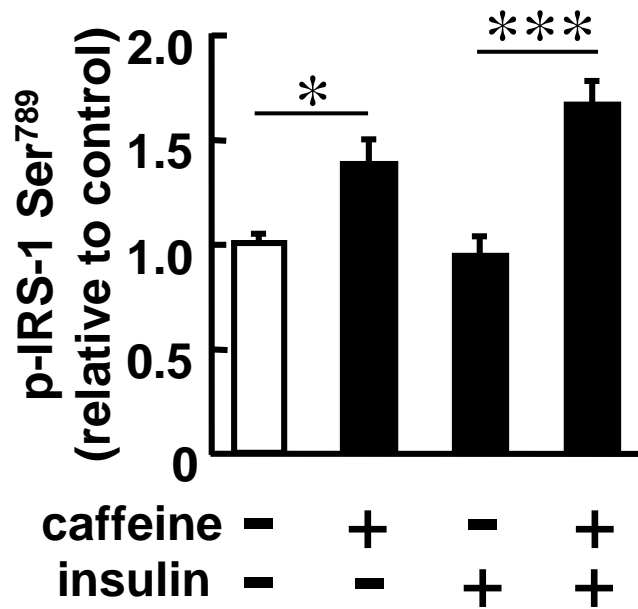
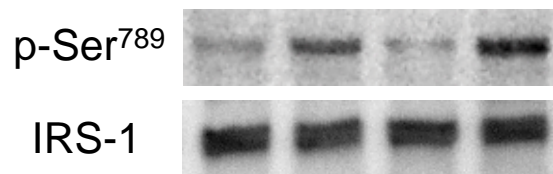


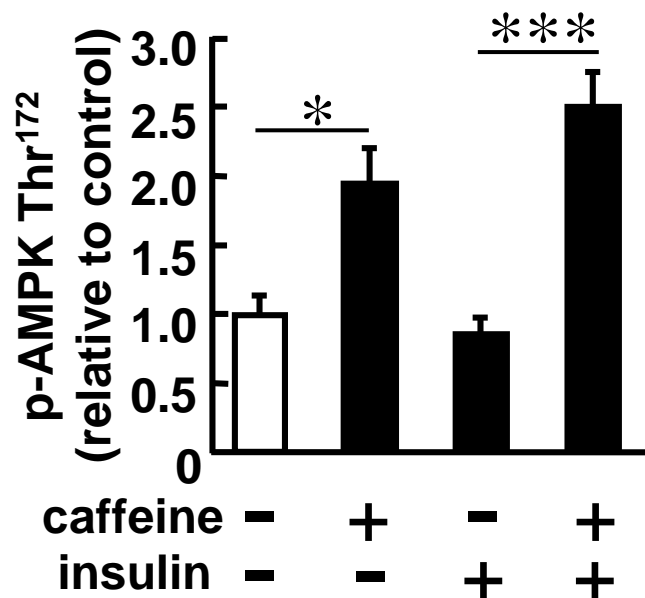
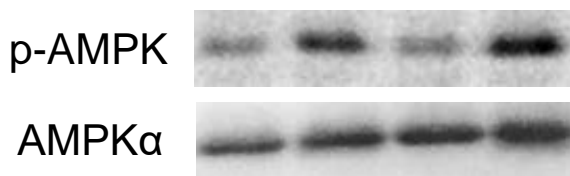
Figure. 4



