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Inhibitory mechanism of caffeine on insulin-stimulated glucose uptake in adipose cells

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Abstract

Caffeine inhibits insulin-induced glucose uptake in rat adipocytes and also decreases insulin sensitivity, including whole-body glucose disposal and glucose uptake in skeletal muscle, during a euglycemic–hyperinsulinemic clamp in human. However, the mechanism by which caffeine decreases the insulin sensitivity is not still clear. We found that pre-treatment with caffeine inhibited the insulin-induced 2-deoxy-D-[1-^{3}H]glucose uptake in a concentration-dependent manner in mouse preadipose MC-3T3-G2/PA6 cells differentiated into mature adipose cells. Caffeine also suppressed insulin-induced GLUT4 translocation in the differentiated cells. Although caffeine did not alter insulin-induced activation of PI3K and protein kinase C-zeta (PKCζ), an isoform of atypical PKC, which is reported to have an important role in insulin-induced GLUT4 translocation, we found that insulin-induced phosphorylation and activation of Akt were blocked by pre-treatment with caffeine. Inhibition of insulin-induced 2-deoxy-D-[1-^{3}H]glucose uptake by caffeine was also observed in primary cultured brown adipocytes in a concentration-dependent manner. These results may, in part, explain the ability of caffeine to decrease insulin sensitivity.

Keywords: Adipocytes; Glucose uptake; Insulin; Phosphatidylinositol 3-kinase; Akt; GLUT4

1. Introduction

Insulin plays a key role in the stimulation of glucose uptake in the tissues, such as muscle and adipose tissue, as well as in the maintenance of glucose homeostasis. Impairment of insulin's ability to stimulate glucose uptake in the tissues is a major factor responsible for insulin resistance associated with type 2 diabetes [1]. Methylxanthines, such as caffeine, have been shown to inhibit insulin-stimulated glucose uptake in rat isolated adipocytes [2] and Chinese hamster ovary cells overexpressing insulin receptors [3], as well as in perfused contracting rat hindquarters [4]. Furthermore, in humans, caffeine decreases insulin sensitivity, including whole-body glucose disposal [5–7] and glucose uptake in skeletal muscle [7], during a euglycemic–hyperinsulinemic clamp. The mechanisms of some of the pleiotropic effects of methylxanthines include inhibition of phosphodiesterase and an antagonistic effect on adenosine receptors. Since adenosine or its receptor antagonists modify insulin sensitivity and glucose tolerance [2,4,8,9], the inhibition of glucose uptake by caffeine has been suggested to result from antagonism at the adenosine receptor. In contrast, Thong et al. [10] have suggested that the insulin-antagonistic effects of caffeine are independent of the adenosine receptor antagonism.

Abbreviations: α-MEM, alpha modification of Eagles minimal medium; ATM, ataxia-telangiectasia-mutated protein kinase; ATR, ATM/Rad3-related protein kinase; aPKC, atypical PKC; cAMP, cyclic adenosine monophosphate; Dex, dexamethasone; DMEM, Dulbeccos modified Eagles medium; Erks, extracellular signal-regulated kinases; FBS, fetal bovine serum; GLUT, glucose transporter; GSK-3, glycogen synthase kinase-3; IBMX, 1-methyl-3-isobutylxanthine; IR, insulin receptor; IRS, IR substrate; KRP, Krebs-Ringer phosphate; MAP, mitogen-activated protein; MEK, MAP kinase/Erk kinase; mTOR, mammalian target of rapamycin; p70 S6K, ribosomal p70 S6 protein kinase; PBS, phosphate-buffered saline; PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-monophosphate; PIKK, PI3K-related kinase; RT-PCR, reverse transcription-polymerase chain reaction

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The primary mechanism of glucose uptake stimulated by insulin in muscle and adipocytes is through the translocation of glucose transporters 4 (GLUT4) from intracellular pools to the plasma membrane [11]. The translocation of GLUT4 to plasma membrane was established to be mediated by phosphatidylinositol 3-kinase (PI3K), based on the use of pharmacological inhibitors and expression of a dominant negative mutant or constitutively active form of PI3K [12–16]. Caffeine is known to potentiate the lethal effects of radiation or genotoxic agents in cultured cells due to, for example, inhibition of DNA repair [17], alteration of checkpoint control in the cell cycle [18,19], or induction of apoptosis [20]. Caffeine-induced alteration of checkpoint control is suggested to be caused by inhibition of the activities of kinases, such as ataxia-telangiectasia-mutated protein kinase (ATM) and ATM/Rad3-related protein kinase (ATR) [18,21]. ATM and ATR are the members of the PI3K-related kinase (PIKK) family [22]. The PIKK family members share a C-terminal kinase domain bearing significant sequence homology to the catalytic domains of mammalian and yeast PI3K. Methylxanthines are also known to have inhibitory effects on phosphoinositide metabolism [3,23,24]. Therefore, the antagonistic effect of caffeine on insulin-induced glucose uptake may be due to the inhibition of PI3K and its downstream effectors, such as Akt (also called protein kinase B) and atypical protein kinase C (aPKC). To test the hypothesis, in this study, we investigated the effects of caffeine on insulin-induced PI3K signaling and glucose uptake using the mouse preadipose cell line, MC3T3-G2/PA6, which differentiates into mature adipocytes.

