Original Article

Single oral administration of quercetin glycosides prevented acute hyperglycemia by promoting GLUT4 translocation in skeletal muscles through the activation of AMPK in mice

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Quercetin is a natural flavonol and has various health beneficial functions. Our pervious study demonstrated that long-term feeding (13 weeks) of quercetin and its glycosides, isoquercitrin, rutin, and enzymatically modified isoquercitrin, which is a mixture of quercetin monoglycoside and its oligoglycosides, prevented hyperglycemia and adiposity in mice fed a high-fat diet but not standard diet. It is, however, unclear whether a single administration of these compounds prevent postprandial hyperglycemia or not. In the present study, we estimated their prevention effect on acute hyperglycemia by an oral glucose tolerance test in ICR mice and investigated its mechanism. It was found that quercetin glycosides, but not the aglycone, suppressed acute hyperglycemia and isoquercitrin showed the strongest effect among the glycosides. As the underlying mechanism, quercetin glycosides promoted translocation of glucose transporter 4 to the plasma membrane of skeletal muscle of mice through phosphorylation of adenosine monophosphate-activated protein kinase and its upstream Ca^{2+}/calmodulin-dependent protein kinase kinase β without activating the insulin- and JAK/STAT-signal pathways. In conclusion, single oral administration of quercetin glycosides prevented a blood sugar spike by promoting glucose transporter 4 translocation through activating the CAMKKβ/AMPK signaling pathway.

***Key Words*: quercetin glycosides, blood sugar spike, GLUT4 translocation, AMPK**

Chronic hyperglycemia has become a serious problem in many countries, and is attributed to over-eating and physical inactivity.[1] Therefore, preventing hyperglycemia and improving obesity are important issues for health promotion. Glucose transporter (GLUT) 4 plays a pivotal role in regulating insulin-stimulated glucose transport in absorptive tissues, such as skeletal muscle and adipose tissue.[2] The conditional depletion of GLUT4 in skeletal muscle or adipose tissue can cause insulin resistance and chronic hyperglycemia,[2–4] suggesting that GLUT4 is involved in the maintenance of glucose homeostasis.

Dietary intake of quercetin improved hyperglycemia and hyperinsulinemia by decreasing oxidative stress and expression of peroxisome proliferator-activated receptor-γ and sterol regulatory element-binding protein-1 in the liver of mice fed a Western-style diet.[5] Onion-peel extracts ameliorated hyperglycemia by modulating the uptake and metabolism of glucose.[6] We also reported that long-term feeding of quercetin and its glycosides, isoquercitrin, rutin, and enzymatically modified isoquercitrin (EMIQ), which is a mixture of quercetin monoglycoside and its oligoglycosides, prevented hyperglycemia and adiposity in male ICR mice fed a high-fat diet but not standard diet for 13 weeks.[7] However, we also found that intake of EMIQ, but not quercetin, for 2 weeks in male C57BL/6 mice promoted GLUT4 translocation in the skeletal muscle, though we did not measure blood glucose level in this experiment.[8] These results suggest that anti-hyperglycemic action of quercetin varies depending on its intake period and this discrepancy may be due to the bioavailability of compounds.

Quercetin is a major dietary flavonoid and has various health beneficial functions.[9–11] Quercetin is mainly containing in the plants as the glycoside forms, such as quercetin-3-*O*-β-glucoside (Q3G; also termed isoquercitrin) and quercetin-3-*O*-β-rutinoside (also known as rutin). Quercetin glycosides are absorbed primarily as an its aglycone form from the small and large intestines. It has been reported that the bioavailability of quercetin glycosides is influenced by the type of sugar moiety. For instance, isoquercitrin has reported to be hydrolyzed to an aglycone by lactase phlorizin hydrolase on the outside of brush-border membranes, and then absorbed in the small intestine.[12] After absorption, quercetin aglycone receives methylation, sulfation, glucuronidation, and their combination in the small intestine and/or liver.[13] Rutin is scarcely hydrolyzed and absorbed in the small intestine. It has been reported that rutin is hydrolyzed to an aglycone and its phenolic degradation products by colonic microflora and then absorbed from the large intestine.[13] Recently, EMIQ, a mixture of quercetin monoglycoside and its oligoglycosides, has been paid attention to its high bioavailability. It was reported that EMIQ was hydrolyzed to quercetin and isoquercitrin by incubation with homogenates of rat intestinal epithelia in an *in vitro* study, and that absorption rate of EMIQ was higher than that of quercetin.[14] Indeed, EMIQ was absorbed efficiently in humans as compared with other quercetin glycosides, such as isoquercitrin and rutin.[15] These results indicate results indicate that bioavailability of quercetin aglycon is lower than its glycosides and absorption rate of glycosides differ the structures of sugar moieties.

Many researchers reported that quercetin and its glycosides have anti-hyperglycemic effects.[16,17] Although the prevention effects of quercetin and its glycoside were reported, results from

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long-term feeding experiment revealed efficiency of these compounds showed the almost same extent. Comparison of anti-hyperglycemic effect of them after a single oral administration is unclear yet. In the present study, we compared the anti-hyperglycemic effect of single administration of quercetin, isoquercitrin, rutin and EMIQ by the oral glucose tolerance tests (OGTT) and further investigated GLUT4 translocation and its upstream signaling pathways in the skeletal muscle of mice to understand mechanism of their acute anti-hyperglycemic effect.

Materials and Methods

Compounds. Quercetin, isoquercitrin and rutin were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). EMIQ and isoquercitrin were products of San-Ei Gen FFI (Toyonaka, Japan). Glucose level in the plasma was measured using a commercial kit (Labassay® Glucose kit; FUJIFILM Wako Pure Chemical Corporation). Anti-GLUT1 goat polyclonal antibody (#SC-1605), anti-GLUT4 goat polyclonal antibody (#SC-1608), Anti-Ca^{2+}/calmodulin-dependent protein kinase kinase β (CAMKKβ) rabbit polyclonal antibody (#SC-50341), horseradish peroxidase-conjugated anti-goat (#SC-2020), anti-rabbit (#SC-2030), and anti-mouse (#SC-2005) immunoglobulin-G antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-AMPKα (#2532), anti-phospho-AMPKα (#2531), anti-phospho-CAMKKβ (#12919), anti-liver kinase B1 (LKB1) (#3047), anti-phospho-LKB1 (#3051), anti-insulin receptor substrate-1 (IRS-1) (#2390), anti-phospho-IRS1 (#2381), anti-phosphoinositide 3-kinase (PI3K) (#4257), anti-phospho-PI3K (#4228), anti-Akt1 (#9272), anti-phospho-Akt1 serine 473 (#9271) and threonine 308 (#9275), anti-Janus kinase (JAK) 2 (#3230), anti-phospho-JAK2 (#3776), anti-signal transducers and activator of transcription (STAT) 3 (#4904), anti-phospho-STAT3 (#9145), anti-insulin receptor beta (IR-β) (#3025), and β-actin (#4967) were purchased from Cell Signaling Technology (Danvers, MA). All other reagents used were of the highest grade available from commercial sources.

