Original paper

Anti-Prediabetic Effect of 6-O-Caffeoylsophorose in Prediabetic Rats and Its Stimulation of Glucose Uptake in L6 Myotubes

Gonzalo MIYAGUSUKU-CRUZADO1, Naoki MORISHITA1, Keiichi FUKUT3, Norihiko TERAHARA3 and Toshiro MATSUI1*

1Faculty of Agriculture, Graduate School of Kyushu University, Fukuoka 812-8581, Japan
2Miyazaki JA Food Research & Development Inc., Miyazaki 880-0943, Japan
3Faculty of Health and Nutrition, Minami-Kyushu University, Miyazaki 880-0032, Japan

Received November 10, 2016 ; Accepted January 30, 2017

The aim of this study was to evaluate the anti-prediabetic effect of 6-O-caffeoylsophorose (CS) isolated from red vinegar in prediabetic spontaneously diabetic Torii (SDT) rats. The effect of CS on glucose uptake in rat skeletal muscle L6 myotubes was also investigated. By the supplementation of CS at a dose of 100 mg/kg/day for 8 weeks to 8-week-old SDT rats, impaired glucose tolerance in 12- and 16-week-old rats was improved in CS group, along with promoted insulin secretion. In a glucose uptake study using 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG), > 50 μM CS stimulated uptake of 2-NBDG and translocation of glucose transporter 4 (GLUT4) to the plasma membrane. In CS-treated L6 cells, western blot analysis showed phosphorylation of AMP-activated protein kinase (AMPK) but not of Akt. Consequently, we concluded that CS showed an in vivo anti-prediabetic effect, and promoted GLUT4 translocation in L6 myotubes possibly through the activation of the AMPK signaling pathway.

Keywords: 6-O-caffeoylsophorose, anti-prediabetes, SDT rat, glucose uptake, AMPK, L6 cell

Introduction

Diabetes is one of the most common and prevalent lifestyle-related diseases, but an appropriate postprandial control of blood glucose level (BGL) has been proven to be effective in preventing the development of diabetes in borderline hyperglycemic subjects (Chiasson et al., 2002). Alternative medicinal foods or physiologically functional foods inhibiting glucose production from dietary carbohydrates in the gut or promoting glucose uptake into organs are in demand for improving or preventing diabetes. We have previously reported that acylated anthocyanins (Matsui et al., 2001, 2002) and condensed catechins (Matsui et al., 2007; Toshima et al., 2010) have the ability to retard an excessive postprandial BGL increase by inhibiting intestinal α-glucosidase.

Tsuda (2012) has reviewed the health-benefits of a variety of anthocyanins showing anti-diabetic and anti-obesity effects. In our earlier studies, we also reported the anti-hyperglycemic effect, in vivo, of a new natural polyphenol, 6-O-caffeoylsophorose (CS), which was attributable to its ability to inhibit intestinal α-glucosidase (maltase) (Matsui et al., 2004). CS, a type of glycosylated hydroxycinnamic acid, was our first identified natural caffeic acid analog from red vinegar produced by the acetic acid fermentation of the storage root of purple-fleshed sweet potato, Ipomea batatas L. cv. Ayamurasaki (Terahara et al., 2003). Reports have shown the absorption of CS into rat blood system in its intact form through passive paracellular transport along with comparable absorption of its glucuronide and/or sulfate conjugates (Qiu et al., 2011). The intact absorbable CS led us to investigate its anti-hyperglycemic potential by analyzing its ability to promote glucose uptake in skeletal muscle, since absorbable polyphenols such as theasinensins (Qiu et al., 2014) and catechins (Ueda et al.,
2008) were reported to promote glucose uptake in skeletal muscle cells. Thus, in this study, we primarily evaluated the in vivo potential of CS to prevent the progressive diabetes in pre-diabetic Spontaneously Diabetic Torii (SDT) rats. As reported previously, SDT rats displayed a long-term prediabetic stage showing impaired glucose tolerance and no fasting blood glucose level (BGL) rise at 10 to 20 weeks of age (Chen et al., 2015). Experiments using rat skeletal muscle L6 cells were also performed in order to obtain insights into promoting glucose uptake of CS in muscles.