2. Materials and methods

2.1. Materials

Alpha modification of Eagle's minimal medium (α-MEM) and Dulbecco's modified Eagle's medium (DMEM) were from ICN Biomedicals, Inc. (Irvine, CA, USA). The anti-actin antibody, fetal bovine serum (FBS), insulin, wortmannin, rapamycin, CGS-15943 and caffeine were from Sigma–Aldrich Corp. (St. Louis, MO, USA). Kanamycin was from EMD Biosciences (Calbiochem; San Diego, CA, USA). The Akt kinase assay kit, phospho-specific Akt (Ser 473) antibody, phospho-specific p70 S6 kinase (Thr 389) antibody, and PhosphoPlus p44/42 MAP kinase antibody kit were from Cell Signaling Technology, Inc. (Beverly, MA, USA). The anti-Akt1/2 antibody, anti-glucose transporter 1 (GLUT1) antibody, anti-GLUT4 antibody, anti-PKCζ antibody and agarose conjugated with monoclonal anti-phosphotyrosine antibody (PY99) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Dexamethasone (Dex), 8-phenyltheophylline and 1-methyl-3-isobutylxanthine (IBMX) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The cAMP EIA system, 2-deoxy-D-[1-H^3]glucose and [γ-^{32}P]ATP was purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA).

2.2. Cell culture

The mouse preadipocyte cell line, MC3T3-G2/PA6 [25], was maintained in α-MEM supplemented with 10% heat-inactivated FBS and 60 μg/ml kanamycin in an atmosphere of 5% CO_2 at 37 °C. Differentiation was induced by treating confluent cells with α-MEM containing 0.5 mM IBMX, 0.25 μM Dex and 10% FBS for 4 days. The cells were refed with α-MEM supplemented with 10% FBS every other day for the following 4–6 days.

Brown adipocytes were obtained from interscapular brown adipose tissue of 3-week-old male C57BL/6J mice (Nippon SLC Corp., Hamamatsu, Japan) as described previously [26,27]. The tissue was minced in an isolation solution (123 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 5 mM glucose, 1.5% BSA, 100 mM Hepes, pH 7.4) containing 0.2% collagenase type II (Wako Pure Chemical) and digested for 30 min at 37 °C. The digest was successively filtered through 250- and 25-μm nylon filters to remove undigested material and mature cells. The brown adipocytes were pelleted by centrifugation (10 min, 2000 rpm), washed and resuspended in DMEM, and seeded into 24-well plates for culture in DMEM supplemented with 10% heat-inactivated FBS and 100 μg/ml kanamycin in an atmosphere of 5% CO_2 at 37 °C.

2.3. Glucose uptake

The cells cultured in 24-well plates were incubated in α-MEM containing 0.1% FBS for 24 h at 37 °C. Then, the medium was changed to Krebs–Ringer phosphate (KRP) buffer (123 mM NaCl, 4.94 mM KCl, 1.23 mM $MgSO_4 \cdot 7H_2O$, 0.84 mM $CaCl_2 \cdot 2H_2O$, 4.99 mM glutamine, 20 mM $NaH_2PO_4 \cdot 2H_2O$, 15 mM Hepes, pH 7.4) and incubation was continued for 2 h at 37 °C. Before the cells were exposed to insulin, they were treated or not treated with caffeine or adenosine receptor antagonists in KRP buffer for 1 h. After incubation with or without insulin (1 μM) for the indicated period, 2-deoxy-D-[1-^{3}H]glucose (0.5 μCi) [specific activity; 14.0 Ci/mmol (518 GBq/mmol)] was added, and incubation was continued for 5 min. The cells were washed twice with ice-cold PBS and then solubilized with 0.1% SDS in 1N NaOH. After neutralization with 1 N HCl, the radioactivity incorporated into the cells was measured by liquid scintillation spectroscopy.

2.4. Immunoblotting

The differentiated MC3T3-G2/PA6 cells cultured in 24-well plates were incubated in α-MEM containing 0.1% FBS for 24 h at 37 °C. The medium was changed

to α-MEM containing 0.1% FBS and incubation was continued for another 2–4 h at 37 °C. Before the cells were exposed to insulin, they were treated or not treated with caffeine for 1 h. Then, insulin (1 (M) was added and incubation was continued for an additional 5 min at 37 °C. The cells were lysed, and immunoblot analysis was performed with antibody against actin or the phospho-specific antibodies against Akt, ribosomal p70 S6 protein kinase (p70 S6K) and extracellular signal-regulated kinases (Erks).

The differentiated 3T3-G2/PA6 cells pre-treated with caffeine or wortmannin for 1 h were incubated with insulin (1 μM) for 20 min. The cells were washed with ice-cold PBS and scraped in homogenization buffer (20 mM Tris–HCl, pH 7.4, 2 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM β-mercaptoethanol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). After incubation for 10 min, the cells were homogenized with 30 strokes of a Dounce homogenizer using a tight-fitting pestle, and centrifuged at 500 × *g* for 5 min. The supernatant was centrifuged at 100,000 × *g* for 30 min. The pellet was washed three times, then extracted in ice-cold homogenization buffer containing 1% Triton X-100 for 60 min, and the extract was centrifuged at 100,000 × *g* for 15 min. The quantity of protein in the supernatant (plasma membrane fraction) was normalized against the untreated control, and immunoblotting was performed with anti-GLUT1 and anti-GLUT4 antibodies.

