RS4-type resistant starch prevents high-fat diet-induced obesity via increased hepatic fatty acid oxidation and decreased postprandial GIP in C57BL/6J mice

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RS4-type resistant starch prevents high-fat diet-induced obesity via increased hepatic fatty acid oxidation and decreased postprandial GIP in C57BL/6J mice.

The aim of this study was to investigate the effects of dietary RS4 supplementation on the development of diet-induced obesity in mice. Weight- and age-matched male C57BL/6J mice were fed for 24 wk on a high-fat diet containing unmodified starch, hydroxypropylated distarch phosphate (RS4), or HACS (RS2). Those fed the RS4 diet had significantly lower body weight and visceral fat weight than those fed either unmodified starch or the RS2 diet. Those fed the RS4 diet for 4 wk had a significantly higher hepatic fatty acid oxidation capacity and related gene expression and lower blood insulin than those fed either unmodified starch or the RS2 diet. Indirect calorimetry showed that the RS4 group exhibited higher energy expenditure and fat utilization compared with the RS2 group. When gavaged with fat (trioleate), RS4 ingested results in increased fatty acid oxidation in the liver and lowers the postprandial increase of blood glucose-related gene expression and lower blood insulin than those fed either unmodified starch or the RS2 diet.

Indirect calorimetry showed that the RS4 group exhibited higher energy expenditure and fat utilization compared with the RS2 group. When gavaged with fat (trioleate), RS4 stimulated a lower postprandial glucose-dependent insulinotropic polypeptide (GIP; incretin) response than RS2. Higher blood GIP levels induced by chronic GIP administration reduced fat utilization in high-fat diet-fed mice. In conclusion, dietary supplementation with RS4-type resistant starch attenuates high-fat diet-induced obesity more effectively than RS2 in C57BL/6J mice, which may be attributable to lower postprandial GIP and increased fat catabolism in the liver.
obtained from Nippon Starch Chemical (Osaka, Japan). Waxy corn starch (WCS) and HDP from tapioca (HPdTSP), corn (HPdCSP), and WCS (HPdWSCSP) were obtained from National Starch Food Innovation (Tokyo, Japan). A mixture of safflower, rapeseed, and perilla oil was used as the standard triglyceride (TG) oil. The fatty acid composition was 5.2% C16:0, 17.5% C18:0, 36.5% C18:1, 46.9% C18:2, 7.7% C18:3, and 2.0% others (Summit Oil Mill, Chiba, Japan).

**In Vitro Determination of Starch Availability**

Test starch (1 g) was gelatinized by autoclaving in distilled H2O (15 ml) at 120°C for 15 min. Gelatinized starch was digested according to the method of Åkerberg et al. (2) with slight modification. Briefly, an amount of product corresponding to 1 g of total starch was placed in a beaker containing 5 ml of α-amylase solution [α-amylase (EC3.2.1.1, from Bacillus subtilis), Wako Pure Chemical Industries, Osaka, Japan]. After mixing for 30 s, 1 ml of pepsin solution [pepsin (EC3.4.23.1, from porcine stomach mucosa) = 50 g/l, 2,000 FIB-U/g, Sigma-Aldrich Japan] and 10 ml of distilled H2O were also added into the beaker. The pH was adjusted to 1.5 with 2 mol/l HCl, and the samples were incubated at 37°C for 30 min. During incubation, the samples were stirred continuously.

After the pepsin incubation, 10 ml of a sodium acetate buffer (pH 5.0, 0.5 mol/l) was added and the pH was adjusted to 5.0 with 1 mol/l NaOH. The following were added to the beakers: 125 µl of a MgCl2-CaCl2 solution (0.06 mol/l MgCl2, 0.3 mol/l CaCl2), 125 µl of pancreatin (from porcine pancreas, 40 g/l), 400 µl of amyloglucosidase (EC3.2.1.3, from Aspergillus niger, 1.5 g/l), and 100 µl of isopropanol. Distilled water was then added to a final volume of 0.05 liter. The beakers were covered with double layers of Parafilm, secured with a rubber band, and incubated for 16 h at 40°C with constant stirring (100 rpm).

After incubation, an amount of 95% (vol/vol) ethanol corresponding to four times the incubation volume was preheated to 60°C and added to the samples. The RS was then allowed to precipitate for 60 min at ambient temperature and filtrated through a glass filter (type 1G2, pore size 40–50 µm, AGC Techno Glass, Chiba, Japan). The filtrates were collected for analysis of the potentially available starch fraction.