Materials and Methods

Reagents  
CS, 6-O-(E)-caffeoyl-(2-O-(6-O-(E)-feruoyl)-β-D-glucopyranosyl)-α-D-glucopyranose (FCS), and 6-O-(E)-caffeoyl-(2-O-(6-O-(E)-caffeoyl)-β-D-glucopyranosyl)-α-D-glucopyranose (CCS) were obtained according to our previous methods (Terahara et al., 2003; Matsui et al., 2004). Caffeic acid (CA) was purchased from Tokyo Chemical Industries (Tokyo, Japan) and chlorogenic acid (CGA) was purchased from Nacalai Tesque (Kyoto, Japan). Compound C was purchased from Calbiochem-EMD Biosciences (La Jolla, CA). AICAR was purchased from Wako Pure Chemical Industries (Osaka, Japan). Insulin and sophorose were purchased from Sigma-Aldrich (St. Louis, MO). 2-[6-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxyglucose (2-NBDG) was purchased from Life Technologies Co. (Molecular Probes, OR). The primary antibodies of Akt and phospho (Ser473)-Akt (p-Akt) were purchased from Cell Signaling Technology Inc. (Tokyo, Japan). Antibodies for glucose transporter 4 (GLUT4), insulin receptor β subunit (IR-β), AMP-activated protein kinase (AMPK) α, and phospho (Thr172)-AMPKα (p-AMPK) were purchased from Merck Millipore (Bedford, MA). Secondary antibody HRP-conjugated donkey anti-rabbit IgG was purchased from GE Healthcare (Piscataway, NJ). Other reagents were of analytical grade and were used without further purification.

Animals  
Eight male SDT rats (7-week-old), purchased from CLEA Japan (Tokyo, Japan), were acclimatized under laboratory conditions (21 ± 1°C, 55.5 ± 5% humidity, a 12 h light-dark cycle) for 1 week. The individually housed rats were fed with a standard diet (Oriental Yeast Co., Tokyo, Japan), and distilled water ad libitum. The rats in the CS group were daily administered CS at a dose of 100 mg/kg during the protocol period of 8 to 16-week-old (control group was given water). CS solution was prepared fresh everyday using deionized tap water and administered daily by intubation at an oral dose of 100 mg/kg during the protocol period of 8 to 16-week-old. Blood samples were collected from the tail vein every week after 16 h of fasting and BGL was measured. Plasma was separated by centrifugation (3500 g, 15 min, 4°C) and stored at −30°C until insulin analysis. Plasma insulin levels were measured by using a rat insulin ELISA kit (Shibayagi Co., Gunma, Japan) according to the manufacturer’s instructions. Body weight and food consumption were monitored over the experimental period. All animal experiments were conducted in accordance with the Guidance for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105, 1973) and Notification (No. 6, 1980 of the Prime Minister’s Office) of the Japanese Government. All experiments were reviewed and approved by the Animal Care and Use Committee of Kyushu University (Permit Number: A24-051).

Oral glucose tolerance test  
Rats of 8, 12, and 16 weeks of age were subjected to an oral glucose tolerance test (OGTT) to evaluate glucose tolerance. The rats were fasted overnight (16 h) prior to receiving a single oral administration of a glucose solution (2 g/kg). BGL was measured at 0, 30, 60, 90, and 120 min after administration using a blood glucose test meter with a disposable blood glucose sensor (Glutest Pro, Sanwa Chemical Research Co., Tokyo, Japan).

Cell culture  
L6 myoblasts (American Type Culture Collection) were grown in DMEM supplemented with 10% FBS, 1% nonessential amino acids, 2 mM l-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1.7 mM insulin, in a humidiﬁed atmosphere containing 5% CO₂ and 95% air at 37°C. Differentiation was induced by changing the medium from 10% FBS to 2% horse serum, and renewing the medium every day for 4–5 days. The extent of the differentiation was determined by observing the formation of elongated and multinucleated myotubes. Fully differentiated myotubes were used for all experiments.