Animal treatment. The protocol for animal experiments was approved by the Animal Care and Use Committee of Kobe University (1-27-05-09; Kobe, Japan) and carried out according to the guidelines set by this institution. Male ICR mice (4 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and maintained in a room at 23 ± 2°C with a 12-h light–dark cycle (lights on at 09:00). The mice were acclimatized for 7 days with free access to a commercial standard mouse diet consisting of 76% carbohydrate, 15% protein and 9% fat (3.850 kcal/g diet; Research Diets, Tokyo, Japan) and tap water. To examine the effect of single administration of quercetin and its glycosides on acute anti-hyperglycemia and its molecular mechanisms, the mice were subjected to following three experiments.

Experiment 1: For the OGTT, 120 mice were divided into 24 groups of five each. Quercetin, isoquercitrin, rutin, and EMIQ at 1.0^{-6}, 1.0^{-5}, 1.0^{-4}, 1.0^{-3}, and 1.0^{-2} g/kg body weight, or water alone (5.0 ml/kg body weight) as a vehicle control, were orally administered to mice after 18 h-fasting. Sixty minutes after the oral administration of each compound, mice received glucose (1.0 g/kg body weight, p.o.) solution or water (5 ml). Blood was collected from the tail vein in heparinized tubes at 0 (before administration), 15, 30, 60, and 120 min after the glucose-loading and centrifuged at 9,600 × *g* for 10 min at 4°C.[18] Plasma was collected in a heparinized tube and the glucose level measured using a commercial kit (Labassay® Glucose kit).

Experiment 2: To examine GLUT4 translocation and its signaling pathways, 45 mice were assigned randomly to nine groups of five each. Quercetin, isoquercitrin, rutin, and EMIQ in water were orally given to the mice at 1.0^{-6} g/kg body weight or 1.0^{-3} g/kg body weight after 18 h-fasting. Mice in the control group received water alone (5 ml/kg body weight). Mice were sacrificed 60 min after oral administration of quercetin and its glycosides under anesthesia using sevoflurane (inhalational anesthetic) and sodium pentobarbital (analgesic). Plasma was prepared and its glucose level was measured as described above. The soleus muscle was collected from the hind legs and used for measurement of GLUT4 translocation and its related signaling pathways.

Experiment 3: Another 50 mice were assigned randomly to ten groups of five each. Nine groups of mice were given each compound or water as the same protocol as described in Experiment 2. Mice were given glucose (1.0 g/kg body weight, p.o.) 60 min after he oral administration of each compound. Remaining one group of mice was given water alone (5 ml/kg body weight) without glucose loading. These mice were sacrificed 15 min after the glucose-loading under anesthesia. Preparation of plasma for measurement of the glucose level and collection of the soleus muscle for measurement of GLUT4 translocation were performed as described below.

Experiment 4: To confirm the activation of adenosine monophosphate-activated protein kinase (AMPK) was involved in GLUT4 translocation, 20 mice were assigned randomly to five groups of four each. Two groups of mice were given 1.0^{-3} g of isoquercitrin via p.o. administration and another two groups of mice were single intraperitoneally injected 0.85 g/kg body weight of 5-amino oimidazole-4-carboxamide ribonucleoside (AICAR; FUJIFILM Wako Pure Chemical Corporation) as an AMPK activator. One of each two groups of mice were intraperitoneally injected with 20 mg/kg body weight of 6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1, 5-α] pyrimidine (Compound C; abcam, Cambridge, UK), a specific inhibitor of AMPK, 30 min before administration of isoquercitrin or AICAR. Remaining one group of mice was used for vehicle control. These mice were sacrificed 60 min after the administration of isoquercitrin or AICAR under anesthesia. The soleus muscle was collected and used for measurement of GLUT4 translocation and phosphorylation of AMPK as described below.

Experiment 5: To examine upstream of CAMKKβ, W-13 [*N*-(4-aminobutyl)-5-chloronaphthalene-2-sulfonamide hydrochloride], a calmodulin inhibitor, was introduced. Twenty mice were assigned randomly to five groups of four each. Two groups of mice were given 1.0^{-3} g of isoquercitrin via p.o. administration and another two groups of mice were single orally administered 0.1 mg/kg body weight of piperine (Sigma-Aldrich, St. Louis, MO), a CAMKKβ activator. One of each two groups of mice were intraperitoneally injected with W-13 at 30 nmol/kg body weight once a day for 3 days 30 min before the administration of isoquercitrin and piperine. Remaining one group of mice was used for vehicle control. These mice were sacrificed 60 min after the administration of isoquercitrin or 240 min after the administration of piperine under anesthesia. The soleus muscle was collected and used for measurement of GLUT4 translocation and phosphorylation of CAMKKβ as described below.

Preparation of the plasma-membrane fraction and tissue lysate. The plasma membrane fraction and tissue lysate were prepared from skeletal muscle as described previously.[19] Briefly, skeletal muscle was chopped into small pieces and homogenized in buffer A [10 mM Tris–HCl (pH 7.8), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 μg/ml of aprotinin, and 10 μg/ml of leupeptin] containing 0.1% Nonidet P-40, passed through a 22-G needle thrice, and centrifuged at 1,000 × *g* for 10 min at 4°C. The recovered pellet was suspended in buffer A and centrifuged at 1,000 × *g* for 10 min at 4°C. The pellet was re-suspended again in buffer A containing 0.1% Nonidet P-40 and then centrifuged at 10,000 × *g* for 20 min at 4°C. The pellet was solubilized into buffer A containing 1% Nonidet P-40 and centrifuged under the same conditions stated above to obtain the plasma-membrane fraction.