Antibody-bound proteins were detected by luminescence (ECL Western Blotting Kit, Amersham Biosciences) and analyzed using the Las-1000 lumino-imaging analyzer (Fuji Film, Tokyo, Japan). The bands of Western blots were quantified by using NIH image 1.61 software. The intensity of each phospho-specific band was normalized to the corresponding band of actin. Then, the inhibitory effect of caffeine on expression or phosphorylation of proteins increased by insulin was expressed as the expression (%) or phosphorylation (%) calculated by the following formula; $(X - C/I - C) \times 100$, where X is the intensity of expression or phosphorylation of each protein increased by insulin in the presence or absence of caffeine, C is that of the protein band of untreated control, and I is that of expression or phosphorylation of the protein increased by insulin alone.

2.5. PI3K assay

The differentiated MC3T3-G2/PA6 cells pre-treated with caffeine for 1 h were exposed to insulin (1 μM) for 5 min. The cells were lysed in 400 μl of lysis buffer (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μM aprotinin, 10 μM leupeptin), and the lysate was sonicated and centrifuged. The quantity of protein in the supernatant fraction was normalized against the untreated control and incubated with 20 μl of agarose conjugated with a monoclonal anti-phosphotyrosine antibody (PY99) with gentle rocking for 2 h at 4 °C. Then, PI3K activity was determined as described previously [28]. The radiolabeled spot of phosphatidylinositol 3-monophosphate (PI3P) that indicates PI3K activation was quantified using a Bas-2000 bio-imaging analyzer (Fuji Film).

2.6. Akt kinase assay

Akt kinase assay was carried out by using an Akt kinase assay kit (Cell Signaling Technology) according to the manufacturer's instructions. In brief, the differentiated MC3T3-G2/PA6 cells pre-treated with caffeine for 1 h were exposed to insulin (1 μM) for 5 min. The cells were lysed in 400 μl of lysis buffer, and the lysate was sonicated and centrifuged. The quantity of protein in the supernatant fraction was normalized against the untreated control and incubated with 20 μl of immobilized Akt (1G1) monoclonal antibody beads with gentle rocking overnight at 4 °C. The enzyme immune complex was washed twice with 0.5 ml of lysis buffer, and once with 500 μl of kinase buffer. The enzyme immune complex was suspended in 50 μl of kinase buffer supplemented with 1 μl of 10 mM ATP and 1 μl of glycogen synthase kinase-3 (GSK-3) fusion protein. The reaction was incubated for 30 min at 30 °C and terminated by the addition of 25 μl of 3× SDS sample buffer followed by boiling for 5 min at 95 °C. The Akt kinase activity was determined by immunoblotting with phospho-specific GSK α/β (Ser 21/9) antibody (Cell Signaling Technology).

2.7. PKCζ kinase assay

The differentiated MC3T3-G2/PA6 cells pre-treated with caffeine for 1 h were exposed to insulin (1 μM) for 5 min. The cells were lysed in 400 μl of lysis (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 1 mM $MgCl_2$, 10% glycerol, 1% Nonidet P-40, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μM aprotinin, 10 μM leupeptin), and the lysate was sonicated and centrifuged. The quantity of protein in the supernatant fraction was normalized against the untreated control and imunoprecipitated with anti-PKCζ antibody (Santa Cruz Biotechnology). The enzyme immune complex was washed three times with 0.5 ml of lysis buffer, and twice with 500 μl of kinase buffer (35 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM EGTA, 1 μM sodium orthovanadate). The reaction was performed in the kinase buffer containing 1 μCi of [γ-^{32}P]ATP, 60 μM ATP and 1 μg of myelin basic protein (MBP) (Upstate Inc., Charlottesville, VA, USA) as a substrate for 30 min at 30 °C, and terminated by the addition of 5× SDS sample buffer followed by boiling for 5 min at 95 °C. Samples were resolved in 12% SDS-PAGE, and gels were dried out and subjected to autoradiography.

2.8. cAMP content

The differentiated MC3T3-G2/PA6 cells cultured in 24-well plates were incubated in α-MEM containing 0.1% FBS for 24 h at 37 °C. The medium was changed to α-MEM containing 0.1% FBS, and incubation was continued for another 2–4 h at 37 °C. Before the cells were exposed to insulin, they were treated or not treated with caffeine or isoproterenol (10 μM) for 1 h. Then, insulin (1 μM) was added and incubation was continued for an additional 20 min at 37 °C. The medium was aspirated, and the cells were lysed using a lysis buffer (Amersham Biosciences). Cyclic adenosine monophosphate (cAMP) content was determined with a cAMP EIA system according to the manufacturer's instructions.

3. Results

3.1. Effects of caffeine on insulin-induced uptake of 2-deoxy-D-[1-^{3}H]glucose

We assessed the effect of caffeine on insulin-induced 2-deoxy-D-[1-^{3}H]glucose uptake in MC3T3-G2/PA6 cells differentiated into mature adipose cells. As shown in Fig. 1A, caffeine inhibited insulin-induced 2-deoxy-D-[1-^{3}H]glucose uptake in a concentration-dependent manner (IC50 value; 0.24 mM), while it had no effect on the basal glucose uptake without insulin.

The primary mechanism of glucose uptake stimulated by insulin in muscle and adipocytes is through the translocation of GLUT4 from intracellular pools to the plasma membrane [11]. Therefore, we determined the effect of caffeine on GLUT4 translocation to the plasma membrane induced by insulin stimulation. As shown in Fig. 1B, immunoblotting analysis with anti-GLUT4 antibody indicated that caffeine inhibited the insulin-induced GLUT4 translocation to plasma membrane (IC50 value; 0.15 mM), as did wortmannin (100 nM). In contrast, the amount of GLUT1 in the plasma membrane was unaffected by insulin, caffeine and wortmannin.

