Triterpene alcohols and sterols from rice bran lower postprandial glucose-dependent insulinotropic polypeptide release and prevent diet-induced obesity in mice

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Fukuoka D, Okahara F, Hashizume K, Yanagawa K, Osaki N, Shimotoyodome A. Triterpene alcohols and sterols from rice bran lower postprandial glucose-dependent insulinotropic polypeptide (GIP) levels. GIP is a gut hormone that is secreted from enteroendocrine K cells, which are predominantly located in the intestinal epithelium of the proximal duodenum (42). The secretion of GIP is stimulated by the presence of secretagogues such as dietary fat, carbohydrates, and proteins to the luminal surface of K cells (42). The primary action of GIP is to potentiate glucose-stimulated insulin secretion from pancreatic β-cells. In addition to pancreatic β-cells as the classical target of GIP, the GIP receptor (GIPR) is located on various extrapancreatic organs, including adipose tissue (34). Previous studies demonstrated that GIP mediated various anabolic effects on adipocytes, including the stimulation of glucose uptake (13, 39), lipoprotein lipase (LPL) activity (19–21), and fatty acid synthesis (29). Furthermore, recent studies in mice implicated GIP in the regulation of energy metabolism and subsequent obesity-related phenotypes (11, 23, 24, 25). The prevention of high-fat diet-induced obesity accompanied by increased fat utilization has been observed in genetically GIPR-ablated mice (24). Furthermore, a daily injection of the specific GIPR antagonist, (Pro3)GIP, to high-fat diet-fed obese mice resulted in a decrease in body weight and adipose tissue mass accompanied by improvements in insulin sensitivity and the normalization of glucose tolerance (11, 23).

Not only the inhibition of GIP signaling but also a decrease in the secretion of GIP have been shown to prevent diet-induced obesity. The genetic ablation of GIP-secreting K cells enhanced energy expenditure and prevented high-fat diet-induced obesity in mice (3). Previous studies also revealed that the administration of dietary components that lowered postprandial blood GIP levels, such as diacylglycerol (35), 1-monooolein (36), and RS4-type resistant starch (37) increased fat catabolism and prevented high-fat diet-induced obesity in mice. Postprandial fat utilization was higher with the intake of 1-monooolein (36), and RS4-type resistant starch (37) increased fat catabolism and prevented high-fat diet-induced obesity in mice. Postprandial fat utilization was higher with the intake of either diacylglycerol (35) or RS4 (37) than with its equivalent control oil or carbohydrate in humans. These findings indicated that reducing postprandial GIP levels may contribute to the attenuation of obesity due to enhanced fat utilization and reduced anabolic signaling on adipocytes. Accordingly, we have examined food components that regulate the postprandial secretion of GIP.

Rice is consumed as a staple food in Asian countries. Unpolished brown rice contains various nutrients in its bran and germ layer, all of which are removed in the process of...
producing polished white rice. In modern lifestyles, white rice is preferably eaten over brown rice because of its mild flavor and soft texture. Nevertheless, due to the recent increase in the rate of obesity and relevant diseases, brown rice and its components have started to attract a lot of attention as a natural food that is considered to be beneficial for health. Rice bran contains various nutrient components such as minerals, fibers, fatty acids, and oil-soluble nutraceuticals (5). Triterpene alcohols and plant sterols, as well as their ferulic acid esters (γ-oryzanol), are characteristic components of rice bran oil, which has been shown to exhibit several physiological actions, such as hypcholesterolemic (33), antioxidant (17), anti-inflammatory (2), and anticarcinogenic (44) effects. Even though rice bran oil has been a research focus for its physiological actions and application to healthy foods, little is known about the effects of fat-soluble rice bran components on energy metabolism and obesity.

Therefore, we examined the effects of fat-soluble components from rice bran on postprandial GIP secretion and high-fat diet-induced obesity in C57BL/6J mice to determine their beneficial effects on energy metabolism.

**MATERIALS AND METHODS**

**Materials.** All reagents for experiments were purchased from Sigma-Aldrich Japan (Tokyo, Japan), unless otherwise stated. A commercially available triterpene alcohol and sterol preparation (TASP) of food grade was purchased from Oryza Oil & Fat Chemical (Oryza Triterpenoid-P Lot B-702, Aichi, Japan). The composition of TASP was determined by gas chromatography. The triterpene alcohol and sterol composition in TASP was cycloartenol (CA)/24-methylene cycloartanol (24Me)/β-sitosterol (SS)/campesterol (CS) = 19/33/13/16 (%). Triterpene alcohols (CA and 24Me) or their ferulic acid esters [cycloartenol ferulate (CA-F) and 24-methylene cycloartenol ferulate (24Me-F)] were purified from TASP or γ-oryzanol (Tsuno Rice Fine Chemicals, Wakayama, Japan) by silica gel and octadecylsilyl HPLC column chromatography. The oleic acid esters [cycloartenol oleate (CA-O) and 24-methylene cycloartenol oleate (24Me-O)] were synthesized from triterpene alcohols and oleoyl chloride. SS and CS were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sulfo-N-succinimidyl oleate (SSO), an inhibitor of FAT/CD36 and CS, was purchased from Sigma-Aldrich Japan (Tokyo, Japan), unless otherwise stated. A commercial animal diet containing [3H]oleate (cat. no. NET289, 0.14 Ci/ml; PerkinElmer, Tokyo, Japan) was used in the present study.