A



B



C

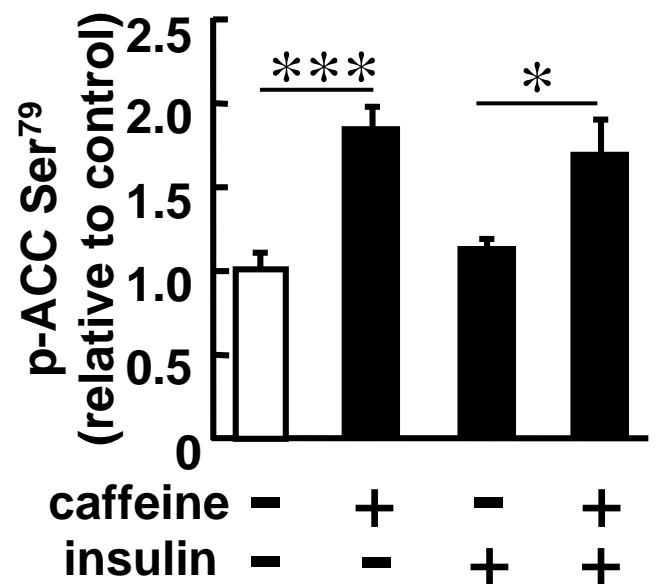
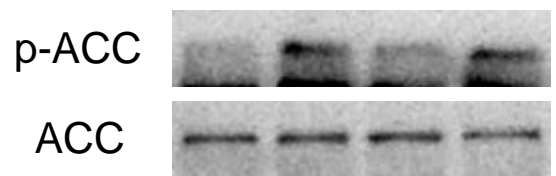