To obtain the tissue lysate, part of the homogenate in buffer A

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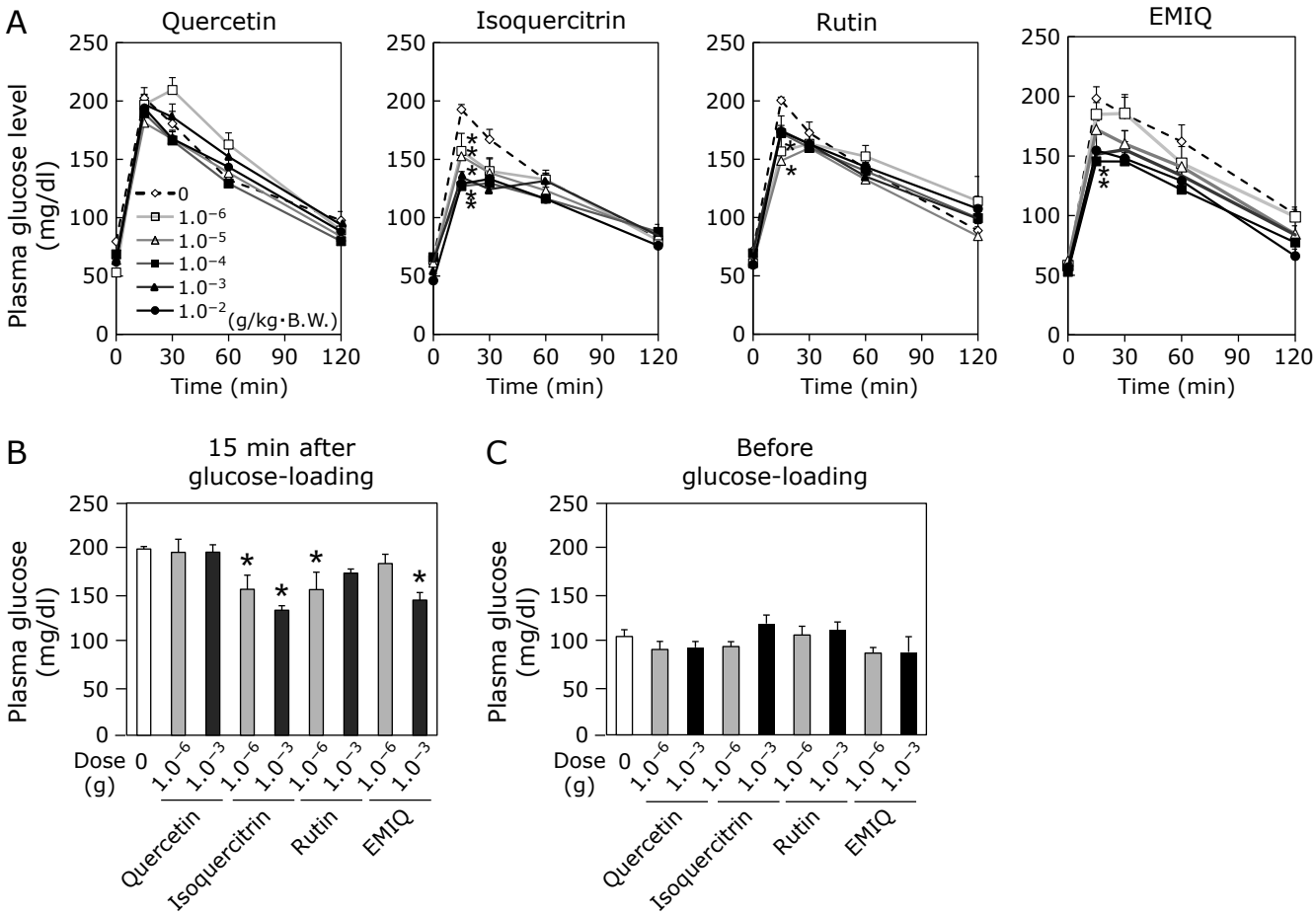

Fig. 1. Effects of quercetin and its glycosides on the plasma glucose level in an oral glucose tolerance test (Experiment 1). (A) Results of OGTT are shown after treatment with different doses of quercetin, isoquercitrin, rutin, or EMIQ. (B) Plasma glucose levels 15 min after glucose administration (1.0 g/kg body weight, p.o.) are shown from (A). (C) Plasma glucose levels are shown from (A) 60 min after quercetin and glycosides administration (before glucose-loading). Values are the mean ± SE (n = 5). *significant difference from the control group by Dunnett's multiple comparison test (p<0.05).

was lysed with buffer B [10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml of aprotinin, and 10 μg/ml of leupeptin] and centrifuged at 16,000 × g for 20 min at 4°C. Obtained plasma membrane fraction and tissue lysate were used for following immunoblotting.

Immunoblotting. Immunoblotting was carried out according to the method of Yamamoto and colleagues.[19] Briefly, proteins in the tissue lysate of muscles were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After treatment with Blocking One solution (Nacalai Tesque, Kyoto, Japan) to block non-specific binding, the polyvinylidene difluoride membrane was incubated with each primary antibody (1:5,000 dilution) overnight at 4°C, followed by the corresponding horseradish peroxidase-conjugated secondary antibody (1:20,000 dilution) for 1 h at room temperature. Proteins were visualized using the ImmunoStar® LD luminescence system (FUJIFILM Wako Pure Chemical Corporation) and detected using the Light-Capture II imaging system (ATTO, Tokyo, Japan).

Statistical analyses. Data are expressed as the mean ± SE (n = 4 or 5). The statistical significance of experimental observations was determined using Dunnett's multi-comparison test (Fig. 1–5) and Tukey-Kramer multiple comparison test (Fig. 6 and 7) by JMP statistical software ver. 11.2.0 (SAS Institute. Cary, NC). The level of statistical significance was set as p<0.05.

Results

Effects of quercetin and its glycosides on acute hyperglycemia. In the first experiment (Experiment 1), the OGTT was carried out to evaluate the effect of a single-oral administration of quercetin and its glycosides on anti-hyperglycemia after the glucose-loading. The glucose level increased in plasma of the control mice (in which glucose alone was given), reached a maximum 15 min after glucose administration, decreased with time, and almost recovered to a normal level by 120 min. Pre-administration of EMIQ, isoquercitrin and rutin suppressed acute hyperglycemia (Fig. 1A). Isoquercitrin (1.0^{-6} g/kg body weight to 1.0^{-2} g/kg body weight) showed a significant reduction in the glucose level 15 min after glucose-loading. Rutin (1.0^{-6} and 1.0^{-5} g/kg body weight) and EMIQ (1.0^{-4} and 1.0^{-3} g/kg body weight) also showed a significant reduction in the plasma glucose level at the same time point. Quercetin failed to affect the plasma glucose level at all doses used in this experiment (Fig. 1A). Isoquercitrin had the strongest effect on preventing acute hyperglycemia among the used compounds. The plasma glucose levels of compounds at 1.0^{-6} g/kg body weight and 1.0^{-3} g/kg body weight after 15 min of glucose-loading are selected and shown in Fig. 1B. These data suggested that quercetin glycosides, but not quercetin *per se*, prevented acute hyperglycemia after single oral administration. Before the glucose-loading, the basal plasma glucose concentration was not decreased by the administration of