**Animals.** Male C57BL/6J mice (7–10 wk old; CLEA Japan, Tokyo, Japan) were housed at four cages in plastic cages. Animals were fed a standard chow diet consisting of 3.47 kcal/g, with 4.6% fat, 51.4% carbohydrate, and 24.9% protein (CE-2; CLEA Japan). The composition of the commercially available triterpene alcohol and sterol preparation was based on our previous study (26). A dome-type semipurified powder diet (Table 1) was used in the present study. Food and water were provided ad libitum in a room maintained at 23°C, with a relative humidity of 55 ± 10% and a daily photoperiod from 0700 to 1900. All animals were housed for 1–2 wk before use. All animal experiments were conducted in the Experimental Animal Facility of Kao Tochigi Institute. The Animal Care Committee of Kao Tochigi Institute approved the present study. All experiments strictly followed the guidelines of this committee. The handling of animals, administration of drugs, tissue sampling, and euthanasia were monitored by officially qualified animal care personnel.

**Oral carbohydrate and fat load study.** The effects of the major triterpene alcohols and sterols, and their esters from rice bran on the postprandial GIP response was examined in an oral carbohydrate and fat load study using C57BL/6J mice. Mice were assigned to nine groups (n = 16 in each group): carbohydrate plus fat (control) and controls with CA, CA-O, CA-F, 24Me, 24Me-O, 24Me-F, SS, or CS (175 μM, respectively). Starch (Amioka; National Starch, Tokyo, Japan) was gelatinized in distilled H2O by an autoclave treatment (121°C, 2 atm for 20 min) and used as a carbohydrate. Glyceryl trioleate (TO) was used as fat. Lecithin [from egg yolk, 0.04 mg/g body weight (BW)], 0.2% in administered samples; Kanto Chemical, Tokyo, Japan] and albumin (0.2 mg/g BW, 1% in administered samples) were included in all test solutions, which were subsequently sonicated three times for 60 sec with a 1-min interval of cooling on ice to obtain stable emulsions (Sonifier 450, Branson Ultrasonics, Danbury, CT). The administration volume was adjusted to 20 mg/g BW. Overnight-fasted mice were anesthetized through the inhalation of isoflurane (Abbott Laboratories, North Chicago, IL) and gastrically administered 2 mg/g BW of carbohydrate and 1 mg/g BW of fat with or without triterpene alcohols, sterols, or their esters.

To examine the dose-dependence of CA, mice were assigned to five groups (n = 8 in each group): carbohydrate alone and carbohydrate plus fat without (control) or with CA (0.5, 2.5, and 12.5 μg/g BW). Boiled Japonica white rice (Koshikihari) was homogenized with a handy micro homogenizer (Physcion SN-310E, Microtec, Chiba, Japan) and used as carbohydrate. TO was used as fat. Lecithin (0.08 mg/g BW, 0.2% in administered samples) was included in all test solutions, which were subsequently sonicated as described above to obtain stable emulsions. The administration volume was adjusted to 20 mg/g BW. Overnight-fasted mice were anesthetized through the inhalation of isoflurane and gastrically administered 2 mg/g BW of carbohydrate and 2 mg/g BW of fat with or without CA.

In the fat load study, mice were assigned to three groups (n = 8 in each group): fat alone (control) and fat with CA (2.5 and 5 μg/g BW). TO was used as fat. Lecithin (0.08 mg/g BW, 0.2% in administered samples) was included in all test solutions, which were subsequently sonicated as described above to obtain stable emulsions. The administration volume was adjusted to 20 mg/g BW. Overnight-fasted mice were anesthetized through the inhalation of isoflurane and gastrically administered 2 mg/g BW of fat with or without CA.

Blood samples were collected from the orbital sinus under anesthesia with isoflurane inhalation immediately before and at the indicated times after gastric gavage with a heparinized capillary tube (75-mm length) (Drummond Scientific, Broomall, PA). Blood samples were kept on ice until plasma preparation. After centrifugation at 3,500 g for 5 min at 4°C, plasma was stored at −80°C until analysis.

**In vitro fatty acid uptake study with HuTu-80 cells.** The human intestinal epithelial cell line, HuTu-80 cells (Cell Lines Service, Eppelheim, Germany) transfected with human SGLT-1 (36), was used in the present study. Cells were seeded into 12-well plates at 5 × 10^4 cells/well and grown to 95–100% confluence. The cells were then cultured in glucose- and FBS-free DMEM for 1 h before oleate uptake. Cells were washed twice with Dulbecco’s PBS (DPBS) (GIBCO, Invitrogen, Carlsbad, CA) and then incubated in DPBS containing CaCl2 (1.8 mM) and MgCl2 (1 mM) (PBS (+)) buffer with or without 0.5 mM SSO for 1 h at 37°C. To determine the effects of CA on oleate uptake, cells were incubated in PBS (+) buffer containing [1H]oleate (cat. no. NET289, 0.14 μM, 1 μCi/ml; PerkinElmer, Tokyo, Japan), 50 μM oleate, 1% TO, and 0.2% BSA with or without 2 mM of CA for 30 min at 37°C. The cells were then washed three times with DPBS and solubilized with 0.5 ml of 0.1 N NaOH. A total of 10 ml of Scintillation Cocktail (Hionic-Fluor, PerkinElmer) was added to the resultant cell lysate. The radioactivity of[^H] in the cell lysate was measured using an automatic liquid scintillation spectrometer (Tri-Carb 2550 TR/LL, Packard, Rungis, France).