Assay for glucose uptake  
L6 myoblasts were seeded on 96-well plates (10⁴ cells/well) and allowed to attach for 24 h. After differentiation, cells were starved in serum-free DMEM for 16 h prior to incubation with test sample (insulin, CS, CA, CGA, FCS, and CCS) in Krebs-Ringer phosphate HEPES (KRPH) buffer [NaCl 118 mM, KCl 5 mM, KH₂PO₄ 1.2 mM, CaCl₂ 1.3 mM, MgSO₄ 1.2 mM, and HEPES 30 mM (pH 7.4)] for 1 h at 37°C. Inhibition experiments were performed using an AMPK inhibitor (20 μM compound C). L6 cells were treated with the inhibitor for 30 min before incubating with the test sample. Cells were then, incubated with 100 μM 2-NBDG with or without test sample and inhibitor in KRPH for 20 min, followed by washing with ice-cold KRPH buffer. The increase of fluorescence intensity (excitation/emission: 485/535 nm) was measured using a fluorescence spectrophotometer (Wallac ARVO SX 1420 Multilabel Counter, Perkin Elmer Life Sciences, Tokyo, Japan). Results were expressed as a percentage of relative fluorescence intensity against that of control (L6 myotubes incubated with 2-NBDG without test sample).

Preparation of plasma membrane (PM) of L6 cells for GLUT4 expression  
In order to measure the amount of GLUT4 protein in the PM, PM fractions were separated from the cell lysate following the method of Nishiumi and Ashida (2007) with minor modifications. Briefly, after L6 cells were seeded and differentiated on 100-mm cell culture dishes (1 × 10⁵ cells/mL), cells were starved in serum-free DMEM for 16 h and then treated with either 100 μM CS or KRPH alone for 3 h. Positive control cells were...
incubated with insulin (0.1 μM) in KRPH for 15 min. Cells were then washed with ice-cold KRPH and harvested in buffer A [50 mM Tris, pH 8.0, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, 5 μg/mL aproptinin, 10 mM NaF, and 1 mM Na3VO4] containing 0.1% Nonidet P (NP)-40. The cells were homogenized by a microtube homogenizer (Polytron PT1200E, Bohemia, NY) and centrifuged at 1 000 × g for 10 min at 4℃. The precipitate was resuspended in NP-40-free buffer A and kept on ice for 10 min followed by centrifugation at 1 000 × g for 10 min at 4℃. The precipitate was suspended once again in buffer A containing 1% NP-40 on ice for 1 h with occasional mixing. The suspension was then centrifuged at 16 000 × g for 20 min at 4℃ to collect PM fraction. For the whole cell lysates, cells were harvested in buffer B [buffer A + 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] and homogenized using the microtube homogenizer on ice and kept for 1 h, followed by a centrifugation at 16 000 × g for 20 min at 4℃. The supernatant was referred as cell lysate. Protein content in each fraction was quantified with a Bio-Rad DC Protein Assay Kit using BSA as a standard.

Preparation of cell lysates for Akt and AMPK phosphorylation assays L6 myoblasts were seeded and differentiated on 6-well plates (1 × 105 cells/mL), incubated with CS or KRPH alone for 1 h, and harvested in radio-immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium sulfate, 0.1% NP-40, and 50 mM Tris-HCl, pH 8.0) with a phosphatase inhibitor mixture (PhosSTOP, Roche Applied Science, Tokyo, Japan). Positive control cells for Akt and AMPK were incubated with 0.1 μM insulin for 15 min or 0.5 mM AICAR for 1 h.