Figure. 5

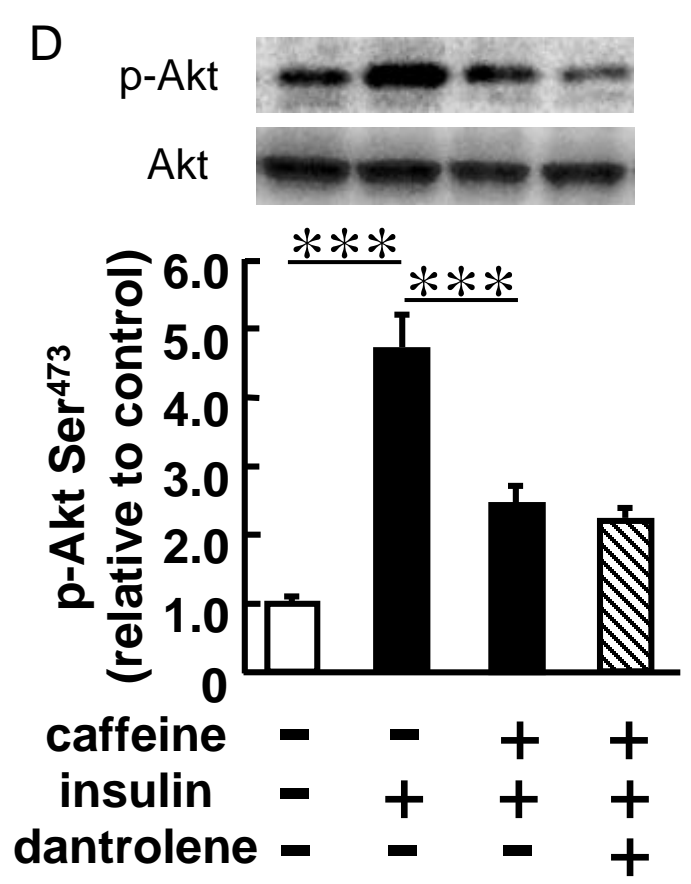
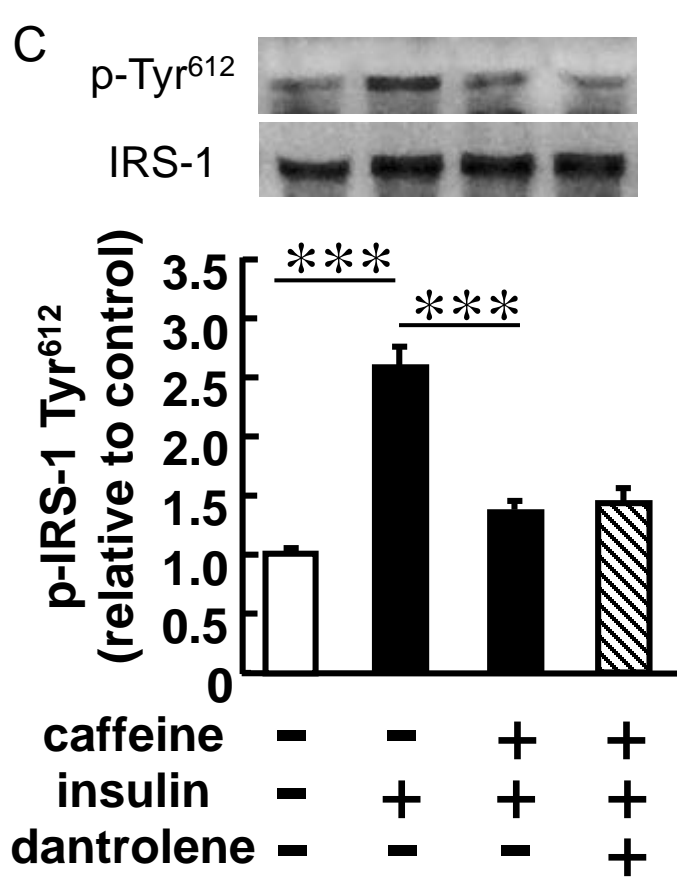
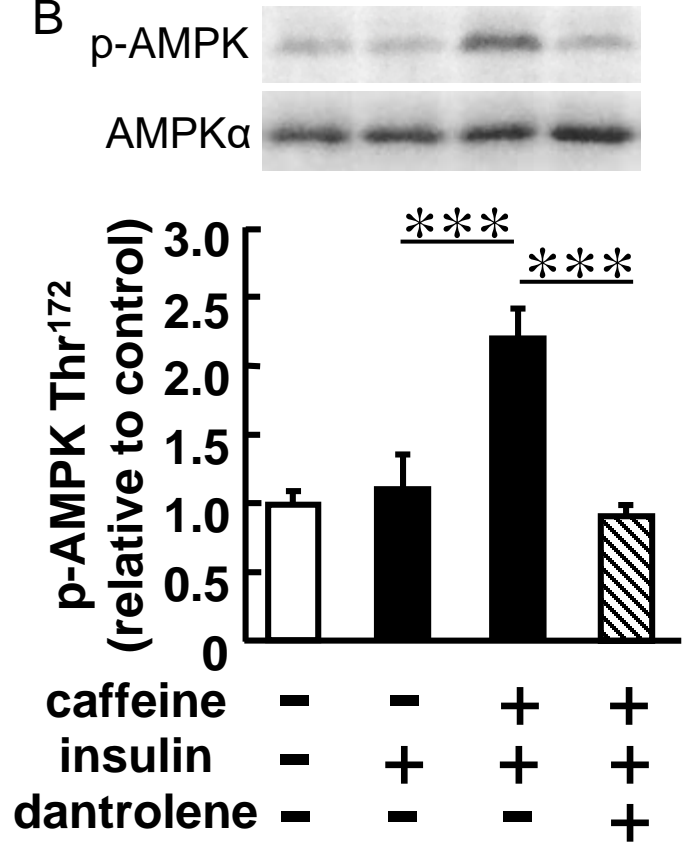