**High-Fat diet-induced obesity.** Mice (8 wk old) were assigned to each of the indicated groups (n = 12) (Table 1). Average body weights were adjusted among each group. Each group had free access to the semipurified powder diet (Table 1). The composition of the respective diets was based on our previous study (26). A dome-type cover on the feeding dish (Rodent Cafe, Oriental Yeast, Tokyo, Japan) was used to avoid scattering of the powdered diet in the cage. The energy values for each diet were calculated from the macronutrient composition using values of 4, 4, and 9 kcal/g for carbohydrate, protein, and fat, respectively.

Mice were maintained for 23 wk. Individual body weights were monitored weekly. Food intake was measured on a per cage basis.
Table 1. Composition of the semipurified experimental diet

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LF, low-fat diet; HF, high-fat diet; TASP, triterpene alcohol and sterol prepa-
ration. a1 The TASP composition of triterpene alcohols and sterols was CA/24Me/SS/CS/ in each group), high-fat diet supplemented with 0.5% TASP. Each group had free access to the diet in Roden Cafe. Data were collected con-
sequently for 39 h with a settling time of 15 sec and a measuring time of
throughout the study every 3 or 4 days. Food intake was determined by subtracting the remaining food weight from the initial food weight on the previous feeding day. Energy intake was calculated from the food intake and macronutrient composition of each diet. Under non-
fasting conditions, mice were anesthetized by the inhalation of iso-
flurane. Blood samples were collected from the abdominal vein into capillary blood collection tubes (Capiject with EDTA-2Na; Terumo Medical, Tokyo, Japan) and maintained on ice until the preparation of plasma. After centrifugation at 3,500 g for 10 min at 4°C, plasma samples were stored at −80°C until analysis. White adipose tissue (WAT) (epidymal, mesenteric, retroperitoneal, and perirenal), skel-
etal muscle (soleus and gastrocnemius), and the liver were removed and weighed. Total liver lipids were extracted by a previously de-
scribed procedure (9), then dried under a stream of nitrogen and re-
dissolved in 2-propanol/Triton X-100 (9:1) solution. Liver triglyc-
erides (TG) content was determined by spectrophotometric methods
(Triglyceride E-test; Wako Pure Chemical Industries).

Indirect calorimetry. Respiratory metabolic performance in the
sedentary condition was measured with an indirect calorimetric sys-
tem equipped with a sixteen-chamber airtight metabolic cage (Arco
System, Chiba, Japan). Airflow through the metabolic
cage was adjusted to 20 mg/g BW. Overnight-fasted mice
were anesthetized through the inhalation of isoflurane and adminis-
tered 2 mg/g BW of carbohydrate and 2 mg/g BW of fat, added with
50 μg/g BW of TASP. The collection of blood from the abdominal vein was performed as described above, immediately before and at the
indicated times after the administration to acquire plasma samples. The liver was removed and weighed, then stored at −80°C until analysis.

CA and 24Me concentrations in the plasma and liver were deter-
mined using a liquid chromatography-electrospray ionization-tandem
mass spectrometer (LC-ESI-MS/MS). Purified CA or 24Me was used as
a standard. Cholesterol-d7 (Avanti Polar Lipids, Alabaster, AL) was used as an internal standard. To prepare plasma samples for
LC-ESI-MS/MS, 100 μl of a plasma sample, 100 μl of cholesterol-d7
(100 ng/ml), 400 μl of methanol (Kanto Chemical), 500 μl of chloroform, and 200 μl ultrapure water (Milli-Q, Millipore, Tokyo, Japan) were added to a polypropylene tube and vortexed briefly. The mixture was then centrifuged at 4,500 g for 10 min at 5°C. The chloroform phase was collected and evaporated under a stream of nitrogen. The remaining residue was dissolved in 3 ml of 80% methanol, then loaded onto a silica gel cartridge column (InertSep
C18, GL Science, Torrance, CA). The cartridge was rinsed with 80% ethanol and eluted with 2 ml of methanol/acetonitrile (2:1, v/v). After evaporation of the solvent under a stream of nitrogen, the residue was reconstituted in 1 ml of 2-fluoro-1-methylpyridinium
p-toluenesulfonate [0.5% in acetonitrile (v/v); Tokyo Chemical Industry, Tokyo, Japan] and 50 μl of triethylamine (Wako Pure Chemical Industries), then incubated for 1 h at 80°C. After evaporation under a stream of nitrogen, the residue was reconstituted in 0.1 ml of the mobile phase [0.5% formic acid/60% acetonitrile in water (v/v/v)]. To prepare liver samples, whole liver, 100 μl of cholesterol-d7 (100 ng/ml), and 2.4 ml of methanol were added to polypropylene tubes and homogenized. A total of 2.5 ml of chloroform and 1 ml of ultrapure water were added to the homogenate, which was vortexed briefly, then centrifuged at 4,500 g for 10 min at 5°C. After the chloroform phase was collected, the rest of the procedure was carried out as described above for plasma samples. Ten microliters of each
prepared sample was used for LC-ESI-MS/MS analysis. The LC condi-
tions were as follows: LC system, UltiMate 3000 system (Di-
noxon, Germering, Germany); Column, Inertsil ODS-3 (2.1 mm × 150
mm, 3 μm; GL Science); column temperature = 40°C; Mobile phase, 0.5% formic acid/60% acetonitrile in water (v/v/v); flow rate = 0.2
ml/min; auto sampler temperature = 5°C; total runtime = 25 min per
sample. The MS/MS conditions were as follows: MS system, QTRAP
5500 (AB SCIEX, Toronto, Canada); ionization, ESI positive; analy-
sis mode, MRM; CAD = 7; CUR = 50 ps; GS1 = 50 ps; GS2 = 50 ps; IS = 5,500 V.