Western blot analysis Equal volumes of protein extracted from L6 myotubes (30 μg of protein for GLUT4 and IR-β or 20 μg of protein for AMPK and Akt) and a buffer (20% glycerol, 4% SDS, 3% DTT, 0.002% bromophenol blue and 0.125 M Tris-HCl, pH 6.8) were mixed and separated using a 10% SDS polyacrylamide gel electrophoresis for 1.5 h at 40 mA. The separated proteins were transferred onto a PVDF membrane (Hybond-P, GE Healthcare) for 1.5 h at 80 mA. The membranes were blocked with 5% non-fat dried milk in TBS-T (Tris buffered saline containing 0.05% Tween 20) for 1 h at 25℃ and then incubated with the respective primary antibody overnight at 4℃ followed incubation with the respective secondary antibody for 1 h at room temperature. Detection was carried out by using an ECL plus detection reagent and ImageQuant LAS 4000 (GE Healthcare). The difference in band intensity was quantified using ImageQuant TL 7.0 software (GE Healthcare) and differences in protein levels expressed as a percentage relative to control.

Statistical analysis The results were expressed as the mean ± standard error of the mean (SEM). Statistical differences among groups were evaluated using one-factor analysis of variance (ANOVA), followed by Tukey-Kramer’s t-test for post hoc analysis. Other statistical evaluations were performed using Student’s t-test. Analyses were performed with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). A P value < 0.05 was considered significant.

Results Effect of CS on glucose tolerance and insulin secretion During the protocol periods of 8 weeks to 20 weeks of age of SDT rats, body weight, food intake, fasting BGL, and insulin did not differ between control and CS groups (Table 1).

In glucose tolerance (OGTT) experiments performed on 8-week-old SDT rats, no significant differences in BGL and insulin levels were observed between CS and control groups (Fig. 1). Meanwhile, at 12 and 16 weeks of age, CS supplementation induced a pronounced reduction in BGL in response to oral glucose load and increased insulin response compared with those of control group. The area under curve (AUC) of the BGL response (Table 1) and the insulin response (Fig. 1) indicated that glucose tolerance was significantly improved by CS supplementation since 12 weeks of age (P < 0.05). The results confirmed the progression of impaired glucose tolerance and impaired insulin secretion in control SDT rats, as well as the blocked progression of prediabetes in CS group. The prediabetic stage (12- to 16-week-old), characterized by normal fasting BGL and impaired glucose tolerance, was in agreement with our previous report (Chen et al., 2015).

Effect of CS on glucose uptake in L6 myotubes In order to assess the possible role of CS in the anti-prediabetic effect in SDT rats (Fig. 1), we first examined whether CS has the potential to
promote a glucose uptake in skeletal muscle L6 myotubes. The myotubes were exposed to different concentrations of CS (25, 50, 100 μM) for 1 h, followed by 2-NBDG uptake measurements. Since 50 μM CS induced a similar magnitude of uptake between 30 min and 1.5 h of incubation, 1 h-incubation of test samples was performed throughout the present study (data not shown). As shown in Fig. 2B, although CS (structure in Fig. 2A) at 25 μM did not affect glucose uptake (92 ± 10% of control), CS at >50 μM significantly increased glucose uptake (50 μM: 196 ± 17% of control, \( P < 0.05 \); 100 μM: 179 ± 18% of control) to levels comparable with that observed with insulin (0.1 μM, 214 ± 16% of control, \( P < 0.05 \)). Although an apparent concentration-dependent effect was not observed for CS-induced uptake using the present CS concentration range, it seems likely that CS at >50 μM concentrations exerted a glucose uptake promoting effect in L6 myotubes.

**Structure-activity relationship of CS for glucose uptake in L6 myotubes** The promoting effect of CS on 2-NBDG uptake into L6 myotubes was compared with that of several mono-caffeoyl moiety-containing CS analogs (CA, CGA, and FCS) and a di-caffeoyl moiety-containing compound (CCS) at a concentration of 100 μM (Fig. 2A). As shown in Fig. 2C, CS induced a similar magnitude of uptake as mono-caffeoyl compounds (CA, CGA, and FCS) and the di-caffeoyl moiety-containing compound (CCS) at a concentration of 100 μM (Fig. 2A). As shown in Fig. 2C, CS induced a similar magnitude of uptake as mono-caffeoyl compounds (CA, CGA, and FCS). In contrast, the di-caffeoyl compound, CCS, induced about 1.5-fold higher glucose uptake compared to that by CS. Conversely, sophorose, a sugar moiety of CS, showed no glucose uptake promoting activity (Fig. 2C) further confirming that it was the caffeoyl moiety in CS analogs, which play a crucial role in the glucose uptake effect.