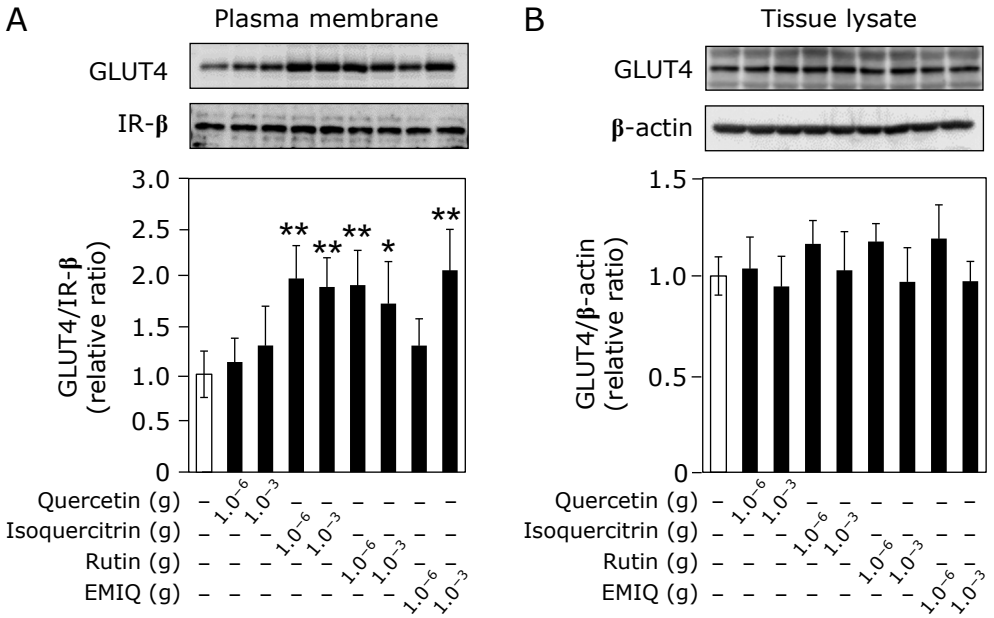

Fig. 2. Quercetin glycosides, but not quercetin, promoted GLUT4 translocation in the skeletal muscle of ICR mice (Experiment 2). Mice were administered (p.o.) 1.0^{-6} and 1.0^{-3} g/kg body weight of quercetin, isoquercitrin, rutin, EMIQ, or water as a vehicle control (5 ml/kg body weight). Plasma membrane fraction and tissue lysate were prepared from the skeletal muscle 60 min after administration of each compound and subjected to Western blotting. (A) GLUT4 and IR-β in the plasma-membrane fraction and (B) GLUT4 and β-actin in the tissue lysate. Each panel shows a typical result from five mice. Density of translocated and expression levels of GLUT4 was analyzed and normalized to IR-β and β-actin levels, respectively. Bar graph shows representative data from five mice. Values are the mean ± SE ($n = 5$). * and ** indicate a significant difference from the control group by Dunnett's multiple comparison test ($p<0.05$ and $p<0.01$, respectively).

quercetin glycosides (Fig. 1C), indicating that quercetin glycosides did not cause hypoglycemia in normal animals.

Quercetin glycosides promoted GLUT4 translocation to the plasma membrane in the soleus muscle. We, next, investigated GLUT4 translocation in the soleus muscle of mice 60 min after oral administration of quercetin and its glycosides at 1.0^{-6} g/kg body weight and 1.0^{-3} g/kg body weight. Rutin and isoquercitrin increased GLUT4 translocation significantly at both doses (Fig. 2A). EMIQ increased GLUT4 translocation significantly at 1.0^{-3} g/kg body weight. However, quercetin did not affect GLUT4 translocation. The level of GLUT1 (which is expressed stably in the plasma membrane) remained unchanged in all groups. In addition, none of the compounds affected GLUT4 expression in the tissue lysate (Fig. 2B).

Effect of quercetin glycosides on the upstream events for GLUT4 translocation in the soleus muscle. It was reported that insulin-, AMPK- and JAK/STAT-signaling pathways are involved in GLUT4 translocation.[20,21] To elucidate the mechanism by which quercetin glycosides promote GLUT4 translocation, phosphorylation of AMPK and its downstream target, acetyl-CoA carboxylase (ACC) was measured in the soleus muscle of mice after administration of each compound at 1.0^{-6} g/kg body weight and 1.0^{-3} g/kg body weight. EMIQ, isoquercitrin and rutin promoted phosphorylation of AMPK and ACC (Fig. 3); isoquercitrin and rutin significantly activated AMPK phosphorylation at 1.0^{-6} g/kg body weight and 1.0^{-3} g/kg body weight, whereas EMIQ activated it at only 1.0^{-3} g/kg body weight. These results were in accordance with those of the OGTT (Fig. 1) and GLUT4 translocation (Fig. 2).

Since CAMKKβ and LKB1 are known as the upstream kinases for AMPK phosphorylation,[22] we investigated weather quercetin glycosides activate CAMKKβ and LKB1. Quercetin glycosides increased phosphorylation of CAMKK but not LKB1 (Fig. 3C and D). With regard to the insulin pathway, phosphorylation of IRS-1, Akt (ser 473 and thr 308) and PI3K was measured in the soleus muscle of mice after administration of each compound. These four compounds did not affect the insulin pathway (Fig. 4). As to the involvement of JAK/STAT pathway in the soleus muscle of the same animals, none of the compounds increased phosphorylation of JAK2 or STAT3 (Fig. 5). These results suggested that quercetin glycosides, but not quercetin, mainly activated the AMPK-signaling pathway to induce GLUT4 translocation in the soleus muscle of mice.

Quercetin glycosides and glucose reveal an additive increase in GLUT4 translocation to the plasma membrane in the soleus muscle. We also examined GLUT4 translocation by quercetin glycosides 15 min after the glucose-loading. It was confirmed that glucose itself induced GLUT4 translocation expectedly. We found that quercetin glycosides and glucose reveal an additive effect on GLUT4 translocation in muscle (Fig. 6A) without affecting its expression level (Fig. 6B). This result strongly suggested that the mode of the action of quercetin glycosides on the GLUT4 translocation was independent of the glucose-induced insulin-signaling pathway.

