RESEARCH ARTICLE

Tiliroside, a glycosidic flavonoid, inhibits carbohydrate digestion and glucose absorption in the gastrointestinal tract

Tsuyoshi Goto[1], Mayuka Horita[1], Hiroyuki Nagai[2], Akifumi Nagatomo[3], Norihisa Nishida[3], Youichi Matsuura[3] and Satoshi Nagaoka[1]

[1] Department of Applied Life Science, Faculty of Applied Biological Sciences, Gifu University, Yanagido, Gifu, Japan
[2] Gifu Prefectural Research Institute for Health and Environmental Science, Naka-Fudougaoka, Kakamigahara, Japan
[3] Research and Development Division, Morishita Jintan Co., Ltd, Tamatsukuri, Chuo-ku, Osaka, Japan

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Scope: Recent studies have reported that tiliroside, a glycosidic flavonoid, possesses anti-diabetic activities. In the present study, we investigated the effects of tiliroside on carbohydrate digestion and absorption in the gastrointestinal tract.

Methods and results: This study showed that tiliroside inhibits pancreatic α-amylase (IC_{50} = 0.28 mM) in vitro. Tiliroside was found as a noncompetitive inhibitor of α-amylase with K_i values of 84.2 μM. In male ICR mice, the increase in postprandial plasma glucose levels was significantly suppressed in the tiliroside-administered group. Tiliroside treatment also suppressed hyperinsulinemia after starch administration. Tiliroside administration inhibited the increase of plasma glucose levels in an oral glucose tolerance test, but not in an intraperitoneal glucose tolerance test. In human intestinal Caco-2 cells, the addition of tiliroside caused a significant dose-dependent inhibition of glucose uptake. The inhibitory effects of both sodium-dependent glucose transporter 1 (SGLT1) and glucose transporter 2 (GLUT2) inhibitors (phlorizin and phloretin, respectively) on glucose uptake were significantly inhibited in the presence of tiliroside, suggesting that tiliroside inhibited glucose uptake mediated by both SGLT1 and GLUT2.

Conclusions: These findings indicate that the anti-diabetic effects of tiliroside are at least partially mediated through inhibitory effects on carbohydrate digestion and glucose uptake in the gastrointestinal tract.

Keywords:
α-Amylase / Flavonoids / GLUT2 / SGLT1 / Tiliroside

1 Introduction

The incidence of type 2 diabetes, which is one of the most serious, chronic diseases in the world, is increasing because of increased obesity and aging in the general population [1]. The disease accounts for substantial morbidity and mortality from adverse effects on the cardiovascular system and disease-specific complications, such as blindness and renal failure [2]. Recent evidence suggests that the postprandial state is an important contributing factor for the development of atherosclerosis. High postprandial plasma glucose concentrations are associated with an increased risk of developing type 2 diabetes and metabolic syndrome [3, 4]. Therefore, the control of postprandial hyperglycemia has

Correspondence: Dr. Satoshi Nagaoka, Department of Applied Life Science, Faculty of Applied Biological Sciences, Gifu University, Gifu 501-1193, Japan
E-mail: nagaoka@gifu-u.ac.jp
Fax: +81-58-293-2931

Abbreviations: AUC, area under the curve; **GLUT2,** glucose transporter 2; **HBSS,** Hanks' balanced salt solution; **ICR,** Institute of Cancer Research; **SGLT1,** sodium-dependent glucose transporter 1

been suggested as an important treatment for diabetes and the prevention of cardiovascular complications.

One therapeutic approach for decreasing postprandial hyperglycemia is to retard or suppress the absorption of glucose in the intestine. In mammals, dietary carbohydrates are hydrolyzed by enzymes, such as α-amylase and α-glucosidase. α-Amylase is an endoglucanase that catalyzes the hydrolysis of internal α-1,4-glycosidic linkages in starch and other related polysaccharides. α-Glucosidase, such as maltase and sucrase, which are located in the brush-border surface membrane of intestinal cells, is an enzyme that catalyzes the final step in carbohydrate digestion. After digestion, the resulting product, glucose, is absorbed into the small intestine. Therefore, the inhibition of α-amylase and α-glucosidase in the digestive organs is an approach for managing postprandial hyperglycemia [5, 6]. In fact, several synthetic α-glucosidase inhibitors are widely used in clinical practice.

In addition to the inhibitors of carbohydrate-hydrolyzing enzymes, a new class of agents that delay or inhibit glucose absorption directly could substantially affect the management of diabetes and obesity. In the intestine, glucose is transported mainly by two transporters, depending on the luminal glucose concentration. At low concentrations, glucose is transported against a concentration gradient by an active transport mechanism involving the high-affinity, sodium-dependent glucose transporter 1 (SGLT1) (Km = 0.2 mmol/L) [7, 8]. At higher concentrations, glucose is transported mainly by the lower affinity facilitated transporter, glucose transporter 2 (GLUT2) (K_m = 40 mmol/L) [7, 8]. GLUT2 is thought to be localized to the membrane only at higher glucose concentrations, whereas SGLT1 is constitutive [9]. Thus, these glucose transporters in the small intestine might be an attractive therapeutic target for diabetes.

Flavonoids are polyphenols that are widely distributed in foods, especially fruits and vegetables [10]. They are characterized by two or more aromatic rings, each bearing at least one aromatic hydroxyl group, which are connected to a heterocyclic pyran [11]. Most flavonoids (with the exception of flavonols) occur naturally in a form that is conjugated, mainly with sugar residues. Epidemiological studies suggest the health benefits of dietary flavonoids in reducing the risk of carcinogenesis, hypertension, inflammation and cardiovascular diseases [12–14]. Several flavonoids reportedly inhibit carbohydrate-hydrolyzing enzymes, such as α-amylase and α-glucosidase [15, 16]. Moreover, some flavonoids can interact with certain sugar transporters, for example, by the competitive inhibition of SGLT1 [17, 18] or the inhibition of GLUT2 [19]. Thus, dietary flavonoids may be candidate agents for managing postprandial hyperglycemia.