3.2. Caffeine inhibited insulin-induced activation of Akt, but not PI3K or PKCζ

We examined the effect of caffeine on the signaling pathway stimulated with insulin. Pre-treatment with caffeine did not affect the insulin-induced PI3K activation (Fig. 2). On the other hand, insulin-induced phosphorylation (IC50 value; 0.22 mM) and activation (IC50 value; 0.28 mM) of Akt were inhibited by pre-treatment with caffeine in a concentration-dependent manner (Fig. 3A and B). Similarly, caffeine also blocked insulin-induced

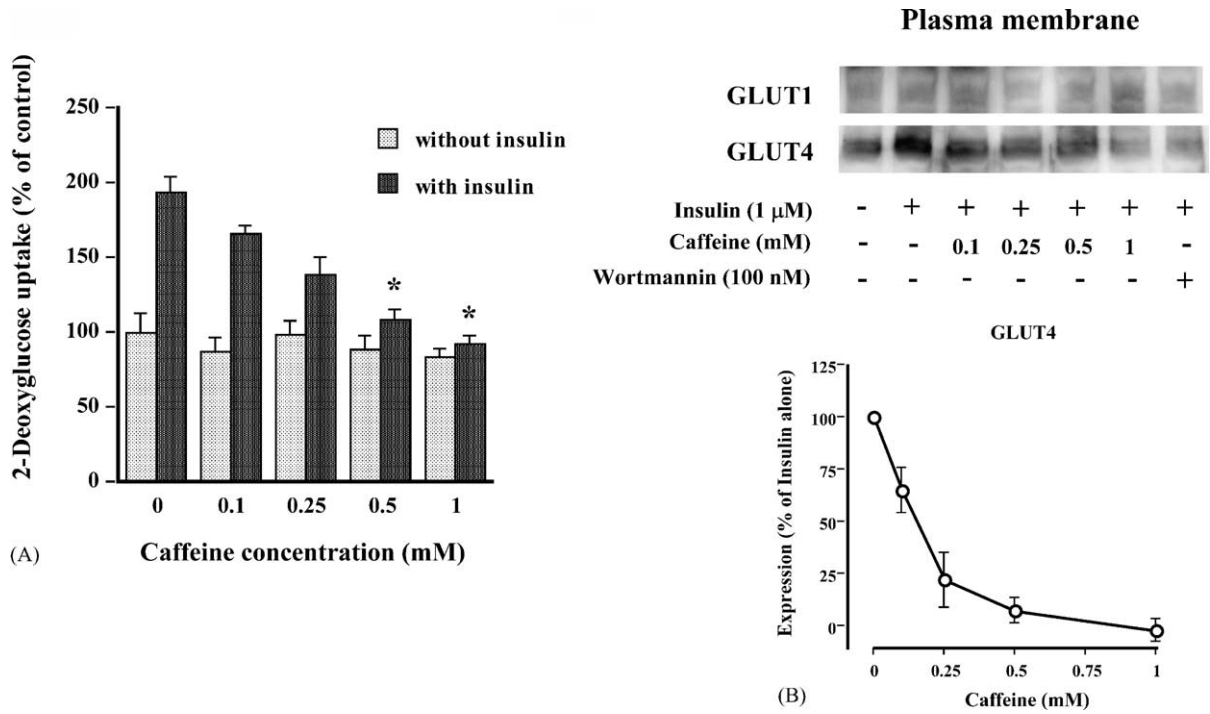

Fig. 1. Inhibition by caffeine of insulin-induced glucose uptake by MC3T3-G2/PA6 cells. (A): Differentiated MC3T3-G2/PA6 cells were treated or not treated with caffeine for 1 h. After incubation with or without insulin (1 μM) for 20 min, 2-deoxy-D-[1-^{3}H]glucose was added and incubation was continued for 5 min. Each value is the mean ± S.E. of at least three experiments. (*) Significantly different from insulin alone at $P > 0.01$. (B): Differentiated MC3T3-G2/PA6 cells were treated or not treated with caffeine or wortmannin (100 nM) for 1 h. Then, the cells were incubated with or without insulin (1 μM) for 20 min. The amount of GLUT1 and GLUT4 in plasma membrane was detected by immunoblotting with anti-GLUT1 and anti-GLUT4 antibodies. The inhibitory effect of caffeine on insulin-stimulated GLUT4 expression [expression (%)] was calculated as described in Section 2. Each value is the mean ± S.E. of three experiments.

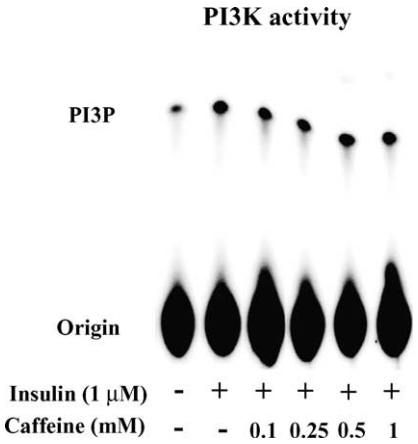

Fig. 2. Effects of caffeine on insulin-induced PI3K activation. Differentiated MC3T3-G2/PA6 cells were treated or not treated with caffeine for 1 h. Then, the cells were incubated with or without insulin (1 μM) for 5 min. The cells were lysed, and the activities of PI3K were determined as described in Section 2. PI3P, phosphoinositol 3-monophosphate.

p70 S6K phosphorylation (IC50 value; 0.22 mM) (Fig. 3A). The inhibition of insulin-induced Erks phosphorylation by caffeine was much less potent (Fig. 3A). Although wortmannin inhibited the insulin-induced activation of PKCζ, an isoform of atypical PKC (aPKC), which is suggested to play an important role in insulin-induced GLUT4 translocation [29], caffeine did not do so (Fig. 4).