Compound C as an inhibitor for AMPK, but not W-13 as a calmodulin inhibitor, canceled isoquercitrin-induced GLUT4 translocation to the plasma membrane in the soleus muscle. To confirm whether CAMKKβ/AMPK pathway was involved in the intake of quercetin glycosides-induced GLUT4 translocation, we used Compound C (Experiment 4) and W-13 (Experiment 5) as the AMPK and calmodulin inhibitors. Among quercetin glycosides used in this study, we selected isoquercitrin because this glycoside is the most effective to prevent postprandial hyperglycemia. Compound C canceled isoquercitrin-induced GLUT4 translocation (Fig. 7A) and phosphorylation of AMPK (Fig. 7B) in the muscle of mice. As expected, AICAR induced GLUT4 translocation through phosphorylation of AMPK, and Compound C canceled them (Fig. 7A and B). These results strongly suggest that quercetin glycosides promoted GLUT4 translocation is mainly driven by the AMPK phosphorylation in

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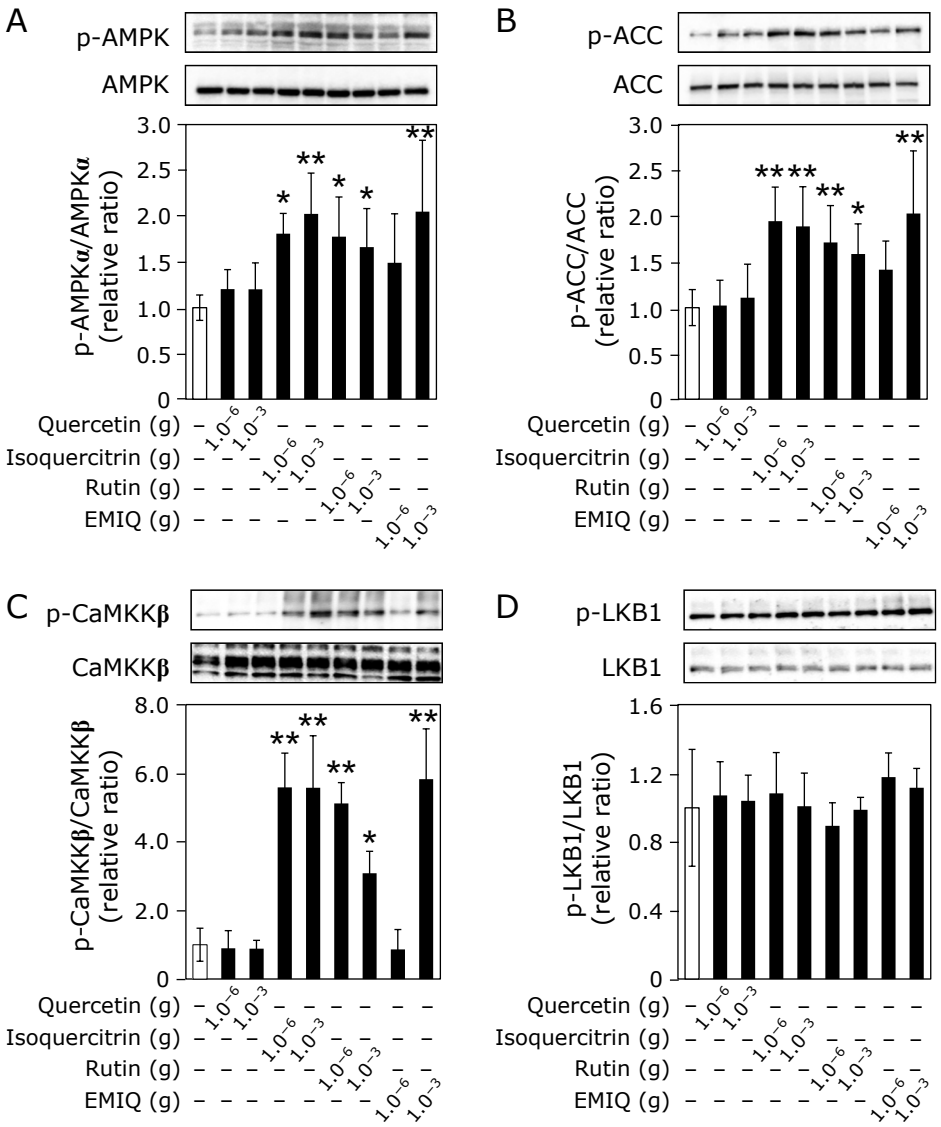

Fig. 3. Quercetin glycosides, but not quercetin, promoted AMPK and CAMKKβ phosphorylation in the skeletal muscle of ICR mice (Experiment 2). Animal treatment was the same protocol as described in Fig. 2. Tissue lysate from the skeletal muscle was prepared and subjected to Western blotting to determine (A) phosphorylated-AMPK and expression of AMPK, (B) phosphorylated-ACC and expression of ACC, (C) phosphorylated-CAMKKβ and expression of CAMKKβ, and (D) phosphorylated-LKB1 and expression of LKB1 in the muscle lysate. The density of each phosphorylation level of AMPK and ACC was analyzed and normalized to that of the corresponding expression level. Each bar graph shows a typical result from five animals. Values are the mean ± SE ($n = 5$). * and ** indicate a significant difference from the control group by Dunnett's multiple comparison test ($p<0.05$ and $p<0.01$, respectively).

the muscle of mice.

Since quercetin glycosides activated CAMKKβ as upstream event of AMPK, we introduced W-13 as a calmodulin inhibitor to estimate whether isoquercitrin activated Ca^{+}/calmodulin dependent pathway. As shown in Fig. 7C and D, pretreatment with W-13 did neither cancel isoquercitrin-induced GLUT4 translocation (Fig. 7C) nor phosphorylation of CAMKKβ (Fig. 7D). However, W-13 suppressed pinerine-induced GLUT4 translocation and phosphorylation of CAMKKβ. In addition, quercetin and its glycosides used in this study did not increase intracellular Ca^{+} concentration in cultured L6 myotubes (data not shown). These results indicate that phosphorylation of CAMKKβ by quercetin glycosides may be independent of Ca^{+}/calmodulin pathway.

Discussion

Chronic and acute hyperglycemia lead to serious health problems in many countries,[1] as it is a risk factor for the onset of cardiovascular diseases.[23] Many researches have focusing on bioactive compounds including polyphenols in food that possess anti-hyperglycemic effects.[24–26] Previous studies have reported that quercetin promotes glucose metabolism and prevents hyperglycemia.[27–29] Moreover, our previous report demonstrated that long-term feeding (13 weeks) of quercetin and its glycosides prevented hyperglycemia and adiposity accompanied by the activation of AMPK in mice fed a high-fat diet but not standard diet.[7] We also found that intake of EMIQ, but not quercetin, for 2 weeks in male C57BL/6 mice promoted GLUT4 translocation and AMPK phosphorylation in the skeletal muscle.[8] However, prevention effects of single administration of quercetin glyco-