The filtrates were diluted to a concentration suitable for analysis. After centrifugation at 1,800 g, the glucose concentration was measured by the mutarotase-GOD method (the glucose C-II test WAKO, Wako Pure Chemical Industries). The possible effect of ethanol on the starch fraction was ruled out.

**Animals**

Male C57BL/6J mice (6–7 wk old, CLEA Japan, Tokyo, Japan) were housed at five per cage in plastic cages. Animals were fed a standard chow consisting of 3.47 kcal/g, with 4.6% fat, 51.4% carbohydrate, and 24.9% protein (CE-2, CLEA Japan). Food and water were provided ad libitum in a room maintained at 23 ± 2.0°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 that committee. The handling of animals, administration of drugs, tissue sampling, and euthanasia were monitored by officially qualified animal care personnel.

**In Vivo Determination of Starch Availability**

Overnight-fasted mice were anesthetized through the inhalation of diethyl ether and administered gelatinized starch [2 mg/g body weight (BW)] through a gastric tube. The administration volume was adjusted to 40 µl/g BW. Blood samples (~50 µl) were collected from the tail vein immediately before and at 30, 60, and 120 min after gastric gavage. Blood glucose was determined with a blood glucose self-monitoring device (Accu-Check Comfort, Roche Diagnostics, Tokyo, Japan) immediately after blood collection. Starch availability in vivo was assessed by calculating the incremental area under the curve (AUC) of blood glucose from 0 to 120 min after starch administration with the trapezoid rule.

**High-Fat Diet-Induced Obesity**

At the age of 8 wk, mice in groups of 10 were assigned to one of the indicated groups (Supplemental Table S1, A and B).1 Average body weight was adjusted among each group. Each group had free access to the powdered diet. A dome-type cover on the feeding dish (Rodan Cafe; Oriental Yeast) was used in order to avoid scattering of the powdered diet in the cage. The energy values for each diet were calculated from the macronutrient composition with values of 4, 4, and 9 kcal/g for carbohydrate, protein, and oil, respectively.

Mice were maintained for 24 wk, unless otherwise stated. Individual body weights and cumulative energy intakes were monitored over the experimental period. Food intake was measured on a per-cage basis throughout the study every 2 or 3 days. Food intake (g·cage−1·day−1) was determined by subtracting the remaining food weight from the initial food weight on the previous feeding day. The energy intake (kcal·mouse−1·day−1) was calculated from the food intake and the macronutrient composition of each diet. Mice were anesthetized by inhalation of sevoflurane (Sevofrane, Maruishi Pharmaceutical, Osaka, Japan). Blood samples were collected from the abdominal vein into capillary blood collection tubes (Capject with EDTA-2Na, Terumo Medical, Tokyo, Japan) and maintained on ice until plasma preparation. The epididymal, mesenteric, perirenal, and retroperitoneal white adipose tissues (WAT) and liver were removed and weighed. Blood samples were centrifuged at 3,500 g for 15 min at 4°C. Plasma samples were stored at −80°C until analysis.

**Indirect Calorimetry**

Respiratory metabolic performance in the sedentary condition was measured with an indirect calorimetric system equipped with an eight-chamber airtight metabolic cage (Oxymax Equal Flow 8 Chamber/Small Subject System, Columbus Instruments, Columbus, OH) (56). Airflow through the metabolic cage was adjusted to 0.4 l/min.

Mice were adapted in metabolic cages for 24 h (day 0) with free access to food and water. Data were collected continuously for 3 days (days 1–3) with a settling time of 30 s and a measuring time of 90 s, and the reference exchange ratio (RER) and substrate utilization were calculated from the measured values of oxygen consumption (VO2) and carbon dioxide production (VCO2) with the following equations (28):

\[
\text{RER} = \frac{\text{VCO}_2}{\text{VO}_2}; \text{carbohydrate utilization (mg·g BW}^{-1}·\text{min}^{-1}) = (4.51 \times \text{RER} - 3.18) \times \text{VO}_2; \text{fat utilization (mg·g BW}^{-1}·\text{min}^{-1}) = 1.67 \times (1 - \text{RER}) \times \text{VO}_2.
\]

**Chronic Insulin Treatment**

Mice (8 wk old) were divided into four groups: the saline-treated low-fat (saline-LF) group; the insulin-treated low-fat (insulin-LF) group; the saline-treated high-fat (saline-HF) group; and the insulin-treated high-fat (insulin-HF) group. Average body weight was adjusted among each group. Saline- or insulin-treated groups received saline or insulin (2 µg g−1 day−1), respectively, by continuous infusion from a subcutaneously implanted osmotic pump (Alzet micro-osmotic pump model 1002; Durect, Cupertino, CA). Saline-LF and insulin-LF or saline-HF and insulin-HF groups had free access to the LF or HF diet (Supplemental Table S1C), respectively. The four

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1 The online version of this article contains supplemental material.
groups of mice were maintained for 2 wk. Plasma, WAT, and liver samples were obtained in the same manner as described above. The epididymal, mesenteric, perirenal, and retroperitoneal WAT and liver were removed and weighed.