Tiliroside (kaempferol 3-*O*-(6″-*O*-*p*-coumaroyl)-β-D-glucopyranoside) is a glycosidic flavonoid found in several medicinal and dietary sources, such as linden, rose hips and strawberries [20–22]. The administration of tiliroside significantly inhibited body weight gain and visceral fat accumulation after fasting in nonobese mice [21]. We recently reported that tiliroside ameliorated obesity-induced metabolic disorders, including insulin resistance, through the enhancement of adiponectin signaling in obese diabetic mice [23]. Therefore, tiliroside seems to be a useful flavonoid for managing metabolic syndrome, but the mechanisms underlying its anti-diabetic activity are not fully understood.

In the present study, we investigated the effects of tiliroside on carbohydrate digestion and absorption in the gastrointestinal tract. We found that tiliroside inhibited pancreatic α-amylase-mediated carbohydrate digestion and SGLT1- and GLUT2-mediated glucose uptake in enterocytes. These effects may contribute to its anti-diabetic activity, at least in part.

2 Materials and methods

2.1 Materials

Tiliroside was extracted and purified from the seeds of *Rosa canina* L., which is commonly known as dog rose, as previously described in [21]. Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Nacalai Tesque (Kyoto, Japan) and were of guaranteed reagent grade or tissue culture grade.

2.2 α-Amylase activity assay

α-Amylase activity was assayed as previously described with a slight modification [15]. Briefly, porcine pancreatic α-amylase was dissolved in PBS (0.2 U/μL) containing 0.2% BSA and 0.02% NaN_3. The substrate solution consisted of 0.625–10 mM 4-nitrophenyl-α-D-glucopyranoside in PBS. Then, 50 μL of enzyme solution and 10 μL of test compounds that were dissolved in DMSO were mixed in 1 well of 96-well plate. After 5 min of incubation at room temperature, substrate solution (40 μL) was added and incubated for another 10 min at room temperature. The absorbance of liberated *p*-nitrophenol was measured at 405 nm using a microplate reader. We measured the absorbance of each sample at zero time and used the increase in absorbance from zero time was used to filter out the background absorbance. α-Amylase activity was expressed as moles of *p*-nitrophenol liberated per second by a unit of α-amylase. Inhibitory activity was expressed as 100 minus the relative absorbance difference (%) of the test compounds to the absorbance change of the vehicle control sample. K_i values were calculated from Dixon and double reciprocal plots.

2.3 α-Glucosidase (maltase and sucrase) activity assays

A crude rat intestinal α-glucosidase solution was prepared according to the method of Hanamura et al., with a slight modification [24]. Briefly, rat intestinal acetone powder was suspended in a 10-fold w/v of 100 mM maleate buffer

(pH 6.0) containing 0.5% BSA and 0.02% NaN_3 and then resuspended by vortexing, which was followed by sonication in an ice bath. After centrifuging at 3000 rpm for 20 min, the supernatant was diluted with a 1.5-fold volume of maleate buffer for the sucrase assay or in a ratio of 1:9 for the maltase assay. The substrate solutions consisted of 74 mM sucrose and 40 mM maltose in maleate buffer for the sucrase and maltase assays, respectively. A total of 25 μL of enzyme solution, 15 μL of maleate buffer, and 10 μL of test compounds dissolved in DMSO were mixed and incubated at 37°C for 5 min. We added 50 μL of the substrate solutions and incubated the mixture at 37°C for 30 min. The reaction was stopped by heating at 95°C for 5 min. The liberated glucose levels were determined enzymatically using a Glucose CII test (Wako Pure Chemical Industries, Osaka, Japan). The inhibitory effects of the α-glucosidases were evaluated by calculating the percentage of glucose content of the reacting supernatant with and without the test compounds.

2.4 Animal studies

Nine-wk-old male ICR (Institute of Cancer Research) mice (Japan SLC, Hamamatsu, Japan) were tested for starch and glucose tolerance. Before testing, all mice were maintained in a temperature-controlled (23°C) facility with a constant 12-h light/12-h dark cycle and given free access to food (Oriental Yeast, Tokyo, Japan) and water. After overnight fasting (with free access to water), the mice were divided. For the oral starch or glucose tolerance tests, a gastric feeding tube was used to administer soluble corn starch (nacalai tecque) (2 g/kg body weight) or d-glucose (2 g/kg body weight) suspended with or without tiliroside (450 and 600 mg/kg body weight) or acarbose (100 mg/kg body weight) in 0.5% carboxymethyl cellulose. For the intraperitoneal glucose tolerance test, d-glucose (2 g/kg body weight) dissolved in sterile PBS was administered intraperitoneally immediately after the oral administration of tiliroside (600 mg/kg body weight), acarbose (100 mg/kg body weight) or vehicle control. Blood samples were collected from the tail vein before and 15, 30, 60, 90 and 120 min after the injection of glucose or starch. Plasma glucose levels were determined by the glucose CII test in accordance with the manufacturer's protocols. The area under the curve (AUC) was calculated using the trapezoidal rule. During the starch tolerance test, plasma insulin levels were also measured 60 min after starch injection with an ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan) in accordance with the manufacturer's instructions. Animal care procedures and methods were approved by the Animal Care and Use Committee of Gifu University (permission number: 09027).