3.3. cAMP and adenosine receptor antagonism are not involved in insulin-induced glucose uptake

Because caffeine has adenosine receptor antagonistic activity and inhibits phosphodiesterase activity, we examined whether caffeine modifies the cAMP content of differentiated MC3T3-G2/PA6 cells. As shown in Fig. 5A, the cAMP content was not changed in cells treated with caffeine (1 mM) and/or insulin (1 μM), whereas

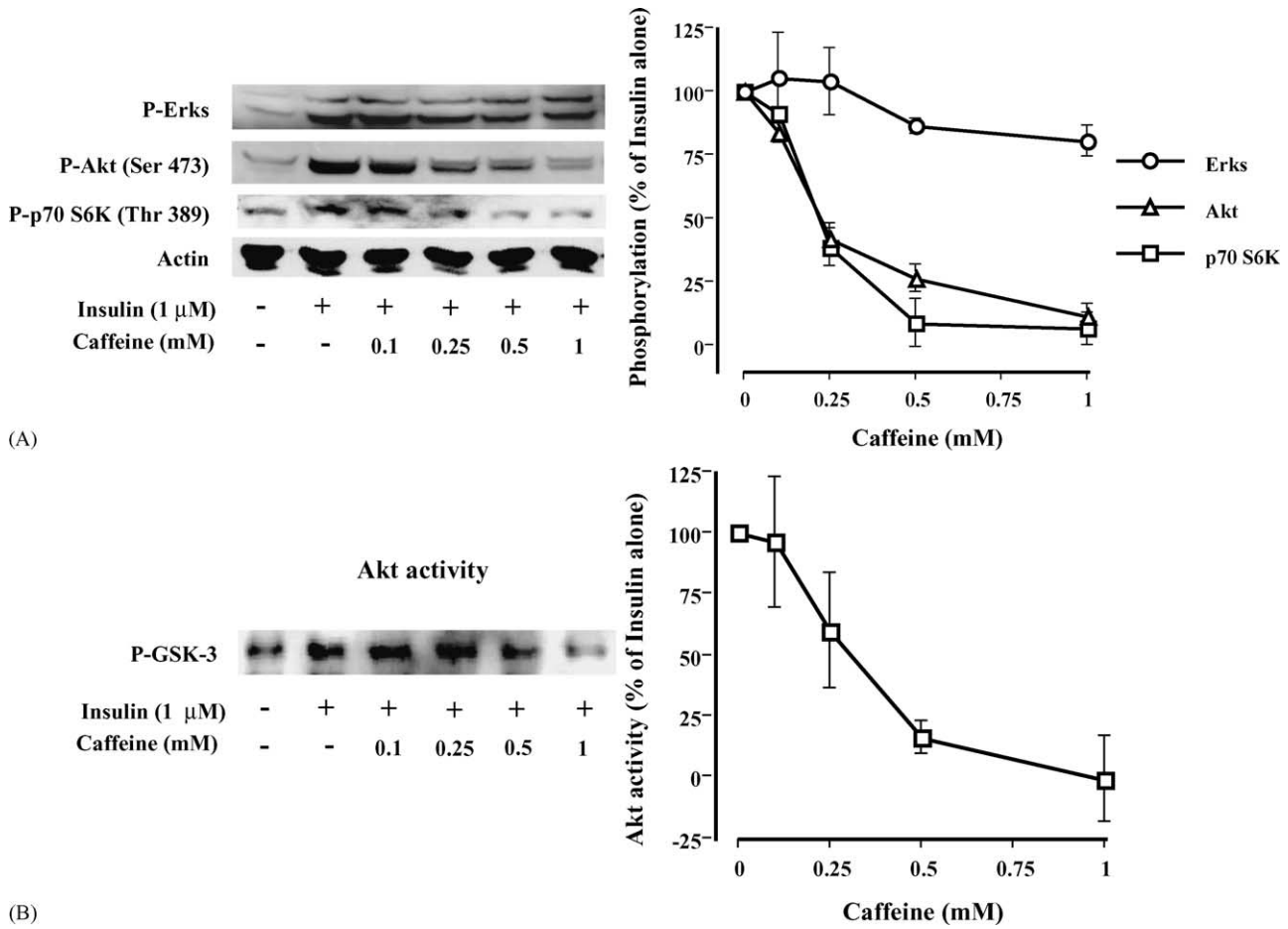

Fig. 3. Inhibition by caffeine on insulin-induced phosphorylation and activation of Akt. Differentiated MC3T3-G2/PA6 cells were treated or not treated with caffein for 1 h. Then, the cells were incubated with or without insulin (1 μM) for 5 min. (A): The cells were lysed and the phosphorylation levels were estimated by immunoblotting with phospho-specific antibodies against p44/42 MAP kinase (Erks), Akt (Ser 473), or p70 S6K (Thr 389). The effect of caffeine on insulin-stimulated phosphorylation of Erks, Akt and p70 S6K [phosphorylation (%)] was calculated as described in Section 2. Each value is the mean ± S.E. of three experiments. (B): The cells were lysed, and the lysate was immunoprecipitated with immobilized Akt (1G1) monoclonal antibody beads. The enzyme immune complex was reacted with ATP and GSK-3 fusion protein. The Akt kinase activity was determined by immunoblotting with phospho-specific GSK α/β (Ser 21/9) antibody. The inhibitory effect of caffeine on insulin-stimulated Akt activation [Akt activity (%)] was calculated as described in Section 2. Each value is the mean ± S.E. of three experiments.