**Effect of CS on GLUT4 translocation in L6 myotubes** Blood glucose is incorporated into skeletal muscle via GLUT4 translocated from the cytosol to PM. In order to confirm increased GLUT4 expression in PM by CS, western blot analyses of the PM were performed using IR-β as loading control as it is present in both the cell lysate and the plasma membrane fraction after separation (Qiu et al., 2014). Absence of IR-β in the post plasma membrane fraction was considered proof of efficient separation (data not shown). As shown in Fig. 3, incubation with 100 μM CS increased the relative expression of GLUT4 (\( P < 0.05 \)) in the PM, without affecting the relative GLUT4 content of the whole cell lysate. This indicates that CS promotes the translocation of GLUT4 from intracellular vesicles to the PM, while having no effect on the total cellular content of GLUT4 in L6 myotubes.

**Effect of CS on IR/Akt signaling pathways** We studied the effect of CS on the IR/Akt signaling pathways leading to GLUT4 translocation. As shown in Fig. 4, 0.1 μM insulin significantly (\( P < 0.05 \)) increased the phosphorylation of Akt at the Ser\(^{473}\), whereas the treatment with CS did not show any effect.

**Effect of CS on AMPK signaling pathways** We next investigated the role of the AMPK signaling pathway in CS-induced GLUT4 translocation and glucose uptake. Fig. 5A shows the effect of compound C, an AMPK inhibitor, on 100 μM CS-induced 2-NBDG uptake. CS-stimulated glucose uptake (182 ± 10% versus control) was significantly (\( P < 0.05 \)) reduced by 20 μM compound C (87 ± 13% versus control). The result that CS promoted the phosphorylation of AMPK at Thr\(^{172}\) (\( P < 0.05 \), Fig. 5B) strongly suggested that CS was involved in the activation of AMPK/GLUT4 signaling pathway in L6 myotubes.
Anti-Prediabetic Effect of 6-\textit{O}-Caffeoylsophorose in Rats

Discussion

CS, our first identified naturally occurring polyphenol, was obtained from the acid decomposition of a diacylated anthocyanin during the acetic fermentation of purple-fleshed sweet potato (Terahara \textit{et al}., 2003). Thus far, we have demonstrated that acute administration of doses (100 mg/kg of CS) exerted a significant reduction of 11.1\% of the maximum BGL in Sprague–Dawley (SD) rats through the suppression of intestinal maltase activity (Matsui \textit{et al}., 2004). It was also demonstrated that administration of 400 mg/kg CS in SD rats resulted in a maximum plasma concentration (C\textsubscript{max}) of 27.5 ± 5.2 nmol/mL with a t\textsubscript{max} at 25 min (Qiu \textit{et al}., 2011) through a carrier (monocarboxylic acid transporter)-mediated transport pathway (Phuong \textit{et al}., 2013). In this study, thus, we investigated the effects of the long-term daily administration of CS at a dose of 100 mg/kg on glucose tolerance and insulin secretion during prediabetic stages in SDT rats. We demonstrated the first physiological role that CS can prevent the progression of diabetes during prediabetic stages by improving impaired glucose tolerance and promoting insulin secretion in SDT rats (Fig. 1). Experiments using rat skeletal muscle L6 cells also revealed that CS can act as a promoter in incorporating glucose into L6 cells (Fig. 2). However, we failed to demonstrate the \textit{in vivo} translocation of GLUT4 in soleus muscles taken from 16-week-old fasting SDT rats in CS group (data were not shown), probably due to fasting prediabetic stages, at which fasted SDT rats showed no BGL difference between control and CS groups (Table 1 and Fig. 2). It has been shown that in skeletal muscle, fasting reduces the GLUT4 presence on the cell surface (Kraegen \textit{et al}., 1993); this can explain the lack of significant differences on the amount of translocated GLUT4 in the soleus muscle isolated from SDT rats. Further experiments, thus, are needed to evaluate \textit{in vivo} GLUT4 translocation in soleus muscles after OGTT or glucose-stimulated animal experiments.