In vitro gene expression study with HepG2 cells. The human
hepatocellular liver carcinoma cell line (HepG2) was purchased from
the American Type Culture Collection (Manassas, VA). Cells were
cultured in DMEM supplemented with 10% FBS and 1% antibiotics.
Cells were then seeded in 12-well plates at a density of 5 × 10^4
cells/well, then incubated to reach subconfluence (70–80%). After an
overnight incubation with serum-free DMEM, the cells were washed
twice with DPBS, then DMEM containing 10% FBS, 1% antibiotics,
and 0.5% DMSO with or without 7 μM of CA or 24Me was added and
cells were incubated at 37°C. After the 24-h incubation, the cells were
washed three times with DPBS and lysed with a lysis solution
(RNeasy Mini Kit, Qiagen, Tokyo, Japan). Total RNA was then
extracted as described below for plasma samples. Ten microliters of each
prepared sample was used for LC-ESI-MS/MS analysis. The LC condi-
tions were as follows: LC system, UltiMate 3000 system (Di-
noxon, Germering, Germany); Column, Inertsil ODS-3 (2.1 mm × 150
mm, 3 μm; GL Science); column temperature = 40°C; Mobile phase, 0.5% formic acid/60% acetonitrile in water (v/v/v); flow rate = 0.2
ml/min; auto sampler temperature = 5°C; total runtime = 25 min per
sample. The MS/MS conditions were as follows: MS system, QTRAP
5500 (AB SCIEX, Toronto, Canada); ionization, ESI positive; analy-
sis mode, MRM; CAD = 7; CUR = 50 ps; GS1 = 50 ps; GS2 = 50 ps; IS = 5,500 V.

Plasma analysis. Plasma insulin was determined with a Rat/Mouse
Insulin ELISA kit and rat insulin as standard (Morinaga Institute of
Biological Science, Kanagawa, Japan). Plasma total GIP and leptin
were measured with a Milliplex Kit for mouse gut hormones (Mouse
Gut Hormone Milliplex Kit, Millipore). Plasma total cholesterol
(T-Chol), TG, and nonesterified fatty acids (NEFA) were determined
by spectrophotometric methods [N-assay L T-CHO-H (Nitto, To-
kyo, Japan), Triglyceride E-test, and NEFA-C test (Wako Pure Chem-
ical Industries), respectively]. Blood glucose was determined with a
blood glucose self-monitoring device (Accu-Chek Aviva, Roche Di-
agnostics, Tokyo, Japan) immediately after blood collection.

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Quantitative RT-PCR. Total RNA was extracted from the mouse liver, skeletal muscle, and HepG2 cells with the RNaseq Mini Kit (Qiagen) according to the manufacturer’s instructions. Total RNA was extracted from mouse WAT with the RNaseq Lipid Mini kit (Qiagen) according to the manufacturer’s instructions. cDNA was produced from 1 μg of total RNA with the SuperScript First-Strand Synthesis System for RT-PCRs (Invitrogen). Quantitative real-time PCR was performed with the Applied Biosystems 7500 Real-Time PCR System and TaqMan Gene Expression Assay ( Applied Biosystems, Tokyo, Japan) according to the manufacturer’s instructions. A predesigned primer and fluorescent probe sets (Table 2) were purchased from Applied Biosystems. The same amount of total RNA was consistently used for each expression analysis to ensure quantitative precision, and the expression level of each gene was normalized to the expression of a housekeeping gene, ribosomal protein, large, P0 (Rplp0/36B4).

Statistical analysis. Numerical data are expressed as means ± SE. Statistical analysis was conducted with the Student’s t-test or one-way ANOVA followed by Dunnett’s post hoc test. The Jonckheere trend test (18) was conducted to evaluate dose dependencies. Data analyses were performed using the statistical software StatView for Windows (version 5.0, SAS Institute, Cary, NC) and R (version 3.0.2, R Foundation for Statistical Computing, Vienna, Austria). Differences were considered significant when the error probability was smaller than 0.05.

RESULTS

Triterpene alcohols and sterols decreased the diet-induced GIP response in C57BL/6J mice. After the carbohydrate and fat had been administered by gavage to overnight-fasted mice, plasma GIP levels peaked by 10 min and thereafter declined, returning close to baseline levels after 120 min (Fig. 1A). The coadministration of CA significantly decreased the elevations in plasma GIP levels observed at 10 min and 30 min (Fig. 1A). CA, but not its oleic acid or ferulic acid ester, significantly decreased the postprandial GIP response [maximum concentration (Cmax)] 10 min after gastric gavage[1] in mice after the gastric gavage of carbohydrate and fat (CA, CA-O, and CA-F, respectively; Fig. 1B). 24Me as well as its oleic acid and ferulic acid ester slightly decreased the postprandial GIP response (24Me, 24Me-O, and 24Me-F, respectively; Fig. 1B). SS and CS also decreased the postprandial GIP response in mice (SS and CS, respectively; Fig. 1B).

CA decreased carbohydrate plus fat-induced postprandial GIP response in mice. After the administration of carbohydrate plus fat by gavage to overnight-fasted mice, plasma GIP levels at 10 min, 30 min, 60 min, and the plasma GIP response for 60 min [area under the curve (AUC) GIP 60 min] were significantly higher than those after the administration of carbohydrate alone (Fig. 2, A and B). However, the coadministration of CA decreased the fat-promoted plasma GIP response (AUC) in a dose-dependent manner (P < 0.05, by the Jonckheere trend test), and a significant decrease was observed following the coadministration of 12.5 μg/g BW of CA (Fig. 2B).