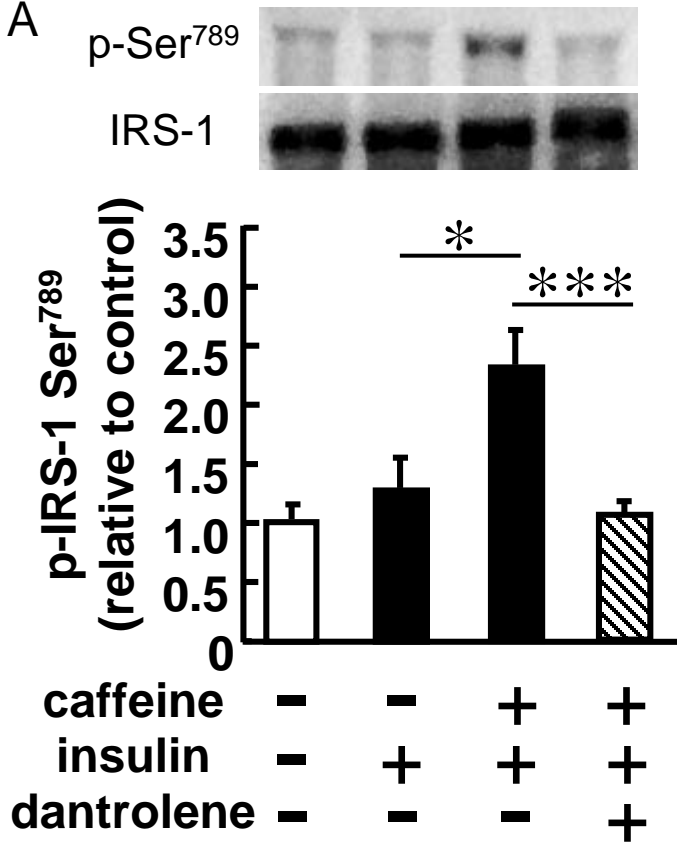
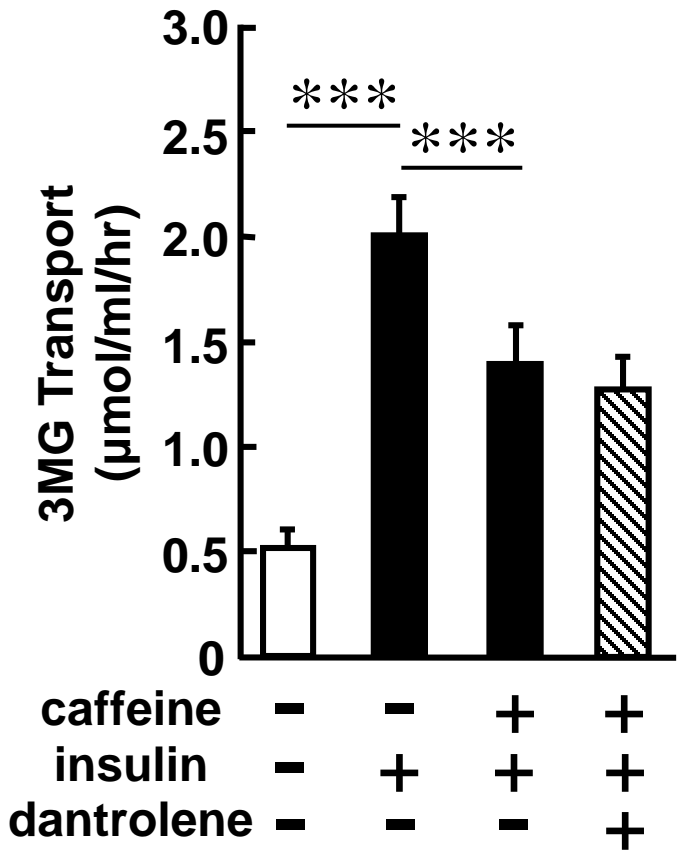
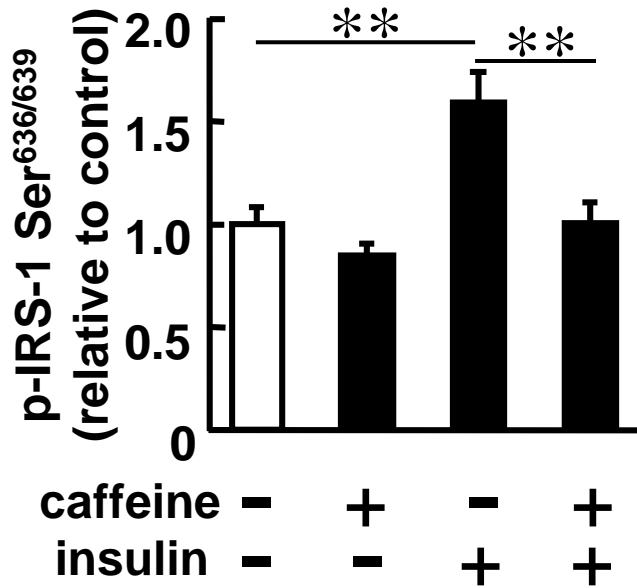
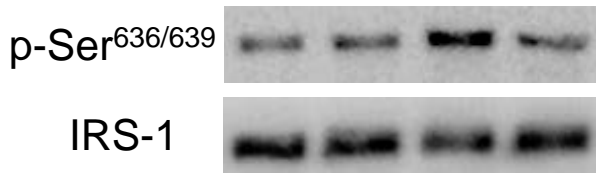