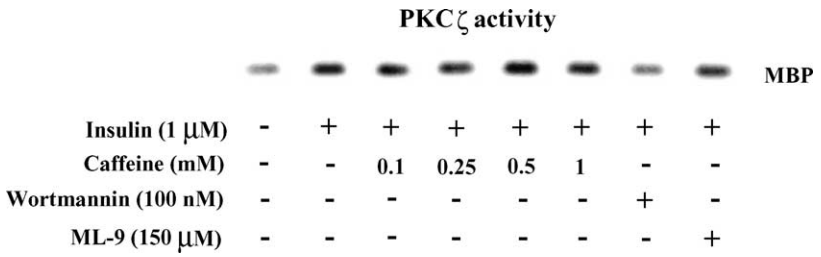

Fig. 4. Effect of caffeine on insulin-induced PKCζ activation. Differentiated MC3T3-G2/PA6 cells were treated or not treated with caffeine, wortmannin or ML-9 for 1 h. Then, the cells were incubated with or without insulin (1 μM) for 5 min. The cells were lysed, and the lysate was immunoprecipitated with PKCζ antibody. The enzyme immune complex was reacted with [γ-^{32}P]ATP and myelin basic protein (MBP). Phosphorylation of MBP was evaluated by autoradiography as a measure of PKCζ kinase activity.

a β-adrenergic agonist, isoproterenol (1 μM), as a positive control drug significantly increased the cAMP content.

We also assessed the effect of 8-phenyltheophylline (Ki; 86 nM against adenosine A1 receptor) [30], a selective A1 adenosine receptor antagonist, and CGS-15943 (Ki; 3.5, 3.5 and 44 nM against adenosine A1, A2A and A2B receptors, respectively) [31], a non-selective adenosine antagonist, on insulin-induced 2-deoxy-D-[1-^{3}H]glucose uptake. Pre-treatment with 8-phenyltheophylline or CGS-15943 did not inhibit 2-deoxy-D-[1-^{3}H]glucose uptake (Fig. 5B).