2-NBDG, a fluorescent deoxyglucose analog, was used to

\textbf{Fig. 2.} Effects of CS and caffeoyl compounds on 2-NBDG uptake into L6 myotubes. (A) Structures of caffeoyl compounds used in this study. (B) Concentration-dependent effect of CS on 2-NBDG uptake. L6 myotubes were incubated with different concentrations of CS (25, 50, and 100 μM) for 1 h. Insulin (0.1 μM, 15 min) was used as a positive control. (C) Effect of CS analogs on 2-NBDG uptake. L6 myotubes were incubated with 100 μM test samples (CS, sophorose, CA, CGA, FCS, and CCS) for 1 h. Results are expressed as mean ± SEM (n = 4). Statistical differences between two groups were evaluated by Student’s \textit{t}-test: *P < 0.05, **P < 0.01 vs. control. Statistical differences among groups were evaluated by Tukey-Kramer’s \textit{t}-test: bars with different letters of the alphabet are significantly different (P < 0.05). CS, 6-\textit{O}-caffeoylsophorose; CA, caffeic acid; CGA, chlorogenic acid; FCS, (6-\textit{O}-((\textit{E})-caffeoyl-(2-\textit{O}-(6-\textit{O}-(\textit{E})-feruoyl)-β-\textit{d}-glucopyranosyl)-α-\textit{d}-glucopyranose; CCS, (6-\text{O-(E)}-caffeoyl-(2-O-(6-O-(E)-caffeoyl)-β-n-glucopyranosyl)-α-n-glucopyranose.
**Fig. 3.** Effect of CS on GLUT4 translocation to the plasma membrane of L6 myotubes. L6 myotubes were incubated with either 100 µM CS for 1 h or 0.1 µM insulin for 15 min. Results are expressed as the mean ± SEM (n = 4). The level of GLUT4 in the plasma membrane was calculated using the ratio of GLUT4/IR-β, and it was expressed as the percentage of total GLUT4 level in the cell lysate. Statistical differences were evaluated by Student’s t-test. *P < 0.05 vs. control.

**Fig. 4.** Effect of CS on the phosphorylation of Akt in L6 myotubes. L6 myotubes were incubated with either 100 µM CS for 1 h or 0.1 µM insulin for 15 min. Results are expressed as mean ± SEM (n = 4). Statistical differences were evaluated by Student’s t-test. **P < 0.01 vs. control.

**Fig. 5.** Role of the AMPK signaling pathway in CS-stimulated 2-NBDG uptake into L6 myotubes. (A) Effect of compound C on CS-stimulated 2-NBDG uptake. L6 myotubes were incubated with either 100 µM CS for 1 h or 0.1 µM insulin for 15 min in the presence or absence of 20 µM compound C. (B) Effect of CS on the phosphorylation of AMPK. L6 myotubes were incubated with either 100 µM CS or 0.5 mM AICAR as positive control for 1 h. Results are expressed as mean ± SEM (n = 4). Statistical differences were evaluated by Student’s t-test. *P < 0.05, **P < 0.01 vs. control.
investigate the effect of CS on glucose uptake into L6 myotubes, as it can be transported into diverse cells such as skeletal muscles, adipocytes, and hepatic cells through glucose transporters (Zou et al., 2005; Manaharan et al., 2013). Our data clearly showed that CS promoted 2-NBDG uptake into L6 myotubes at ≥50 μM (Fig. 2B), and the effect was dependent on the number of caffeoyl groups, but not on sophorose moiety in CS (Fig. 2C). We further investigated the signaling pathway(s) involved in the CS-induced glucose uptake. Since glucose incorporation into muscle cells is triggered by the translocation of GLUT4 to the PM via facilitated diffusion (Huang and Czech, 2007), GLUT4 expression levels in the PM were evaluated by western blot analysis in the presence or absence of CS (Fig. 3). CS increased GLUT4 levels in the PM without changing the total amount of GLUT4 in the whole cell lysate, suggesting that the CS-induced 2-NBDG uptake was achieved by an enhanced GLUT4 translocation, but not by increased GLUT4 gene expression. Although previous studies have demonstrated that moderate increases in the translocated GLUT4 may lead to dramatic increases in glucose uptake (Qiu et al., 2014), the effects of CS on glucose uptake cannot be only restricted to the GLUT4 transporter. It has been hypothesized (Breen et al., 2008) that polyphenols may also stimulate the activity of the transporters already present in the membranes (such as the GLUT1 transporter), which may explain an elevated glucose uptake in response to GLUT4 translocation.