CA decreased the fat-induced postprandial GIP response in mice. Plasma GIP concentrations increased 15 min after the fat emulsion was administrated by gavage to overnight-fasted mice after the administration of the carbohydrate plus fat (2 mg/g BW and 1 mg/g BW, respectively), black circles (control)] or 175 μM of cycloartenol (CA) (white circles) together with the carbohydrate plus fat (2 mg/g BW and 1 mg/g BW, respectively) by gavage to overnight-fasted C57BL/6J mice (A). The plasma response [maximum concentration (Cmax)] of GIP to 175 μM of CA, its oleic acid ester (CA-O) or ferulic acid ester (CA-F), or 24-methylene cycloartenol (24Me), its oleic acid ester (24Me-O) or ferulic acid ester (24Me-F), or β-sitosterol (SS) or campesterol (CS) together with the carbohydrate plus fat (2 mg/g BW and 1 mg/g BW, respectively) in overnight-fasted C57BL/6J mice was determined (B). Each blood sample was collected 0, 10, 30, 60, and 120 min after gavage, and Cmax was defined as the maximum plasma GIP level among the five sampled points. Cmax was equivalent to the concentration observed 10 min after gastric gavage. Data are expressed as means ± SE; n = 16 in each group; *P < 0.05, **P < 0.01, and ***P < 0.001 significantly different from the control group (one-way ANOVA followed by Dunnett’s post hoc test).

![Figure 1](http://jap.physiology.org/)  Plasma GIP elevations were determined after the administration of the carbohydrate plus fat [2 mg/g BW and 1 mg/g BW, respectively, by gavage] to overnight-fasted C57BL/6J mice (A). The plasma response [maximum concentration (Cmax)] of GIP to 175 μM of CA, its oleic acid ester (CA-O) or ferulic acid ester (CA-F), or 24-methylene cycloartenol (24Me), its oleic acid ester (24Me-O) or ferulic acid ester (24Me-F), or β-sitosterol (SS) or campesterol (CS) together with the carbohydrate plus fat (2 mg/g BW and 1 mg/g BW, respectively) in overnight-fasted C57BL/6J mice was determined (B). Each blood sample was collected 0, 10, 30, 60, and 120 min after gavage, and Cmax was defined as the maximum plasma GIP level among the five sampled points. Cmax was equivalent to the concentration observed 10 min after gastric gavage. Data are expressed as means ± SE; n = 16 in each group; *P < 0.05, **P < 0.01, and ***P < 0.001 significantly different from the control group (one-way ANOVA followed by Dunnett’s post hoc test).

![Figure 2](http://jap.physiology.org/)  Plasma GIP elevations were determined after the administration of the carbohydrate plus fat [2 mg/g BW and 1 mg/g BW, respectively, by gavage] to overnight-fasted C57BL/6J mice (A). The plasma response [maximum concentration (Cmax)] of GIP to 175 μM of CA, its oleic acid ester (CA-O) or ferulic acid ester (CA-F), or 24-methylene cycloartenol (24Me), its oleic acid ester (24Me-O) or ferulic acid ester (24Me-F), or β-sitosterol (SS) or campesterol (CS) together with the carbohydrate plus fat (2 mg/g BW and 1 mg/g BW, respectively) in overnight-fasted C57BL/6J mice was determined (B). Each blood sample was collected 0, 10, 30, 60, and 120 min after gavage, and Cmax was defined as the maximum plasma GIP level among the five sampled points. Cmax was equivalent to the concentration observed 10 min after gastric gavage. Data are expressed as means ± SE; n = 16 in each group; *P < 0.05, **P < 0.01, and ***P < 0.001 significantly different from the control group (one-way ANOVA followed by Dunnett’s post hoc test).

Table 2. TaqMan primer and probe sets used in this study

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Fig. 1. Plasma GIP elevations were determined after the administration of the carbohydrate plus fat [2 mg/g BW and 1 mg/g BW, respectively] by gavage to overnight-fasted C57BL/6J mice (A). The plasma response [maximum concentration (Cmax)] of GIP to 175 μM of CA, its oleic acid ester (CA-O) or ferulic acid ester (CA-F), or 24-methylene cycloartenol (24Me), its oleic acid ester (24Me-O) or ferulic acid ester (24Me-F), or β-sitosterol (SS) or campesterol (CS) together with the carbohydrate plus fat (2 mg/g BW and 1 mg/g BW, respectively) in overnight-fasted C57BL/6J mice was determined (B). Each blood sample was collected 0, 10, 30, 60, and 120 min after gavage, and Cmax was defined as the maximum plasma GIP level among the five sampled points. Cmax was equivalent to the concentration observed 10 min after gastric gavage. Data are expressed as means ± SE; n = 16 in each group; *P < 0.05, **P < 0.01, and ***P < 0.001 significantly different from the control group (one-way ANOVA followed by Dunnett’s post hoc test).
mice, and thereafter declined, almost reaching the baseline after 60 min (Fig. 3A). The supplementation of CA significantly decreased the fat-induced plasma GIP response for 60 min (AUC GIP 60 min) in a dose-dependent manner ($P < 0.01$, by the Jonckheere trend test; Fig. 3B).

**CA inhibited fatty uptake into HuTu-80 cells.** The cellular uptake activity of radiolabeled oleate was significantly lower in CA-treated cells than in controls (−SSO; Fig. 3C). The pretreatment of cells with the FAT/CD36 inhibitor SSO reduced the uptake activity of radiolabeled oleate, and uptake activity was further reduced by the addition of CA (+SSO; Fig. 3C).