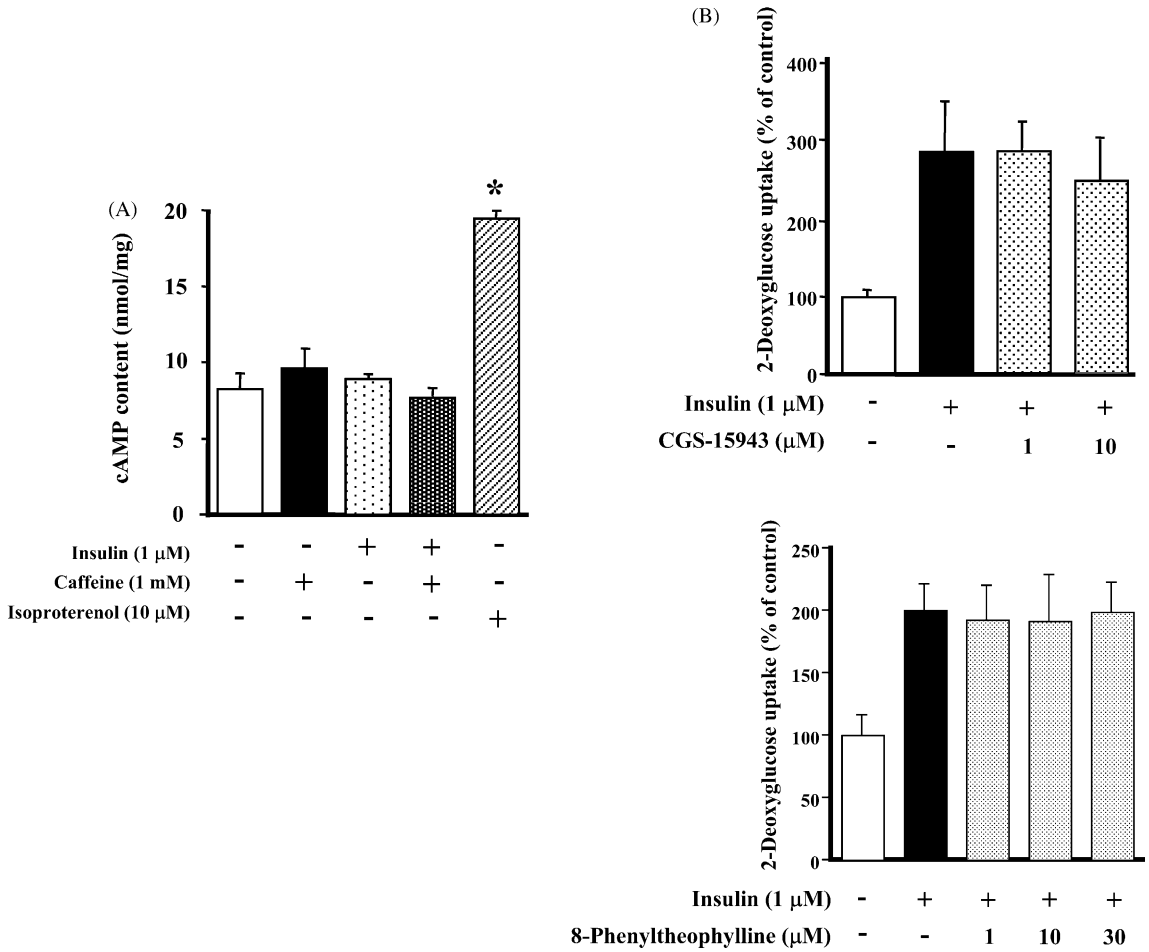

Fig. 5. cAMP and adenosine receptor antagonism are not involved in insulin-induced glucose uptake. (A): Differentiated MC3T3-G2/PA6 cells were treated or not treated with caffeine (1 mM) or isoproterenol (10 μM) for 1 h. Then, the cells were incubated with or without insulin (1 μM) for 20 min. cAMP content was determined as described in Section 2. Each value is the mean ± S.E. of at least three experiments. (*) Significantly different from insulin alone at $P > 0.01$. (B): Differentiated MC3T3-G2/PA6 cells were treated or not treated with the indicated concentration of CGS-15943 or 8-phenyltheophylline for 1 h. After incubation with or without insulin (1 μM) for 20 min, 2-deoxy-D-[1-^{3}H]glucose was added and incubation was continued for 5 min. Each value is the mean ± S.E. of at least three experiments.

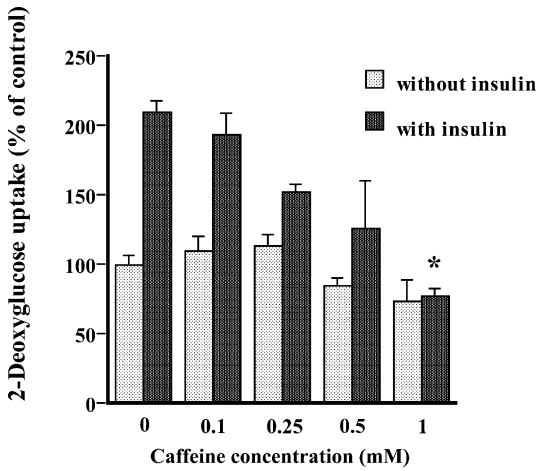

Fig. 6. Effect of caffeine on insulin-induced glucose uptake by primary cultured brown adipocytes. Primary cultured brown adipocytes were treated or not treated with caffeine for 1 h. After incubation with or without insulin (1 μM) for 20 min, 2-deoxy-D-[1-^{3}H]glucose was added and incubation was continued for 5 min. Each value is the mean ± S.E. of at least three experiments. (*) Significantly different from insulin alone at $P > 0.01$.

3.4. Effect of caffeine on insulin-induced glucose uptake in primary-cultured brown adipocytes

Glucose uptake stimulated by insulin in brown adipocytes is also maintained mainly by the activity of GLUT4 [32]. As shown in Fig. 6, pre-treatment of the brown adipocytes with caffeine inhibited the insulin-stimulated 2-deoxy-D-[1-^{3}H]glucose uptake in a concentration-dependent manner (IC50 value; 0.23 mM), as in the case of MC3T3-G2/PA6 cells (Fig. 1A).

4. Discussion

We demonstrated in this study that caffeine inhibits insulin-induced glucose uptake by MC3T3-G2/PA6 cells differentiated into mature adipose cells and by primary cultured brown adipocytes. The inhibition of glucose uptake by caffeine was suggested to be due to loss of GLUT4 translocation to the plasma membrane through blocking of Akt activation (Fig. 7).

Insulin-induced glucose uptake occurs primarily through translocation of GLUT4 to the plasma membrane. This process is initiated by activation of the insulin receptor (IR), and, in turn, leads to activation of the IR substrate (IRS) family and related proteins, including PI3K and its downstream targets [33] (Fig. 7). Numerous studies have shown that PI3K activation provides a pivotal signal for GLUT4 translocation stimulated by insulin [12–16]. Caffeine, like wortmannin, a PI3K inhibitor, inhibited insulin-induced GLUT4 translocation in differentiated MC3T3-G2/PA6 cells. This result suggested that the inhibition of glucose uptake by caffeine occurs through blocking of the PI3K pathway stimulated by insulin.

We then examined the effects of caffeine on the PI3K pathway in differentiated MC3T3-G2/PA6 cells treated with insulin. Caffeine is known to inhibit members of the PIKK family, including ATM and ATR [18,21]. In addition, Foukas et al. [3] have shown by in vitro kinase assay that caffeine directly inhibits PI3K activation. However, in this study, caffeine had no effect on insulin-induced activation of PI3K and its downstream effector PKCζ, an isoform of aPKC. These results suggested that inhibition of insulin-induced glucose uptake by caffeine did not result from inhibition of PI3K or aPKC, and insulin receptor antagonism. In contrast, caffeine precluded insulin-induced activation and phosphorylation of Akt in the same concentration range that inhibited glucose uptake. Akt activation induces glucose uptake via translocation of GLUT4 vesicles to the plasma membrane [12,32,34]. We also have demonstrated that ML-9, which has been characterized as an inhibitor of Akt activation [32,35], inhibits insulin-induced glucose uptake as well as the Akt activation (data not shown). Therefore, blocking of Akt activation by caffeine is likely to contribute to the inhibitory effect on insulin-induced glucose uptake. The inhibition of Akt activation by caffeine is not specific to MC3T3-G2/PA6 cells, because we have observed similar results in a mouse epidermal cell line, JB6 Cl 41 (unpublished data). Furthermore, we confirmed that caffeine also inhibited insulin-induced glucose uptake in primary cultured brown adipocytes.