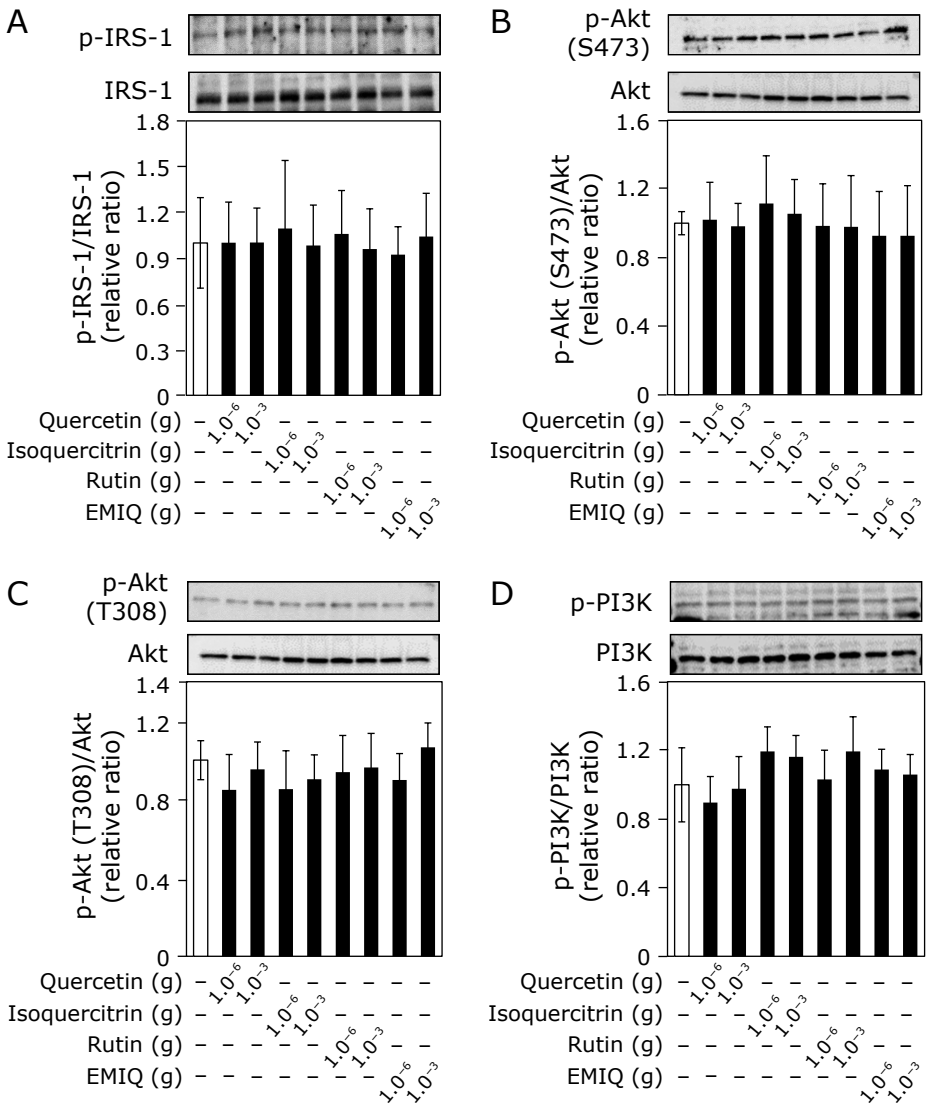

Fig. 4. Neither quercetin nor its glycosides activated the insulin pathway in the skeletal muscle of ICR mice (Experiment 2). Animal treatment was the same protocol as described in Fig. 2. Tissue lysate was prepared and subjected to Western blotting to determine (A) phosphorylation and expression of IRS-1, (B) Akt (S473), (C) Akt (T308), and (D) PI3K. Density of each phosphorylated protein level was analyzed and normalized to corresponding expression level. Each bar graph shows a typical result from five animals. Values are the mean ± SE (n = 5).

sides against postprandial hyperglycemia and its detailed mechanisms including signaling pathways *in vivo* were not fully understood. In addition, most of the previous reports estimated anti-hyperglycemic effect of quercetin and its glycosides at the higher concentration ranges. Thus, the anti-hyperglycemic effect of quercetin and its glycosides at a dietary level is also unclear yet. It was reported that daily intake of quercetin in humans from the meal is about 25–50 mg per day.[30] Therefore, dose range of current study (1.0 mg/kg body weight or less) is relevant to the daily intake level of these compounds from the meal. Results from OGTT revealed that single administration of quercetin glycosides, namely isoquercitrin, rutin and EMIQ, but not quercetin, prevented postprandial hyperglycemia at less than 1.0 mg/kg body weight. Isoquercitrin revealed the strongest anti-hyperglycemic effect and rutin and EMIQ also suppressed postprandial hyperglycemia. These quercetin glycosides promoted GLUT4 translocation through the activation of CAMKKβ/AMPK signaling pathway in skeletal muscle of mice. To our knowledge, this is the first report showing that the single oral administration of quercetin glycosides prevents postprandial hyperglycemia at the dietary level.

Different anti-hyperglycemic effects of used compounds in this study may be due to differences in their bioavailability. Quercetin is poorly absorbed through the small intestine of animals and humans.[15] Quercetin was only slightly absorbed when it was taken as a powder with water.[15] In the same report, it was demonstrated that the maximal plasma levels of quercetin metabolites reached 2.5 μM and 1.0 μM in human plasma 1.5 h after intake of EMIQ and isoquercitrin, respectively, at 2 mg of quercetin aglycone equivalent/kg body weight.[15] In the present study, we attempted to determine the quercetin aglycone and its metabolites by high-performance liquid chromatography system (HPLC), but could not detect them in the plasma of mice after administration of 1.0 mg/kg body weight of quercetin and its glycosides due to the limit of detection and quantification of our HPLC system. Our previous report showed that the plasma level of quercetin aglycone and its conjugated forms were 4.95 ± 0.82 nM and 6.80 ± 2.00 nM, respectively, 1.5 h after the p.o.-

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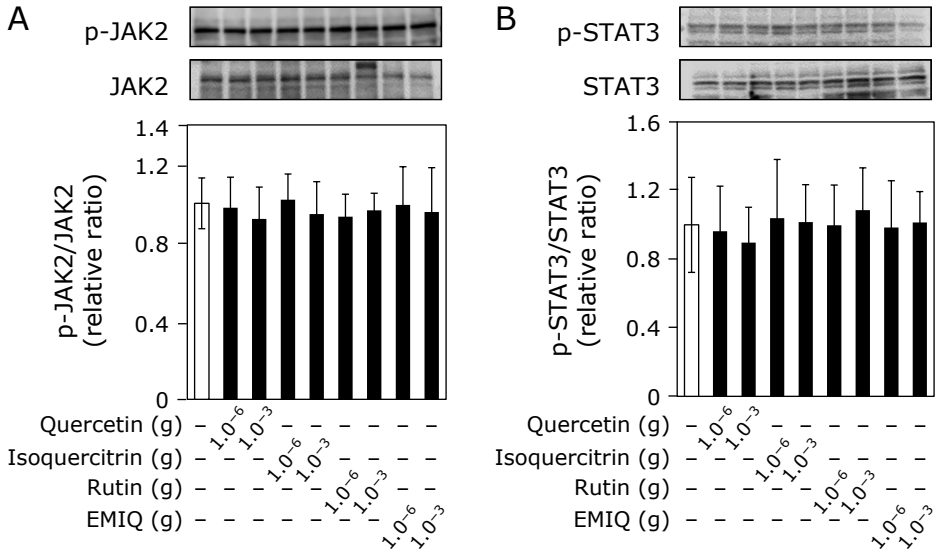