Oral Starch and Fat Loading Study

Overnight-fasted mice were anesthetized through the inhalation of diethyl ether and administered test starch (2 mg/g BW, 5% in administered samples) through a gastric tube with or without the addition of glycercyl trioleate (TO, 2 mg/g BW, 5% in administered samples). Lecithin (from egg yolk, 0.08 mg/g BW, 0.2% in administered samples, Kanto Chemical, Tokyo, Japan) was included in all test solutions containing oil, and these were subsequently sonicated three times for 60 s with a 1-min interval of cooling on ice to obtain stable emulsions (Sonifier 450, Branson Ultrasontics, Danbury, CT). We preliminarily confirmed that the minimal amount of lecithin did not affect blood glucose and insulin responses after gastric starch gavage. Accordingly, we used the starch solution as a control sample. The administration volume was adjusted to 40 μl/g BW. Blood samples (~50 μl) were collected from the orbital sinus under anesthesia with diethyl ether inhalation immediately before and at 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 11,000 rpm for 5 min (Micro Hematocrit Centrifuge model 1002, Kubota, Tokyo, Japan), plasma was stored at ~8°C until analysis.

GIP Treatment

Mice (8 wk old) were divided into two groups: the saline-treated HF (saline-HF) and the GIP-treated HF (GIP-HF) groups. Average body weight was adjusted between the groups. Saline- or GIP-treated groups received saline or GIP [10 pmol·kg⁻¹·min⁻¹ (7.2 mg·kg⁻¹·day⁻¹)], respectively, by continuous infusion from a subcutaneously implanted osmotic pump (Alzet micro-osmotic pump model 1002, Durect). Mice had free access to the HF diet for 3 days for recovery and were adapted to metabolic chambers for 24 h (day 0). Respiratory metabolic performance on the HF diet was measured by indirect calorimetry for 3 days (days 1–3).

Intestinal Preparations

Twenty-hour-fasted male Sprague-Dawley rats (8 wk old; Japan SLC, Shizuoka, Japan) were deeply anesthetized by the inhalation of diethyl ether and killed by cutting the thoracic aorta immediately before the removal of the small intestine. The jejunum was identified (10–50% length) and rinsed with 5 ml of Krebs bicarbonate buffer [pH 7.4, containing (mmol/l) 118.4 NaCl, 4.8 KCl, 19.8 NaHCO₃, 1.2 MgSO₄·7H₂O, 1.2 KH₂PO₄, and 2.5 CaCl₂·6H₂O], and everted. Segments (3 cm; 3 segments/rat; 12 rats/experiment) were cut from the proximal portion and randomized by swirling in oxygenated diethyl ether and killed by cutting the thoracic aorta immediately before and at 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 11,000 rpm for 5 min (Micro Hematocrit Centrifuge model 1002, Kubota, Tokyo, Japan), plasma was stored at ~8°C until analysis.

Glucose and Fatty Acid Transport

The functional integrity of the everted sac preparation was assessed based on its ability to accumulate radiolabeled D-glucose and oleate in the tissue and transfer it to the serosal solution in a time-dependent manner. Test starch (5%), TO (5%), lecithin (from egg yolk, 0.2%, Kanto Chemical), d-[¹⁴C]glucose (0.2 μCi/ml, CFB96; GE Healthcare UK, Chalfont St Giles, UK), and [³H]oleic acid (0.2 μCi/ml, TRK140; GE Healthcare UK) were subsequently sonicated three times for 60 s with a 1-min interval of cooling on ice to obtain stable emulsions (Sonifier 450, Branson Ultrasonics). Everted jejunal sacs were incubated in the emulsion (4 ml). Incubations were carried out for 10 min at 37°C, after which serosal solutions were recovered; the sacs were rinsed with 0.1% N-acetylcysteine (WAKO Chemical, Osaka, Japan) and 0.1% taurocholate for 20 s, followed by gentle tapping between paper towels for 10 s. The sacs were washed five times, cut from their cannulas, and transferred to preweighed glass vials. The tissue was dried (18 h, 95°C), weighed, and subjected to acid hydrolysis (0.4 ml, 11 mol/l HCl, 70°C, 15 min) followed by the addition of 3.6 ml of 0.75 mol/l Trizma base (Sigma, Poole, UK). After mixing, a sample (0.5 ml) was diluted to 1 ml with distilled water and 9 ml of cocktail (Hionic-fluor; PerkinElmer, Boston, MA) added before scintillation counting with an automatic liquid scintillation spectrometer (Tri-Carb 2500 TR/LL; Packard, Rungis, France). Serosal solutions were weighed to assess the volume and diluted to 1 ml with distilled water for scintillation counting.