2.5 Cell cultures

Human intestinal Caco-2 cells (American Type Culture Collection, Rockville, MD, USA) were cultured as previously described in [25]. Briefly, Caco-2 cells were cultured in a humidified CO_2 incubator in an atmosphere of 5% CO_2–95% air v/v at 37°C. Caco-2 cells were grown in DMEM (Nissui Pharmaceuticals, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum, 1% 200 mM L-glutamine, 1% nonessential amino acids (Invitrogen, Carlsbad, CA, USA), 100 U/mL penicillin, 100 μg/mL streptomycin and 50 μg/mL gentamicin. Caco-2 stock cell cultures were maintained in 100-mm plastic dishes. The cells were sub-cultured at confluence by trypsin treatment before use in the experiments. Caco-2 cells used in the glucose uptake experiments were seeded and grown to confluence in 12-well plates. All experiments were performed on cells between passage numbers 65 and 69.

2.6 Assay of glucose uptake in Caco-2 cells

For the assay of glucose uptake in Caco-2 cells, Caco-2 cells were seeded at 0.38×10^5 cells/well. The medium was changed every 2–3 days, and the experiment was conducted within 16 days. During the experiments, the medium was discarded, and the cells were washed twice with 1 mL of Hanks' balanced salt solution (HBSS; 140 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES and 1.0% BSA; pH 7.3) containing 5 mM D-glucose. After washing, 1 mL of HBSS containing 5 mM D-glucose with or without test compounds was added, and the cells were incubated for 30 min at 37°C. After exclusion of the buffer, HBSS buffer containing 0.5 μCi/mL D-[U-^{14}C] glucose and 1 mM total glucose, with or without test compounds, was added. Cells were then incubated for 10 min at room temperature, and the buffer was removed. Glucose uptake was stopped by washing each cell three times with 1 mL of ice-cold PBS, and 400 μL of NaOH solution (0.1 mol/L) was added to solubilize the cells. After overnight incubation at room temperature, aliquots were removed for scintillation counting and protein measurement. Then, 5 mL of scintillation solution (Aquasol-2, PerkinElmer, MA, USA) and 300 μL of the different test solutions were mixed and analyzed using a Packard 1600 TR liquid scintillation analyzer (Packard Instruments, Meriden, CT, USA). Glucose transport values were corrected for protein content, as determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA).

2.7 Statistical analysis

The results are expressed as mean and standard error of mean (SEM). Statistical significance of differences among the group was evaluated using Student's *t*-test or analysis of variance (ANOVA) and the Tukey–Kramer test. Differences were considered significant when $p < 0.05$.

3 Results

3.1 Tiliroside inhibited α-amylase but not α-glucosidases in vitro

Previous reports have shown that tiliroside, a glycosidic flavonoid, has anti-obesity [21] and anti-diabetic [23] effects. Thus, we first asked whether tiliroside has an inhibitory effect on carbohydrate-hydrolyzing enzymes, such as α-amylase and α-glucosidase. The inhibitory effect of tiliroside against α-amylase was determined by an α-amylase activity assay using *p*-nitrophenyl-α-D-maltopentoglycoside as a substrate (Fig. 1A). The addition of tiliroside caused a dose-dependent inhibition of porcine pancreatic α-amylase activity by 30.6, 50.6 and 96.5% at concentrations of 0.1, 0.3 and 1 mM, respectively. In our assay, the IC_{50} value (concentration of sample required for 50% inhibition) for α-amylase inhibited by tiliroside was 0.28 mM. Meanwhile, the addition of tiliroside had the little inhibitory effect against rat intestinal α-glucosidase using maltose and sucrose as substrates (Fig. 1B and C, respectively). Even in the presence of 1 mM tiliroside, maltase and sucrase were inhibited only 25.3 and 33.3%, respectively.

To increase the understanding of the inhibitory effects of tiliroside against α-amylase, we performed a kinetic assessment. K_i, which is the dissociation constant of the enzyme–inhibitor complex, was determined at 84.2 μM from the intercept on a Dixon plot (Fig. 2). Experiments were performed using three substrate concentrations (0.25 mM, circles; 0.5 mM, squares; 1.0 mM, diamonds).

3.2 Tiliroside suppressed hyperglycemia and hyperinsulinemia induced by oral starch administration

In order to investigate whether the inhibitory effect of tiliroside against α-amylase is active in vivo, we performed an oral starch tolerance test on male ICR mice. Postprandial plasma glucose levels were lower in tiliroside-administered mice than in control group mice (Fig. 3A). Plasma glucose levels increased to 381 mg/dL at 30 min after oral starch administration and decreased thereafter (348, 279 and 227 mg/dL at 60, 90, and 120 min, respectively). However, the increase in postprandial plasma glucose levels was significantly suppressed when mice were fed after the administration of tiliroside (450 mg/kg) with plasma glucose levels of 342, 300, 238 and 187 mg/dL at 30, 60, 90 and 120 min, respectively. The inhibitory effects of tiliroside against hyperglycemia induced by starch administration was dose-dependent, and the administration of tiliroside at 600 mg/kg strongly inhibited postprandial hyperglycemia with plasma glucose levels of 266, 227, 172 and 146 mg/dL at 30, 60, 90 and 120 min, respectively. The AUC for the glucose response was significantly lower in the tiliroside-administered group (450 and 600 mg/kg) than in the control group (87 and 69%, respectively) (Fig. 3B). Moreover, the administration of tiliroside at 600 mg/kg significantly suppressed hyperinsulinemia in the oral starch tolerance test. Sixty minute after starch administration, plasma