Fig. 5. Neither quercetin nor its glycosides activated the JAK/STAT pathway in the skeletal muscle of ICR mice (Experiment 2). Animal treatment was the same protocol as described in Fig. 2. Tissue lysate was prepared and subjected to Western blotting to determine (A) phosphorylation and expression of JAK2, and (B) STAT3. Density of each phosphorylated protein level was analyzed and normalized to corresponding expression level. Each bar graph shows a typical result from five animals. Values are the mean ± SE (n = 5).

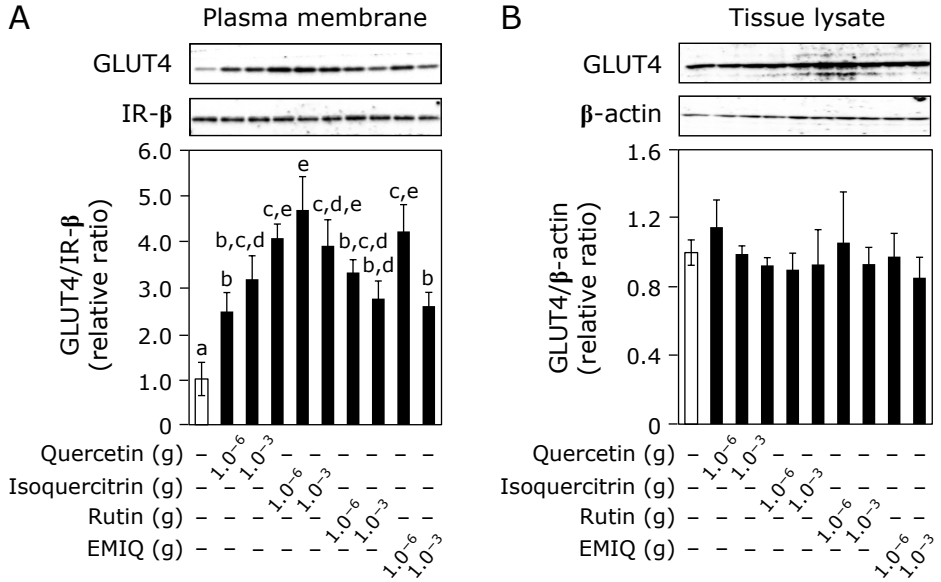

Fig. 6. Effect of quercetin and its glycosides on GLUT4 translocation with glucose-loading in the skeletal muscle of ICR mice (Experiment 3). Mice were administered (p.o.) 1 μg and 1 mg/kg body weight of quercetin, isoquercitrin, rutin, EMIQ, or water as a vehicle control (5 ml/kg body weight). After 60 min, glucose (1.0 g/kg body weight, p.o.) was given to the mice. Plasma membrane fraction and tissue lysate were prepared from the skeletal muscle 15 min after glucose-loading and subjected to Western blotting to determine (A) GLUT4 and IR-β in the plasma-membrane fraction and (B) GLUT4 and β-actin in the tissue lysate. Each panel shows a typical result from five mice. Density of translocated and expression levels of GLUT4 was analyzed and normalized to IR-β and β-actin levels, respectively. Bar graph shows representative data from five mice. Values are the mean ± SE (n = 5). Values with the same letters are not significantly different by Tukey–Kramer multiple comparison test (p<0.05).

administration of EMIQ at 10 mg/kg body weight in ICR mice.[31] These results suggest that quercetin and its metabolites exist in the plasma after intake of quercetin glycosides, but slight amounts of quercetin aglycone also exist in the body and may accumulate in the tissues. Indeed, 13-week feeding of quercetin revealed the anti-hyperglycemic effect,[7] but results from current study and 2-week feeding experiment[8] indicated that quercetin aglycone failed to suppress the blood glucose level and/or promote GLUT4 translocation.

Our finding demonstrated that quercetin glycosides specifically reduced blood glucose level 15 min after glucose-loading. This result indicate that quercetin glycosides are effective for blood sugar spike. Among the glycosides, the antihyperglycemic effect of isoquercitrin was stronger than that of EMIQ. Although EMIQ is a mixture of quercetin-3-*O*-glucosides and contains about 30% isoquercitrin, remaining compounds are α-glucosyl derivatives of quercetin with 2–7 additional linear glucose moieties. It was reported that EMIQ is hydrolyzed to