Analytical Methods

Plasma content and hormone level. Plasma TG, nonesterified fatty acid (NEFA), and glucose levels were determined by spectrophotometric methods (triglyceride E-test, NEFA-C test, and Glucose CII test assay kits, respectively, Wako Pure Chemical Industries).

In the starch and fat loading studies, blood glucose was determined with a blood glucose self-monitoring device (Accu-Check Comfort, Roche Diagnostics) immediately after blood collection. Plasma insulin was determined with a Rat/Mouse Insulin ELISA Kit and rat insulin as standard (Moringa Institute of Biological Science, Yokohama, Japan). Total GIP was measured with an ELISA Kit for total GIP (Linco Research, St. Charles, MO).

Fatty acid oxidation. The hepatic fatty acid oxidative capacity was determined in the liver homogenate as reported previously (39). Briefly, frozen mouse liver was thawed and homogenized with a Micro Multi Mixer (Ieda Trading, Tokyo, Japan) on ice with 1 ml of 250 mmol/l sucrose containing 1 mmol/l EDTA and 10 mmol/l HEPES (pH 7.2) and centrifuged at 600 g for 5 min. The resultant supernatant was used for assay. We preliminarily confirmed that the fatty acid oxidation activity of the tissue homogenate was not transferred into the precipitate in this preparation.

The reaction mixture contained (in mmol/l) 50 Tris-HCl (pH 8.0), 40 NaCl, 2 KCl, 2 MgCl₂, 1 dithiothreitol, 5 ATP, 0.2 ³-t-carnitine, 0.2 NAD⁺, 0.06 FAD, 0.12 CoA, and 3 α-cyclodextrin, with 0.1 μCi of [¹⁴C]palmitic acid, and the extract contained 10 μg of protein in a final volume of 200 μl. Protein concentrations were determined with Cytoskeleton Advanced Protein Assay Reagent (Cytoskeleton, Denver, CO).

The reaction was started by adding the tissue extract and incubating the precipitate at 37°C for 20 min. The reaction was terminated by adding 200 μl of 0.6 mol/l perchloric acid, followed by centrifugation. The supernatant was extracted three times with 1 ml of n-hexane to remove residual radiolabeled palmitate. The ³⁵Cl radioactivity of the aqueous phase was measured with a liquid scintillation counter (2550 TR/LL, Packard, Tokyo, Japan).

Quantitative RT-PCR. Frozen mouse liver samples were homogenized in Iogen (Nippon Gene, Toyama, Japan) with a Handy Micro Homogenizer (Phycosron NS-310E, Microtec, Chiba, Japan). Total RNA was extracted from the homogenate according to the manufacturer’s instructions. cDNA was produced with the SuperScript First-Strand Synthesis System for RT-PCRs (Invitrogen, Carlsbad, CA). Real-time PCR was performed with an ABI-Prism 7700 Sequence Detector and the SYBR Green Master Mix kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer’s instructions. Primers used in this study are shown in Supplemental Table S2. For quantitative precision, the same amount of total RNA was consistently used for each expression analysis, and the expression level of each gene...
was normalized by the expression of a housekeeping gene, acidic ribosomal phosphoprotein P0 (Arbp/36B4).

**Statistical Analysis**

Numerical data are expressed as means ± SE. Statistical analysis was conducted with Student’s t-test or analysis of variance (ANOVA) and, subsequently, Fisher’s protected least significant difference (PLSD) multiple comparison (STATVIEW for Windows v. 5.0, SAS Institute, Cary, NC). Differences were considered significant when the error probability was smaller than 0.05.