**TASP ingestion attenuated high-fat diet-induced body weight gain and visceral fat accumulation in mice.** The body and visceral WAT weights of mice fed a HF diet for 23 wk were significantly higher than those of mice fed a low-fat diet, while those of mice fed a HF diet supplemented with 0.2, 0.5, and 1% TASP were significantly lower than those of the corresponding HF diet mice (Fig. 4, A–C). Liver weights and liver TG contents were also significantly lower in the 0.2, 0.5, and 1% TASP-fed mice than in the corresponding HF diet-fed mice (Fig. 4, D and E). Supplementing the HF diet with 0.2, 0.5, and 1% TASP significantly reduced nonfasting plasma leptin concentrations (Table 3). Supplementing the HF diet with 0.5 and 1% TASP significantly reduced nonfasting plasma T-Cho concentrations and slightly decreased plasma insulin concentrations (Table 3). No significant differences were observed in energy intakes between the TASP-supplemented and corresponding HF diet-fed groups (Fig. 4D). Dietary TASP promoted fat utilization in HF diet-fed mice. Indirect calorimetry revealed that oxygen consumption did not differ between the groups (Fig. 5A). RER was slightly lower in

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**Fig. 2. Plasma GIP concentrations (A) and areas under the curve (AUCs) (B) after the administration of carbohydrate alone (2 mg/g BW, white circles), carbohydrate plus fat [2 mg/g BW each, black circles (control)], or CA [0.5 (white squares), 2.5 (black squares), or 12.5 μg/g BW (white triangles)] together with carbohydrate plus fat (2 mg/g BW each) by gavage to overnight-fasted C57BL/6J mice were determined. Data are expressed as means ± SE; $n = 8$ in each group; *$P < 0.05$ and ***$P < 0.001$ significantly different from the control group (one-way ANOVA followed by Dunnett’s post hoc test). The $P$ value for the trend ($P_{\text{trend}}$) was calculated using the Jonckheere trend test.**

**Fig. 3. Plasma concentrations of GIP (A) and AUCs (B) after the administration of fat alone [2 mg/g BW, white circles (control)], or CA [2.5 (black circles) or 5 μg/g BW (white squares)] together with fat (2 mg/g BW) by gavage to overnight-fasted C57BL/6J mice were determined. Data are expressed as means ± SE; $n = 8$ in each group; *$P < 0.05$ and ***$P < 0.01$ significantly different from the control group (one-way ANOVA followed by Dunnett’s post hoc test). The $P_{\text{trend}}$ was calculated using the Jonckheere trend test. Effects of CA on oleate uptake in the human intestinal epithelial cell line, HuTu-80 (C). Confluent cells were pretreated with or without sulfo-N-succinimidyl oleate (SSO) for 1 h, then incubated in an oleate solution containing $[^{3}H]$oleate plus glyceryl trioleate (TO) with or without CA (2 mM) for 30 min. The amount of $[^{3}H]$oleate transported from the oleate solution was determined from $^{3}H$ radioactivity that accumulated in the cells. Data are expressed as means ± SE; $n = 3$ in each group; means that do not share a letter were significantly different (one-way ANOVA followed by Dunnett’s post hoc test).**
Fig. 4. Effects of the triterpene alcohol and sterol preparation (TASP) on time-course changes in body weights (A), final body weights (B), white adipose tissue (WAT) weights (C), liver weights (D), liver triglyceride (TG) contents (E), and energy intake (F). C57BL/6J mice were fed a low-fat (LF) diet (white circles), high-fat (HF) diet (black circles), or HF diet supplemented with 0.04% (white squares), 0.2% (black squares), 0.5% (white triangles), or 1% (black triangles) TASP for 23 wk. Data are expressed as means ± SE; n = 12 in each group; *P < 0.05, **P < 0.01, and ***P < 0.001 significantly different from the HF diet-fed group (one-way ANOVA followed by Dunnett’s post hoc test).
the TASP-fed group than in the corresponding HF diet-fed control group (Fig. 5B). While total energy expenditure during the experimental period did not differ between the groups (Fig. 5C), fat utilization was significantly higher in the TASP-fed group than in the control group (Fig. 5D). No significant differences were observed in dietary intake during the experimental period between the groups (30.42 ± 0.47 and 30.58 ± 2.34 kcal/mouse, respectively). Furthermore, no significant differences were noted in body weight gain in mice during the experimental period between the groups (0.45 ± 0.09 and 0.38 ± 0.21 g, respectively).

Dietary TASP increased fatty acid oxidation-related gene expression in the skeletal muscle, and decreased fatty acid synthesis-related gene expression in the livers of HF diet-fed mice. After a 7-wk feeding protocol, the expression levels of fatty acid oxidation-related genes, such as acyl-CoA oxidase (ACO) and carnitine palmitoyltransferase I (CPT1), in the soleus muscles were significantly or slightly higher in TASP-fed mice than in the corresponding HF diet-fed mice (Fig. 6, A and B). In contrast, no significant difference was observed in the expression of these genes in the liver between the HF diet-fed groups (Fig. 6, C and D). On the other hand, the expression levels of fatty acid synthesis-related genes, such as sterol regulatory element-binding protein-1c (SREBP-1c) and fatty acid synthase (FAS), in the liver were significantly lower in 0.5% TASP-fed mice than in the corresponding HF-fed mice (Fig. 6, E and F).