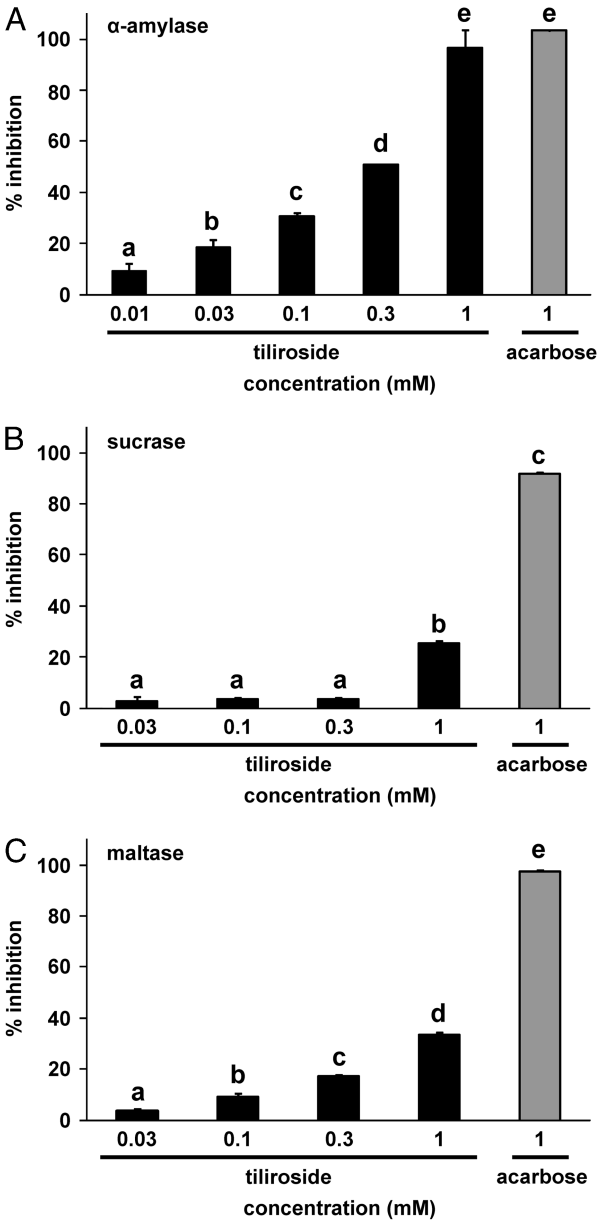

Figure 1. Inhibitory effects of tiliroside against pancreatic α-amylase and intestinal α-glucosidase activity in vitro. (A) Porcine pancreatic α-amylase activity was assayed using *p*-nitrophenyl-α-D-maltopentoglycoside as a substrate. α-Amylase activities were determined for 10 min at room temperature in the presence or absence of compounds. (B) and (C) Rat intestinal α-glucosidase activity was assayed using sucrose (B) and maltose (C) as substrates. α-Glucosidase activities were determined for 30 min at 37°C in the presence or absence of compounds. Acarbose was used as the positive control. The values are mean ± SEM of four tests. Mean values with unlike letters were significantly different ($p < 0.05$; one-way ANOVA and Tukey–Kramer test).

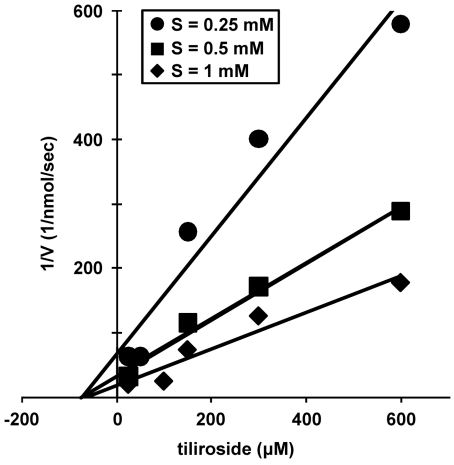

Figure 2. Kinetic analysis of the inhibitory effect of tiliroside against pancreatic α-amylase. Porcine pancreatic α-amylase activity was assayed using *p*-nitrophenyl-α-D-maltopentoglycoside as a substrate. The data were plotted by the Dixon method. Each line represents a different substrate concentration: circles = 0.25 mM, squares = 0.5 mM, and diamonds = 1.0 mM. The intercept value represents the Vmax parameter. The values are mean ± SEM of four tests.

insulin levels in the tiliroside-administered group were reduced by 34% compared with those in the control group (Fig. 3C).

3.3 Tiliroside suppressed hyperglycemia induced by oral glucose administration but not by intraperitoneal glucose administration

In order to assess the contribution of the inhibitory effects of tiliroside against carbohydrate-hydrolyzing enzymes on the suppression of postprandial hyperglycemia, we performed an oral glucose tolerance test with or without tiliroside treatment. In the oral glucose tolerance test, the administration of acarbose (100 mg/kg), a synthetic inhibitor of α-amylase and α-glucosidase, had no effect compared with the vehicle-treated control (Figs. 4A and 4B). However, tiliroside administration (600 mg/kg) significantly inhibited the increase in plasma glucose levels at 15 and 30 min after oral glucose administration (17 and 13% reduction, respectively) (Fig. 4A). The AUC for the glucose response in the tiliroside-administered group was reduced by 13% compared with that in the vehicle-treated control group (Fig. 4B). In contrast, the administration of tiliroside had no effect on plasma glucose levels during the intraperitoneal glucose tolerance test (Fig. 4C and D). These results indicate that tiliroside administration suppressed postprandial hyperglycemia through the inhibition of not only carbohydrate-hydrolyzing enzymes, but also glucose uptake in the intestine.