Figure. 6

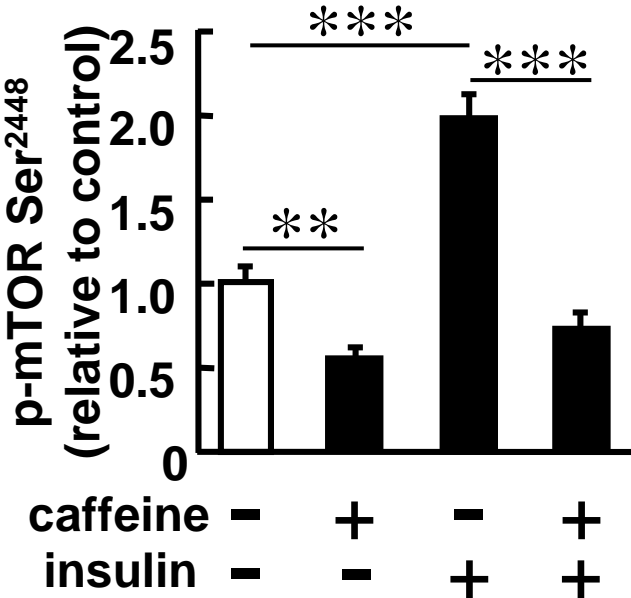
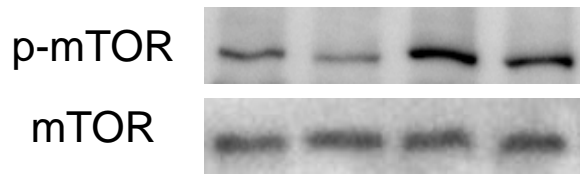
F



A



B



C

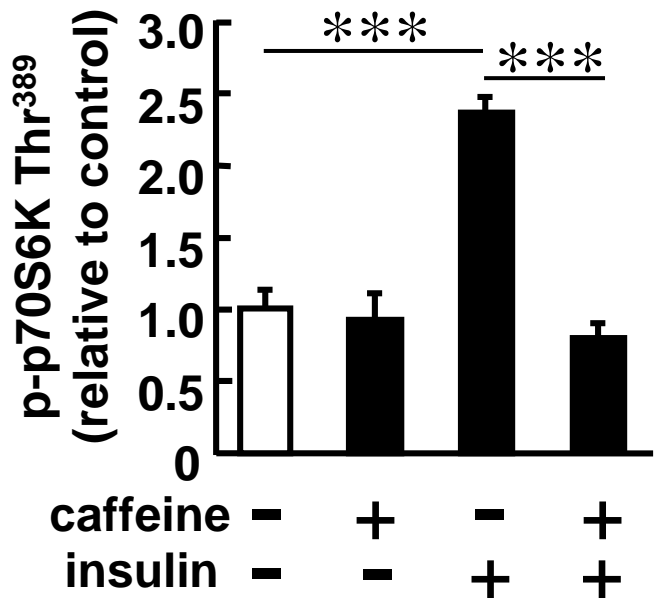
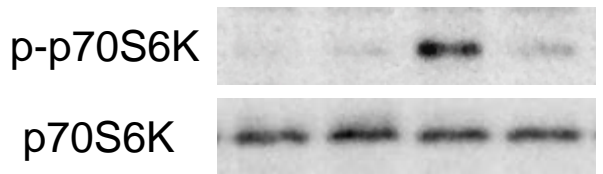