Table 3. Plasma components under nonfasting conditions in C57BL/6J mice fed the LF or HF diet, or HF diets supplemented with TASP for 23 wk

<table>
<thead>
<tr>
<th>Component</th>
<th>LF</th>
<th>0% TASP (control)</th>
<th>0.04% TASP</th>
<th>0.2% TASP</th>
<th>0.5% TASP</th>
<th>1% TASP</th>
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<tbody>
<tr>
<td>Glucose, mg/dl</td>
<td>226.0 ± 13.2</td>
<td>247.2 ± 10.1</td>
<td>272.3 ± 9.4</td>
<td>236.0 ± 10.6</td>
<td>240.7 ± 9.3</td>
<td>238.9 ± 16.4</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>2.419 ± 0.482</td>
<td>2.152 ± 0.460</td>
<td>2.532 ± 0.475</td>
<td>2.370 ± 0.189</td>
<td>1.683 ± 0.424</td>
<td>1.874 ± 0.563</td>
</tr>
<tr>
<td>Leptin, ng/ml</td>
<td>11.52 ± 1.24*</td>
<td>24.92 ± 4.98</td>
<td>22.36 ± 3.36</td>
<td>13.52 ± 2.61*</td>
<td>14.81 ± 3.28*</td>
<td>12.31 ± 2.34**</td>
</tr>
<tr>
<td>T-Chol, mg/ml</td>
<td>47.6 ± 6.2</td>
<td>52.5 ± 3.1</td>
<td>33.6 ± 4.6</td>
<td>25.1 ± 2.7</td>
<td>23.7 ± 2.7</td>
<td>32.2 ± 4.3</td>
</tr>
<tr>
<td>NEFA, mg/ml</td>
<td>0.451 ± 0.036</td>
<td>0.538 ± 0.059</td>
<td>0.747 ± 0.034</td>
<td>0.450 ± 0.041</td>
<td>0.490 ± 0.043</td>
<td>0.576 ± 0.049</td>
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Data are expressed as means ± SE; *P < 0.05, and **P < 0.01 significantly different from the control HF group (one-way ANOVA followed by Dunnett’s post hoc test).

Fig. 5. Effects of dietary TASP on energy homeostasis in mice. Oxygen consumption (VO_{2}) (A), the respiratory exchange ratio (RER) (B), energy expenditure (C), and fat utilization (D) in C57BL/6J mice in metabolic chambers for 39 h, under free access to a HF diet or HF diet supplemented with 0.5% TASP. Data are expressed as means ± SE; n = 5 in each group; *P < 0.05 significantly different between the groups; N.S., not significant (t-test).
Rice Bran Oil Components Prevent Diet-Induced Obesity

Fukuoka D et al.

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Distribution of orally administered CA and 24Me in the blood and liver. The plasma concentration of CA following the oral administration of dietary TASP increased until 6 h, then started to decline (Fig. 7A). Similar results were obtained for the hepatic concentration of CA (Fig. 7B). The plasma and hepatic concentrations of 24Me also peaked at 3 h and 6 h, respectively, then decreased (Fig. 7C and D).

CA and 24Me reduced fatty acid synthesis-related gene expression in HepG2 cells. SREBP-1c gene expression levels in HepG2 cells after a 24-h incubation were significantly lower following the addition of 7 mM of CA or 24Me to the medium than in the nonadditive control (Fig. 8). Between the two triterpene alcohols, the effects of CA were stronger than that of 24Me (Fig. 8).

DISCUSSION

The present study demonstrated that supplementation with TASP from rice bran attenuated postprandial blood GIP increases after the ingestion of macronutrients (Fig. 2) and HF diet-induced body weight gain as well as visceral fat and liver triglyceride accumulation in diet-induced obesity in mice (Fig. 4). Dietary TASP also attenuated high-fat diet-induced hyperleptinemia and hypercholesterolemia (Table 3). Since leptin is secreted by adipose tissues, blood leptin levels are associated with the accumulation of body fat (1), and hypercholesterolemia is closely related to obese phenotypes (7), the lower blood leptin and cholesterol levels observed in the present study may have been due to a reduction in the accumulation of body fat after 23 wk of ingesting TASP.

The results of indirect calorimetry suggested the underlying mechanism for the antiobesity effects of TASP; an increase in fat utilization due to supplementation with TASP may be responsible for its antiobesity activity. Previous studies indicated that GIP may contribute to the regulation of energy expenditure (3, 24, 37). We previously demonstrated that the chronic administration of GIP decreased fat utilization under HF diet-fed conditions in rodents (37). In addition, GIPR ablation increased fat utilization and prevented HF diet-induced obesity in mice (24). The ablation of GIP-secreting K cells enhanced energy expenditure and reduced HF diet-in-
Reduced obesity in mice (3). Our recent study showed that RS4-type resistant starches and 1-monoolein reduced postprandial GIP and insulin responses, resulting in an increase in fat utilization and reduced HF diet-induced obesity in mice (36, 37). The present results are consistent with the findings of previous studies and suggest that the reduction in postprandial blood GIP levels by TASP enhanced fat utilization, thereby preventing HF diet-induced obesity. Increased fat utilization due to the ingestion of TASP may have been due to a decrease in blood insulin levels. However, we previously demonstrated that the chronic administration of GIP decreased fat utilization without changing plasma insulin levels (37). Moreover, the results of the present study showed that dietary TASP did not alter nonfasting blood insulin levels in high-fat diet-fed mice. Therefore, increased fat utilization after the ingestion of TASP was not insulin-dependent.