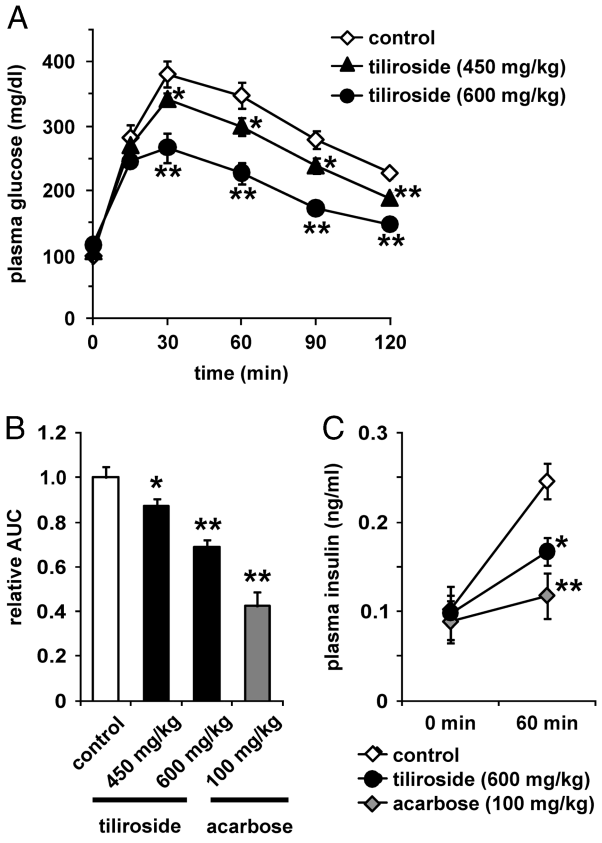

Figure 3. Effects of tiliroside treatment on plasma glucose and insulin levels after oral starch administration in male ICR mice. Tiliroside (450 and 600 mg/kg), acarbose (100 mg/kg), and vehicle control (0.5% carboxymethyl cellulose) were coadministered to fasted 9-wk-old male ICR mice orally with starch (2 g/kg). (A) Plasma glucose levels after starch administration were measured by an enzymatic colorimetric assay. (B) The AUC for glucose response in each group in (A) is shown. (C) Plasma insulin level after starch administration was measured by ELISA. The values are mean ± SEM of 4–7 animals per group. $^{*}p<0.05$, $^{**}p<0.01$ compared with the vehicle control group.

3.4 Tiliroside inhibited both SGLT1- and GLUT2-mediated glucose uptake in Caco-2 cells

In response to the results of the oral and intraperitoneal glucose tolerance tests, we investigated whether tiliroside inhibits glucose uptake in small intestinal epithelial cells. In the presence of Na^+, Caco-2 cells absorbed glucose (1 mM) from the cell culture medium at a rate of 6.72 nmol/min/mg cellular protein. Under this condition, both SGLT1- and GLUT2-mediated glucose uptake was active. The addition of tiliroside to Caco-2 cells led to a dose-dependent inhibition of glucose uptake (Fig. 5A). The IC_{50} value for glucose uptake in the presence of Na^+ inhibited by tiliroside was 97 μM. Under this condition, the glucose uptake rate was decreased by 5.95 nmol/min/mg cellular protein in the presence of 600 μM tiliroside. The above experiments were

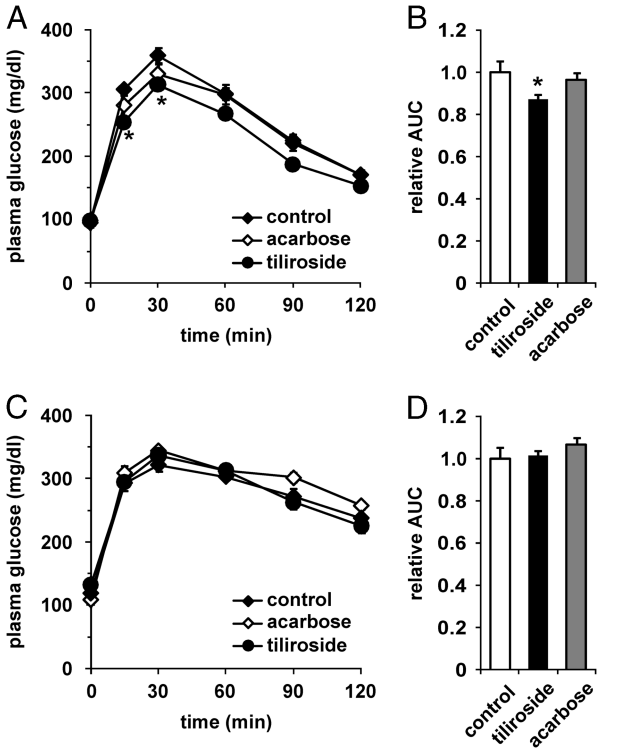

Figure 4. Effects of tiliroside treatment on plasma glucose levels after oral and intraperitoneal glucose administration in male ICR mice. (A) and (B) Tiliroside (600 mg/kg), acarbose (100 mg/kg), and vehicle control (0.5% carboxymethyl cellulose) were coadministered to fasted 9-wk-old male ICR mice orally with glucose (2 g/kg). Plasma glucose levels after oral glucose administration were measured by an enzymatic colorimetric assay (A). The AUC for glucose response in each group in (A) is shown (B). (C) and (D) Glucose was administered intraperitoneally to fasted 9-wk-old male ICR mice immediately after oral administration of tiliroside (600 mg/kg), acarbose (100 mg/kg), and vehicle control (0.5% carboxymethyl cellulose). Plasma glucose levels after intraperitoneal glucose administration were measured by an enzymatic colorimetric assay (C). The AUC for glucose response in each group in (C) is shown (D). The values are mean ± SEM of 4–7 animals per group. $^{*}p < 0.05$ compared with the vehicle control group.

repeated under sodium-free conditions, with KCl and K_2HPO_4 replacing NaCl and Na_2HPO_4, respectively. Under this condition, SGLT1-mediated glucose uptake was inactive, but GLUT2-mediated uptake was active. Glucose uptake was decreased (3.55 nmol/min/mg cellular protein) compared with culture medium containing sodium. Tiliroside also caused a dose-dependent inhibition of glucose uptake under this condition (Fig. 5B), suggesting that tiliroside inhibited GLUT2-mediated glucose uptake in Caco-2 cells. The IC_{50} value for glucose uptake inhibited by tiliroside (240 μM) was higher in the absence of sodium than in the presence of sodium. Moreover, the glucose uptake rate was inhibited by 600 μM tiliroside in the presence of Na^+ (5.95 nmol/min/mg protein) was higher than that mediated by GLUT2 (3.55 nmol/min/mg protein), suggesting that tiliroside also inhibited SGLT1-mediated glucose uptake in Caco-2 cells.