Two possible intracellular signaling pathways involved in GLUT4 translocation in skeletal muscles, namely, the PI3K/Akt and AMPK pathways (Kahn et al., 2005) were studied. Studies have shown the crucial role of IR in phosphorylating and activating Akt via insulin-stimulated IR substrate-1 (IRS-1). In this study, CS did not affect the phosphorylation of Ser473 of Akt (Fig. 4), suggesting that CS did not trigger the IR/GLUT4 signaling pathway. Besides, Fig. 5 clearly showed that CS promoted the phosphorylation of Thr172 of AMPK (Fig. 5B). These results led us to consider the involvement of CS in AMPK activation leading to GLUT4 translocation in L6 myotubes. Lee et al. (2007) reported that caffeic acid phenethyl ester stimulated glucose uptake in L6 cells by activating both the PI3K/Akt and AMPK signaling pathways, suggesting that although compounds bearing a caffeoyl moiety might be a key player in promoting glucose uptake in skeletal muscles, these moieties are not the only determinant affecting GLUT4 translocation.

The activation of AMPK in smooth muscle cells is closely associated with up-stream activation of either liver kinase B1 (LKB1) or Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase (CaMKK). It has been previously reported that natural compounds such as capsaicin (Kim et al., 2013), theasinensins (Qiu et al., 2014), and a dipeptide (Trp-His) (Soga et al., 2014) activate CaMKK/AMPK, but not LKB1/AMPK and PI3K/Akt. In contrast, the report by Tsuda et al. (2012) demonstrated that caffeic acid promoted glucose uptake in skeletal muscle C2C12 cells by inducing AMPK activation possibly through LKB1 signaling. Another report by Cheng and Liu (2000) strongly supported the involvement of caffeic acid in the LKB1/AMPK pathway, in which caffeic acid preferably interacted or bound to α\(_{1}\)-adrenoceptor to activate the phospholipase C (PLC)/protein kinase C (PKC) pathway followed by LKB1/AMPK activation. Although the present study could not clarify the defined CS-induced AMPK activation pathway, PLC/PKC-guided LKB1/AMPK signaling pathway must be taken into consideration as the underlying mechanism of compounds bearing a caffeoyl group including CS, FCS and CCS for promoting glucose uptake. Further studies using CS and CS analogs and α\(_{1}\)-adrenoceptor or LKB1-knockdown L6 cells are in progress to elucidate the exact signaling pathways involved in the stimulation of GLUT4 translocation.

**Conclusion**

We demonstrated for the first time that CS (6-O-cafeoylsophorose, a natural compound found in red wine) obtained from the storage root of purple-fleshed sweet potato, *Ipomea batatas* L. cv. Ayamurasaki) has an in vivo anti-prediabetic potential in SDT rats by improving impaired glucose tolerance. CS also promoted glucose uptake in *in vitro* rat skeletal muscle cell experiments. The 2-NBDG uptake of CCS having two caffeoyl groups was much higher than that of CS analogs having one caffeoyl group such as CA, CS and FCS, suggesting that the caffeoyl moiety in these compounds may play a crucial role in the effect. The result that CS induced Thr\(^{172}\)-AMPK phosphorylation, but not that of Ser\(^{473}\)-Akt phosphorylation supported by the inhibitory effect of compound C on the CS-induced 2-NBDG uptake, indicated that GLUT4 translocation by CS was achieved in an insulin-independent manner by inducing the translocation of GLUT4 to the PM via AMPK activation in L6 cells.

**References**