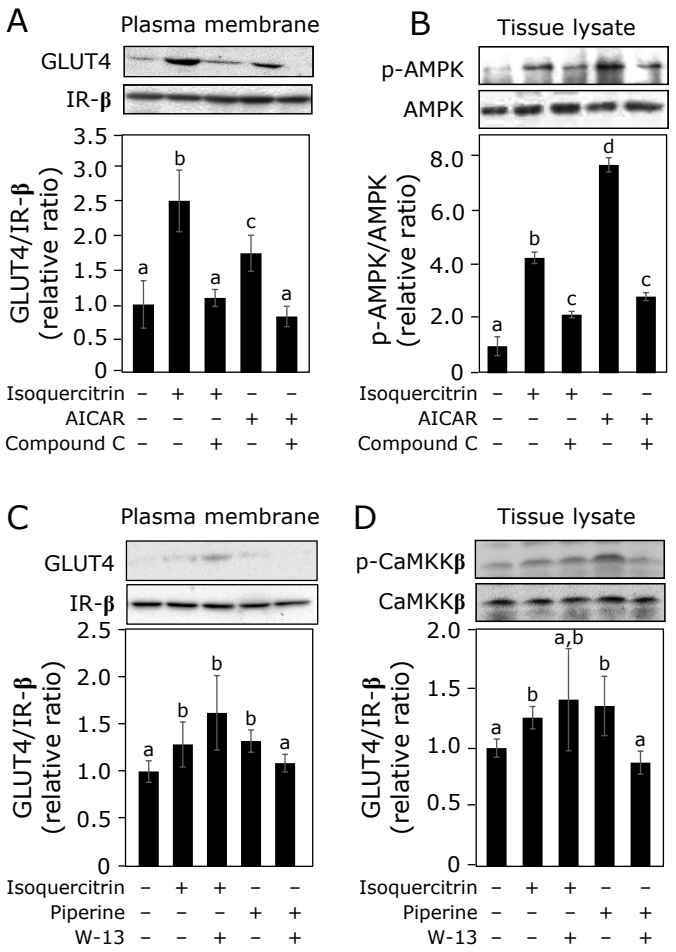

Fig. 7. Compound C as an inhibitor for AMPK, but not W-13 as a calmodulin inhibitor, canceled isoquercitrin-induced GLUT4 translocation to the plasma membrane in the soleus muscle of ICR mice (Experiments 4 and 5). Mice were pretreated with Compound C before administration of isoquercitrin at 1.0^{-3} g/kg body weight. Plasma membrane fraction and tissue lysate were prepared and subjected to Western blotting to determine (A) GLUT4 and IR-β in the plasma-membrane fraction and (B) phosphorylation and expression of AMPK in tissue lysate. Another series of mice were pretreatment with W-13 before administration of isoquercitrin at 1.0^{-3} g/kg body weight. Western blotting was performed to determine (C) GLUT4 and IR-β in the plasma-membrane fraction and (D) phosphorylation and expression of CAMKKβ in tissue lysate. Each panel shows a typical result from four mice. (A, C) Density of translocated level of GLUT4 was analyzed and normalized to IR-β. (B, D) The density of each phosphorylation level of AMPK and CAMKKβ was analyzed and normalized to that of the corresponding expression level. Bar graph shows representative data from four mice. Values are the mean ± SE ($n = 4$). Values with the same letters are not significantly different by Tukey–Kramer multiple comparison test ($p<0.05$).

isoquercitrin by α-glucosidase[14] and further hydrolyzed to aglycone by β-glucosidase in the small intestine after injection of EMIQ in the jejunal loops of male Wistar/ST rats.[32] These results suggest that formation of quercetin aglycone from the EMIQ is slower than that of isoquercitrin and needed higher concentration than isoquercitrin to possess the same extent anti-hyperglycemic effect. It is also reported that formed quercetin aglycone from the glycosides easily and rapidly receive further metabolism such as conjugation and methylation. Recently, 7-*O*-glucuronide-4'-*O*-sulfate and methylquercetin are major metabolites in plasma after oral administration of EMIQ in male Wistar/ST rats.[33] Suppression of blood sugar spike by quercetin glucosides in the current study may be due to the rapid metabolism of the glucosides. As for rutin, it is reported that the absorption and metabolism of rutin differ from those of isoquercitrin: rutin is not the substrates for hydrolytic enzymes and transporters in the small intestine.[34] Rutin is mainly received metabolism by intestinal microflora and forms quercetin aglycone and its degradation products in the large intestine.[13,35] These results suggest that certain polyphenols including rutin may reach the large intestine within one hour. However, it is unclear the reason why rutin prevented postprandial hyperglycemia at the low concentration range, but not at the higher concentrations. We speculate that higher concentration of rutin may cause aggregation and difficult to interact with tissue or intestinal microbiota in the digestive tracts. It needs further experiment to clarify this unknown issue.

Our findings in this study contained inconsistent results: i.e., Rutin induced GLUT4 translocation at 1.0 mg/kg body weight, but the same dose of rutin did not show the anti-hyperglycemic effect. This inconsistency may be explainable as follows: GLUT4 translocation does not completely correlate with increasing glucose uptake, since it was reported that mitogen-activated protein kinase phosphorylation and phosphatidylinositol 3, 4, 5-trisphosphate are needed to increase glucose uptake after GLUT4 translocation.[36,37] In addition, it is demonstrated that suppressor of cytokine signaling 3, Src homology region 2 domain-containing phosphatase 1 and 2, and insulin receptor substrate 2 are involved in promotion of GLUT4 translocation without increasing glucose uptake.[38–40] Further study is needed to clarify the exact action of rutin.

Although a single administration or short-term feeding of quercetin aglycone failed to promote GLUT4 translocation in the skeletal muscle of mice, intake of quercetin glycosides may release the aglycone in the intestinal tissues. Formed slight amount of intracellular quercetin aglycone may promote GLUT4 translocation in the muscle cells. Indeed, our previous results demonstrated that 1 nM quercetin promoted AMPK phosphorylation and subsequent GLUT4 translocation in L6 myotubes[31] In the current study, we confirmed that Compound C as an AMPK inhibitor abolished isoquercitrin-promoted GLTU4 translocation and AMPK phosphorylation in the skeletal muscle of mice. As for the upstream of AMPK, K-13 as an inhibitor of calmodulin failed to cancel both quercetin glycosides-induced phosphorylation of CAMKKβ and downstream GLUT4 translocation. In addition, quercetin and its glycosides did not increase intracellular Ca^{2+} level in cultured L6 myotubes (data not shown). These results suggest that that phosphorylation of CAMKKβ may be independent of Ca^{2+}/calmodulin pathway. Thus, upstream event of quercetin glycosides-caused phosphorylation of CAMKKβ is still unknown. To clarify this important and interesting issue, further experiment is needed in future.

Single oral administration of quercetin glycosides, but not quercetin, at a dietary concentration range prevented postprandial hyperglycemia significantly. This effect was accompanied by promotion of GLUT4 translocation to the plasma membrane through the activation of the AMPK signaling pathway in the skeletal muscle of mice. Among the quercetin glycosides, isoquercitrin revealed the strongest effect. Thus, quercetin glycosides, in particular isoquercitrin, are effective food compounds for prevention of postprandial hyperglycemia and potentially diabetes mellitus.

Author Contributions

HA and YYamashita conducted study concept and design. HJ, TK, FO and YYoshioka performed experiments and statistical analyses. HJ and YYamashita wrote the initial draft of the manuscript. HA critically revise the manuscript. All of authors read and approved the final version of manuscript, and agree to submit it to the Journal of Clinical Biochemistry and Nutrition.

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Abbreviations

ACC	acetyl-CoA carboxylase
Akt	protein kinase B
AMPK	adenosine monophosphate-activated protein kinase
CAMKKβ	Ca^{2+}/CaM-dependent protein kinase kinase
EMIQ	enzymatically modified isoquercitrin
GLUT	glucose transporter
HPLC	high-performance liquid chromatography system
IR-β	insulin receptor beta
IRS-1	insulin receptor substrate-1
JAK	Janus kinase
LKB1	liver kinase B1
LPH	lactase phlorizin hydrolase
OGTT	oral glucose tolerance test
PI3K	phosphoinositide 3-kinase
STAT	signal transducers and activator of transcription

Conflict of Interest

No potencial conflicts of interest were disclosed.

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