In order to increase the understanding of the inhibitory effects of tiliroside against glucose uptake in Caco-2 cells, we performed experiments using inhibitors of SGLT1- and GLUT2-mediated glucose uptake (phlorizin and phloretin, respectively). In the presence of 500 μM phlorizin and 100 μM phloretin, glucose uptake rate was decreased by 2.51 (34.8%) and 3.35 (46.4%) nmol/min/mg cellular protein, respectively (Fig. 5C). At 600 μM tiliroside, the inhibitory effect of 500 μM phlorizin and 100 μM phloretin against glucose uptake was nullified and significantly diminished, respectively (Fig. 5C). The phlorizin- and phloretin-inhibitable fractions of the glucose uptake rate were inhibited 98.5 and 85.9%, respectively, by 600 μM tiliroside (Fig. 5D and E). These results indicate that there are little phlorizin- and phloretin-inhibitable fractions of the glucose uptake rate under tiliroside-existing condition [26]. Because phlorizin- and phloretin-inhibitable fractions of the glucose uptake rate represents SGLT1- and GLUT2-mediated glucose uptake, respectively, these findings strongly suggest that tiliroside inhibited both SGLT1- and GLUT2-mediated glucose uptake in enterocytes.

3.5 Comparison of the inhibitory effects of tiliroside against α-amylase activity and glucose uptake with those of its related compounds

In order to the structural requirements of tiliroside for the inhibitory effects against α-amylase and glucose uptake, the effects of *p*-coumaric acid, kaempferol and kaempferol-3-*O*-glucoside were compared with the effects of tiliroside (Fig. 6A). In the α-amylase activity assay, both *p*-coumaric acid kaempferol-3-*O*-glucoside inhibited α-amylase activity slightly, but the inhibitory effects were much weaker than the effects of tiliroside (Fig. 6B). In contrast, kaempferol showed approximately the same inhibitory effect ($IC_{50} = 0.22$ mM) against α-amylase activity as tiliroside ($IC_{50} = 0.28$ mM) (Fig. 6B). In the glucose uptake assay, glucose uptake was barely inhibited by *p*-coumaric acid at concentrations of 60–600 μM and was slightly inhibited by kaempferol and kaempferol-3-*O*-glucoside (44.3 and 44.4%, respectively) at a concentration of 600 μM (Fig. 6C). However, the inhibitory effects of these compounds were much weaker than the effects of tiliroside (87.6% inhibition at 600 μM) (Fig. 6C). These results suggest that the structural requirements of tiliroside for the inhibitory effects against α-amylase and glucose uptake were not completely consistent.

4 Discussion

Obesity and its associated disorders are major noncommunicable public health problems of the 21st century. Studies

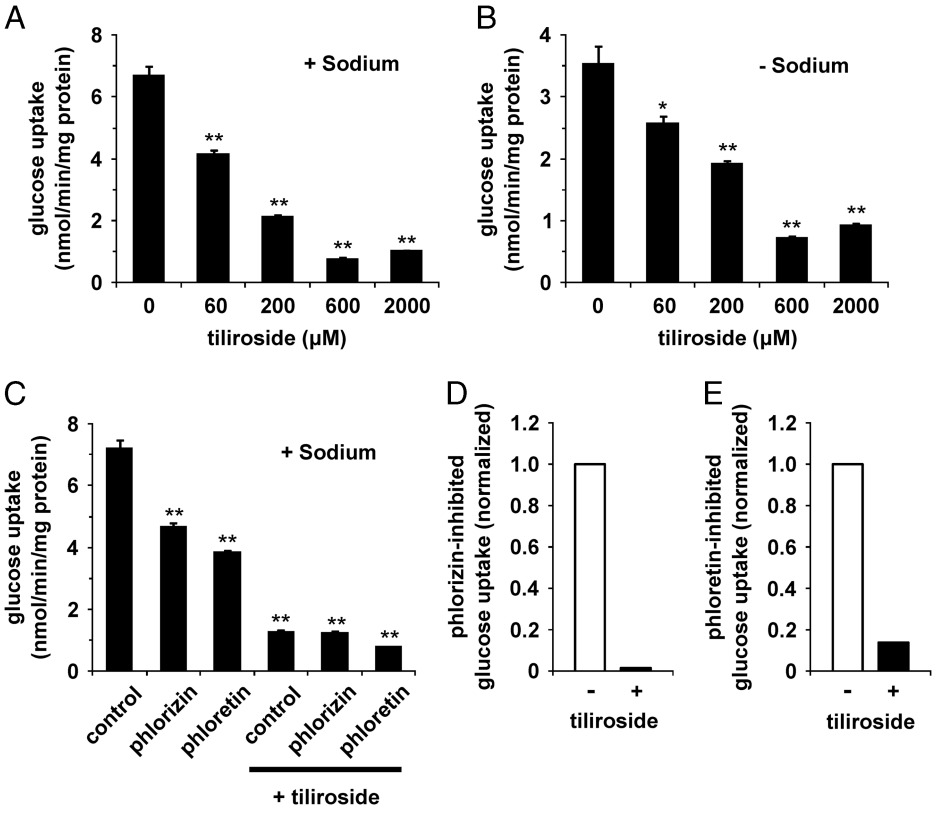

Figure 5. Inhibitory effects of tiliroside against SGLT1- and GLUT2-mediated glucose uptake in Caco-2 cells. Glucose uptake was measured for 10 min in Caco-2 cells treated under sodium-containing (A, C–E) and sodium-free (B) conditions with 1 mM glucose and 0.5 μCi/mL D-[U-^{14}C] glucose in the presence or absence of the indicated compounds. (A) and (B) Dose-response effect of tiliroside on glucose uptake in Caco-2 cells under sodium-containing (A) and sodium-free (B) conditions. (C)–(E) Effect of SGLT1 (500 μM phlorizin) and GLUT2 (100 μM phloretin) inhibitors on glucose uptake in Caco-2 cells in the presence or absence of 600 μM tiliroside (C). Effect of 600 μM tiliroside on phlorizin-inhibited (500 μM) (D) and phloretin-inhibited (100 μM) (E) glucose uptake in (C). The values are mean ± SEM of 3–4 tests. $^{*}p<0.05$, $^{**}p<0.01$ compared with the vehicle control group.