Figure. 7

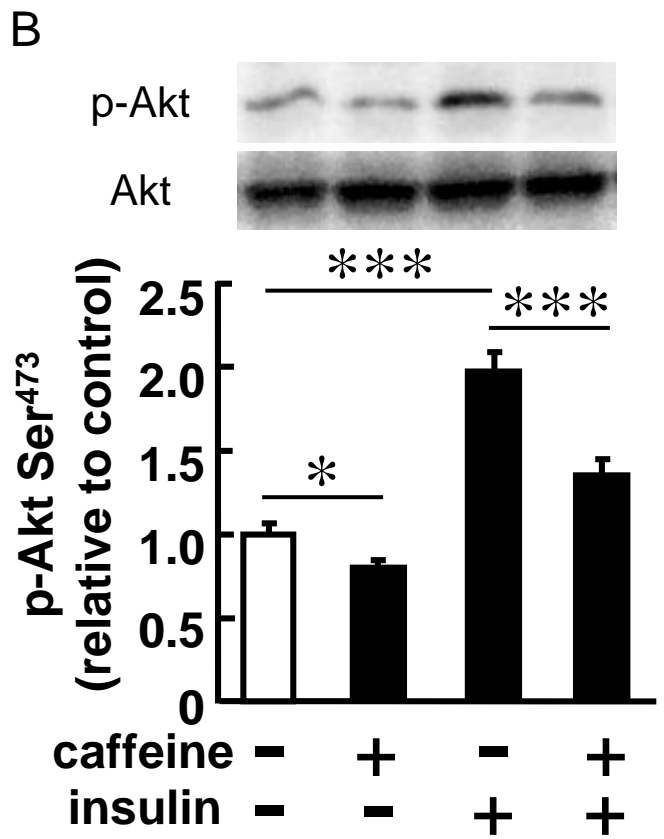
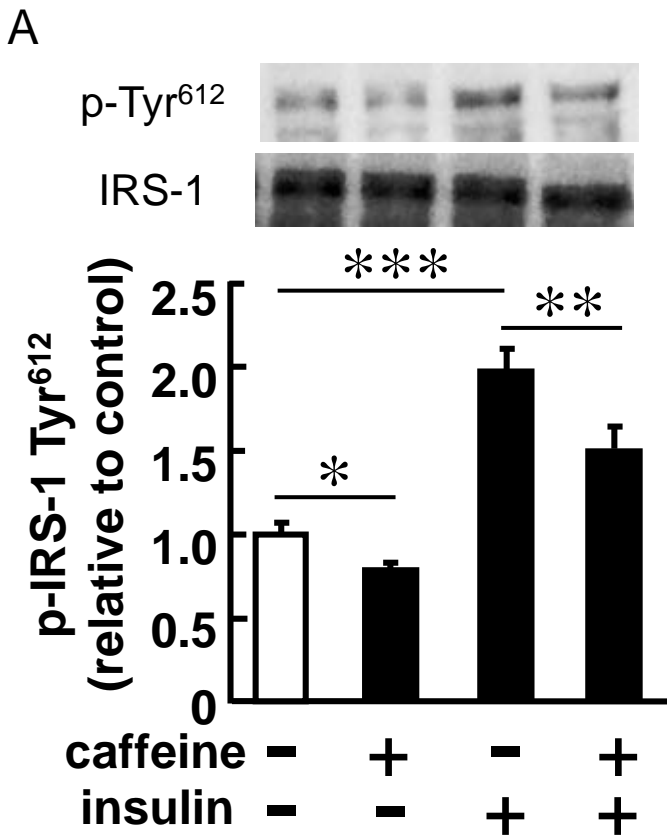


Figure. 8