Gene expression analysis of the skeletal muscle and liver of TASP-fed mice provided a more detailed insight into the mechanisms underlying the antiobesity effects of dietary TASP. Dietary TASP upregulated a fatty acid oxidation-related gene including MCAD (after 2 wk, data not shown) and CPT1 (after 7 wk, Fig. 6B) in the soleus muscle. These changes in skeletal muscular gene expression may have contributed to increased fat utilization due to dietary TASP. Even though GIPR expression was absent in the skeletal muscle of mice (our unpublished data), GIPR ablation increased serum adiponectin levels, which resulted in an increase in fat oxidation in skeletal muscle (27, 46). Although we did not examine adiponectin levels following the ingestion of TASP, the present results suggest that skeletal muscle may be responsible for the increased fat utilization by dietary TASP. Moreover, the fatty acid synthesis-related genes SREBP-1 and FAS were downregulated in the liver. GIPR expression was also shown to be absent in the liver (40, 41, 45). Even though the expression of both the SREBP-1c and FAS genes was previously reported to be directly regulated by insulin (28), a decrease in the expression of these genes due to the ingestion of TASP may also be insulin-independent because nonfasting blood insulin levels were not altered by the ingestion of TASP. The present results demonstrated that dietary triterpene alcohols (CA and 24Me) were absorbed into the circulation. Each triterpene alcohol decreased SREBP-1 gene expression in the human hepatocyte cell line (HepG2), which suggests that dietary triterpene alcohols directly decreased SREBP-1 levels in the liver. CA decreased hepatic SREBP-1 levels more than 24Me. After the ingestion of TASP, the concentration of CA was higher than that of 24Me in the liver. Taken together, these results indicate that CA may be the major active component that decreases hepatic SREBP-1 after the ingestion of TASP. Insulin may also be responsible for enhanced hepatic SREBP-1 and FAS gene expression (10, 31). However, other molecules regulated by insulin, such as ACO, CPT1 in the liver, and fatty acid synthesis-related genes in adipose tissue, remained unchanged. Therefore, reduced insulin levels after the ingestion of TASP may not be responsible for the changes observed in gene expression.

Previous studies reported that fat is a potent secretagogue of GIP release (8, 35). CA was shown to attenuate fat-promoted GIP secretion in mice (Fig. 3). Our previous study also demonstrated that fat-induced GIP secretion was triggered by fatty acid transport via FAT/CD36, which is located in GIP-producing K cells (36). Extracellular CA reduced fatty acid transport in FAT/CD36-expressing human enterocytes (Hutu-80). More importantly, cellular fatty acid transport was further reduced by CA, even when cells were treated with a FAT/CD36 inhibitor (SSO), which suggested that the inhibitory effect of CA on fatty acid transport may be independent of FAT/CD36. G protein-coupled fatty acid receptors, such as GPR40, 119, and 120, were recently suggested to play a role in the regulation of enterocyte hormone release (4, 14, 30). However, the molecular mechanisms responsible for the reduced release of GIP by TASP have yet to be elucidated.

Even though dietary CA reduced fatty acid transport into enterocytes, dietary supplementation with either TASP or CA did not alter postprandial blood TG increases (data not shown) or nonfasting blood TG levels (Table 3). These results suggest that the antiobesity effects of dietary supplementation with TASP were not due to a reduction in the digestibility or absorption of macronutrients.

Several studies in humans and animals clarified the beneficial effects of triterpene alcohols and their derivatives from rice bran. For example, γ-oryzanol, a mixture of the ferulic acid esters of triterpene alcohols and sterols in rice bran, was shown to have various biological effects, such as cholesterol-lowering (33), anti-inflammatory (2), anticancer (44), and antidiabetic effects (38). More recently, γ-oryzanol was reported to reduce dietary fat preferences by attenuating HF diet-induced endoplasmic reticulum stress in the hypothalamus, resulting in the amelioration of HF diet-induced obesity in mice (22). In the present study, the intake of food did not change with the TASP supplementation, which indicated that the antiobese effects of TASP were independent of eating behavior patterns.

To prevent diet-induced obesity, we focused on reducing the postprandial release of GIP. Rice bran–derived, oil-soluble TASP can be added to a wide variety of foods. Therefore, supplementing food with TASP may represent a good approach to improve diet-induced obesity. Given that rice is now a staple worldwide, the enrichment of TASP in brown rice and rice bran. For example, γ-oryzanol, a mixture of the ferulic acid esters of triterpene alcohols and sterols in rice bran, was shown to have various biological effects, such as cholesterol-lowering (33), anti-inflammatory (2), anticancer (44), and antidiabetic effects (38). More recently, γ-oryzanol was reported to reduce dietary fat preferences by attenuating HF diet-induced endoplasmic reticulum stress in the hypothalamus, resulting in the amelioration of HF diet-induced obesity in mice (22). In the present study, the intake of food did not change with the TASP supplementation, which indicated that the antiobese effects of TASP were independent of eating behavior patterns.

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bran by chemical or biological processing may be an alternative way to develop obesity-preventive foods.

In conclusion, the present study demonstrated for the first time that TASP from rice bran increased the postprandial utilization of fat and attenuated diet-induced obesity via GIP-dependent and GIP-independent mechanisms. These results are important for the further development of GIP-based therapies to treat obesity. Therefore, more detailed studies on the kinetics of GIP should be undertaken in relation to energy homeostasis in larger animals and humans.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: D.F., F.O., K.H., K.Y., N.O., and A.S. conception and design of research; D.F., F.O., K.H., and K.Y. performed experiments; D.F. and F.O. analyzed data; D.F., F.O., and A.S. interpreted results of experiments; D.F. and F.O. revised manuscript; D.F., F.O., and A.S. approved final version of manuscript.

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