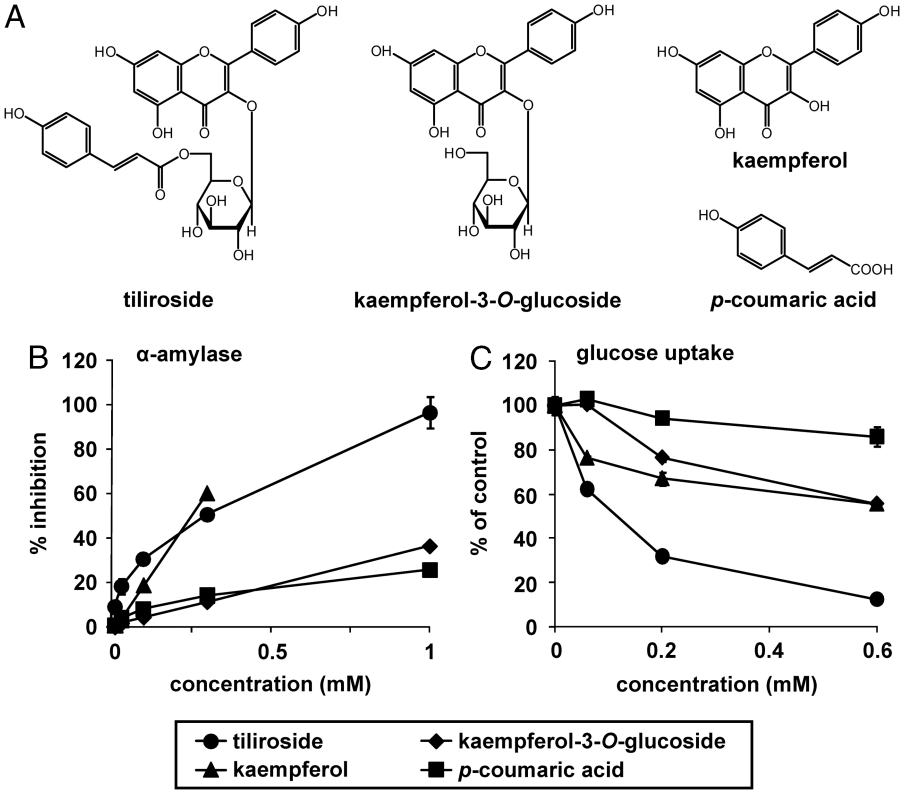

Figure 6. Comparison of the inhibitory effects of tiliroside against α-amylase activity and glucose uptake with those of related compounds. (A) Chemical structures of tiliroside and related compounds (*p*-coumaric acid, kaempferol, and kaempferol-3-*O*-glucoside). (B) and (C) Inhibitory effects of tiliroside and related compounds against pancreatic α-amylase activity (B) and glucose uptake in Caco-2 cells (under sodium-containing condition) (C). α-Amylase activity and glucose uptake were determined as mentioned in Figs. 1 and 5, respectively. The values are mean ± SEM of 3–4 tests.

indicate that high levels of body fat are associated with an increased risk of developing numerous adverse health conditions [27, 28]. Thus, effective therapeutic approaches to obesity and obesity-induced metabolic syndrome are currently of general interest. Previous reports have raised the possibility that several flavonoids, such as catechines and anthocyanins, are useful dietary compounds for managing obesity and obesity-induced metabolic syndrome [13, 14, 29, 30]. In the present study, we examined the effects of the glycosidic flavonoid, tiliroside, which has been reported to improve adiposity and obesity-associated metabolic abnormalities [21, 23], on carbohydrate digestion and glucose absorption in the gastrointestinal tract. Tiliroside treatment inhibited both α-amylase activity and glucose uptake in enterocytes, leading to the suppression of postprandial hyperglycemia and hyperinsulinemia. Because high postprandial plasma glucose and insulin concentrations are associated with an increased risk of developing type 2 diabetes and metabolic syndrome [3, 4, 31], tiliroside may be a novel dietary flavonoid useful for suppressing the development of obesity-induced metabolic diseases.

In this study, tiliroside inhibited porcine pancreatic α-amylase activity more potently than α-glucosidase activity. In previous reports, Kim et al. investigated the effects of various flavonoids and glycosidic flavonoids on pancreatic α-amylase activity [15]. Compared with the results of that report, the inhibitory effects of tiliroside against α-amylase activity seemed to be relatively potent among those flavonoids and glycosidic flavonoids. The type of inhibition and the inhibitor constant for tiliroside were estimated by kinetic analysis. A noncompetitive inhibition pattern was suggested from the Dixon plot, but mixed inhibition may still be possible because the K_m value was slightly increased in proportion with the tiliroside concentration. Previous reports showing that several flavonoids decrease α-amylase activity through mixed inhibition also support our results [16].