Caffeine shows adenosine receptor antagonism and inhibits phosphodiesterase activity. Previous studies have shown that cAMP elevators, such as forskolin and IBMX, inhibit glucose transport in intact adipocytes [36,37]. In addition, Wang et al. [38] showed that cAMP inhibits the Erk and PI3K/Akt pathways through blocking Rap1, a member of the Ras family of small GTP-binding proteins. However, caffeine did not change the cAMP content in differentiated MC3T3-G2/PA6 cells. Therefore, it is unlikely that inhibition of glucose uptake by caffeine is involved in the regulation of cAMP. On the other hand, several studies have indicated that adenosine receptor antagonists decrease insulin-induced glucose uptake [2,4]. However, in this study, the pre-treatment with 8-phenyltheophylline, which is reported to attenuate glucose transport [2], and CGS-15943, a potent adenosine antagonist, had no effect on insulin-induced glucose uptake. It is likely because of which caffeine concentration (IC50 value; 0.24 mM) inhibited insulin-stimulated glucose uptake is much higher than the concentrations (IC50 values; 20–30 μM) that antagonize adenosine receptors [39]. Thus, the effects of adenosine receptor antagonists or the regulation of adenosine on insulin-induced glucose transport may be different in different cells. These results suggested that the effect of caffeine on insulin-induced glucose uptake did not result from its adenosine receptor antagonism or phosphodiesterase inhibition.

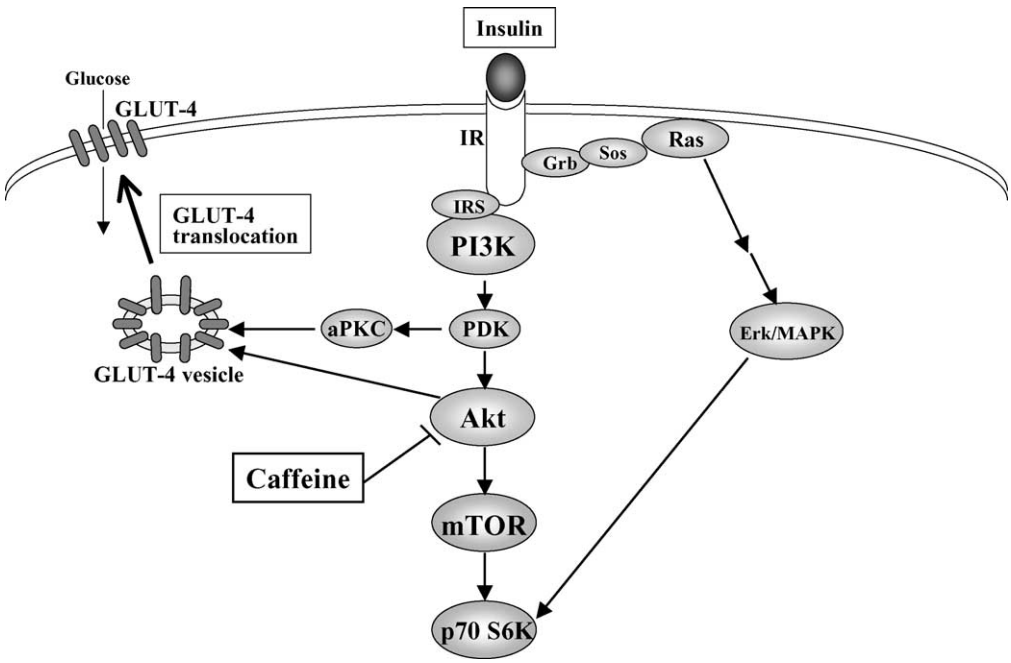

Fig. 7. Inhibitory mechanism of caffeine on insulin-induced glucose uptake. Insulin activates at least two main pathways, the PI3K and Erks cascades [33,40]. PI3K has a major role in insulin functions, including glucose uptake via translocation of GLUT4 vesicles to the plasma membrane, mainly via Akt or aPKC activation [33]. Our data indicated that caffeine inhibited insulin-induced glucose uptake via blocking of GLUT4 translocation to the plasma membrane by inhibition of Akt activation. (→), activation: (⊥), inhibition. aPKC, atypical protein kinase; Erk, extracellular signal-regulated kinase; GLUT4, glucose transporter 4; Grb, growth factor receptor-bound protein; IR, insulin receptor, IRS, IR substrate family; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; p70 S6K, ribosomal p70 S6 protein kinase; PDK, 3-phosphoinositide-dependent protein kinase; PI3K, phosphatydylinositol 3-kinase; SOS, son of sevenless guanine nucleotide exchange factor.

In this study, we have found that caffeine inhibited insulin-induced Akt activation in adipose cells, and thereby caused a decrease of glucose transport as well as GLUT4 translocation (Fig. 7). Therefore, our results provide novel insights into the biological modification by caffeine of insulin actions, including glucose uptake in skeletal muscle and adipocytes.

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