Although tiliroside administration inhibited hyperglycemia induced by both oral starch and glucose administration, the degree of reduction in the AUC for glucose response of the tiliroside-administered group (600 mg/kg) was much larger in the oral starch tolerance test (31%) than in the oral glucose tolerance test (13%). Therefore, the inhibition of α-amylase activity seemed to be important for the inhibitory effect of tiliroside against postprandial hyperglycemia. Chronic tiliroside administration ameliorated obesity-induced metabolic disorders through the activation of adiponectin signaling, which was followed by enhanced fatty acid oxidation in liver and skeletal muscle in obese diabetic mice [23]. Adiponectin is an adipocytokine reported to have anti-diabetic, anti-insulin resistance, and anti-atherosclerotic effects [32–34]. Acarbose, a synthetic α-amylase and α-glucosidase inhibitor reportedly increased serum total adiponectin levels in patients with type 2 diabetes [35]. These studies raise the possibility that the inhibitory effect of tiliroside against α-amylase activity plays an important role in the activation of adiponectin signaling followed by improvement of obesity-associated metabolic disorders.

Intestinal sugar absorption is increased in experimental diabetes [36–38]. Before a meal, glucose concentration in the lumen is much lower than that in plasma. Any glucose is rapidly captured by SGLT1, which is ideal because SGLT1 is a low-capacity, high-affinity transporter and is the only transporter capable of moving glucose against a concentration gradient [7, 8]. GLUT2 is a high-capacity, low-affinity facilitative transporter that equilibrates glucose between plasma and enterocytes [7, 8]. As the concentration of free glucose increases, initial transport across the apical membrane occurs through SGLT1, causing activation of protein kinase C βII. These events result in rapid ($t_{1/2}$∼3.5 min) activation of apical GLUT2 already in the membrane and further insertion of GLUT2 into the apical membrane from underlying intracellular vesicles [39]. The present study suggests that tiliroside treatment inhibits both SGLT1- and GLUT2-mediated glucose uptake in Caco-2 cells, in which both glucose transporters are reportedly to expressed abundantly [40]. In our assay (1 mM glucose), tiliroside inhibited glucose uptake more potently under the sodium-containing condition (IC_{50} = 97 μM) than under the sodium-free condition (IC_{50} = 240 μM). These results indicate that tiliroside inhibited SGLT1-mediated glucose uptake more potently than GLUT2-mediated glucose uptake. However, the expression levels of GLUT2 in apical membranes increase with glucose concentration, whereas those of SGLT1 are unchanged [39]. The exact local concentration of glucose is, of course, unknown, but estimates range from 50 to 300 mM after a meal [41, 42]. Thus, the IC_{50} value for glucose uptake inhibited by tiliroside may vary according to the assay condition, such as glucose concentration. In addition to the acute inhibition of SGLT1- and GLUT2-mediated glucose uptake, chronic administration of tiliroside might suppress the expression levels of glucose transporters. There are few reports about the effects of flavonoids on the expression levels of glucose transporters in the small intestine, but chronic administration of phloridzin (for 2–3 wk), a kind of flavonoids, has been reported to down-regulate SGLT1 mRNA expression in the jejunum of mice fed a high-salt diet [43], and in the small intestine of streptozotocin-induced diabetic mice [44].

Flavonoid glycosides generally are believed to be hydrolyzed to aglycones before being absorbed [45, 46]. Most previous studies have discussed aglycone-type flavonoids [47–51]; the inhibitory effect of a flavonoid complex like tiliroside against α-amylase has rarely been reported. However, recent studies reported the detection of intact flavonoid glycosides in human or rat plasma [52, 54], indicating that some flavonoid glycosides may be absorbed before hydrolysis. Although the absorption mechanism of tiliroside has not been clarified, in our preliminary experiment, unchanged tiliroside in peripheral blood was nearly undetectable after oral tiliroside administration. This result indicates that specific bioactivities of tiliroside are exerted in

the gastrointestinal tract or that metabolites derived from tiliroside play an important role in tiliroside function. In this study, we focused on the function of tiliroside in the gastrointestinal tract. The inhibitory effect of kaempferol, a moiety of tiliroside, against α-amylase activity, was same as that of tiliroside. Kaempferol is a popular flavonol found in foods (e.g. tea, broccoli, cabbage, kale, beans, endive, leek, tomato, strawberries and grapes) and in medicinal plants (e.g. leaves of *Ginkgo biloba*) [55]. Our results prove that the flavonoid frame plays an important role in the inhibitory effect of α-amylase activity. However, the inhibitory effects of kaempferol- 3-*O*-glucoside were much weaker than the inhibitory effect of tiliroside. The, *p*-coumaric acid moiety seems to play an important role in the α-amylase inhibitory activity of tiliroside, although *p*-coumaric acid itself barely inhibits α-amylase activity. In addition to α-amylase inhibitory activity, the *p*-coumaric acid moiety seems to be important for the inhibitory effect of tiliroside on glucose uptake in enterocytes. In our glucose uptake assay, the inhibitory effect of kaempferol and kaempferol-3-*O*-glucoside was much weaker than that of tiliroside. Matsuda et al. showed that kaempferol-3-*O*-glucoside moiety was essential for the hepatoprotective activity of tiliroside, and the *p*-coumaroyl moiety enhanced that activity [20]. These results indicate that, *p*-coumaric acid moiety is essential for the specific bioactivities of tiliroside. Structure analyses of flavonols and amylase by computational ligand docking showed that the inhibitory activity of flavonols and flavones depends on (i) hydrogen bonds between the hydroxyl groups of the polyphenol ligands and the catalytic residues of the binding site and (ii) a conformation that stabilizes the interaction with the active site. [56]. Because the especially unsaturated center ring, -OH, and -CO of the center ring enhance the inhibitory activity of flavonoids [15, 16], the high inhibitory activity of tiliroside is consistent with previous reports.

In conclusion, the present study indicates that tiliroside, a glycosidic flavonoid, inhibited both pancreatic α-amylase-mediated carbohydrate digestion and SGLT1- and GLUT2-mediated glucose uptake in enterocytes. These effects may contribute to its anti-diabetic activity, at least partially, suggesting that tiliroside may be a candidate agent for managing postprandial hyperglycemia.

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