**RESULTS**

**Starch Availability in Vitro and in Vivo**

In Table 1, the available glucose contents determined in vitro are listed for the different starches. For comparison, the glucose availability of identical starches, as estimated in vivo, is included. The available glucose level was >800 mg/g in unmodified starches (TS, CS, and WCS) but <600 mg/g in CMS (HPdTSP, HPdCSP, and HPdWCSP) and HACS. The postprandial glycemic response for 2 h after gavage was significantly lower in CMS as well as HACS compared with those fed the HF diet or 10% HPdWCSP. Observed when the HF diet was supplemented with over 5% CMS compared with corresponding in vitro data, resulting in significantly lower in CMS as well as HACS compared with postprandial glycemic response for 2 h after gavage was significantly lower in CMS as well as HACS compared with those fed the HF diet or 10% HPdWCSP. In CMS supplementation on diet-induced obesity, we examined fat metabolism in mice fed a HF diet supplemented with 10% test starch for 4 wk (Supplemental Table S1C). The hepatic fatty acid oxidative capacity was significantly higher in mice fed the HPdWCSP diet than in those fed the HF diet but not those fed the HACS diet (Fig. 2A). Fatty acid oxidation-related gene expression such as that of medium-chain acyl-CoA dehydrogenase (MCAD) and acyl-CoA oxidase (ACO) was significantly higher in mice fed the HPdWCSP diet than in those fed the HF or HACS diet (Fig. 2, B and C). Enoyl-CoA hydratase 1 (ECH1) and hydroxyacyl-CoA dehydrogenase (HADH) were also expressed at higher levels in the HPdWCSP-fed group, but not in the HACS-fed group, compared with the HF-fed groups (Supplemental Fig. S2, A and B). Although not significant, similar trends could also be observed in the expression of carnitine palmitoyltransferase I (CPT1) and enoyl-CoA hydratase (EHHADH) genes (Supplemental Fig. S2, C and D). Body weights did not differ between HPdWCSP and HF groups, whereas the HACS group showed a significantly higher body weight than the HF group. Total WAT weight and nonfasting plasma insulin level were significantly lower in the HPdWCSP group compared with the HF group but not compared with the HACS group (Table 3).

**Effect of Insulin Treatment on Hepatic Fatty Acid Oxidation**

We examined the effect of blood insulin level on fat catabolism in the liver by chronic insulin treatment of mice. Supplemental Fig. S3 shows fatty acid oxidation and its related gene expression in the livers of mice after insulin treatment on a LF or HF diet for 2 wk. Nonfasting plasma insulin levels were significantly higher in insulin-treated than in saline-treated groups (1.38 ± 0.186, 3.30 ± 0.726, 1.28 ± 0.122, and 1.92 ± 0.268 ng/ml in saline-LF, insulin-LF, saline-HF, and insulin-HF, respectively). Hepatic fatty acid oxidation (Supplemental Fig. S3A) and ACO mRNA expression (Supplemental Fig. S3B) were significantly lower in insulin-treated than in saline-treated mice on the HF diet, whereas they did not differ between groups on the LF diet.

**Indirect Calorimetry**

We examined the effect of 10% CMS or HACS supplementation of the diet on energy consumption in mice. In the HF diet-fed condition, VO2 was significantly higher in the HPdWCSP compared with the HACS diet-fed group (Fig. 3A). RER was significantly lower in the HPdWCSP compared with the HACS group (Fig. 3B). Fat utilization during the experi-

| Table 1. In vitro and in vivo starch availability |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | TS              | CS              | WCS             | HPdTSP          | HPdCSP          | HPdWCSP         | HACS            |
| In vitro availability glucose, mg/g | 827.0 ± 12.0×0.4 | 847.4 ± 4.0×0.4 | 808.4 ± 18.2×0.2 | 554.8 ± 9.4×0.4 | 546.2 ± 4.0×0.4 | 455.0 ± 3.2×0.4 | 593.6 ± 10.8×0.8 |
| In vivo availability glucose, mg/dl per 2 h | 231.9 ± 18.7×0.7 | 257.5 ± 11.4×0.7 | 233.8 ± 24.6×0.4 | 133.5 ± 9.9×0.9 | 159.3 ± 4.6×0.8 | 131.2 ± 7.7×0.7 | 141.7 ± 13.2×0.7 |

Data are expressed as means ± SE; n = 3 (for in vitro availability) or 6 (for in vivo availability) in each group. TS, tapioca starch; CS, corn starch; WCS, waxy corn starch; HPdTSP, HPdCSP, HPdWCSP, hydroxypropyl distarch phosphate from tapioca, corn, and WCS, respectively; HACS, high-amylose corn starch. AUC, area under curve. Statistical analysis was conducted with 1-way ANOVA and, subsequently, Fisher’s protected least significant difference (PLSD) multiple comparison. Means not sharing a given superscript letter differ significantly (P < 0.05).
E656 RS4 PREVENTS DIET-INDUCED OBESITY IN MICE

LF TS CS WCS HPdWSCP HPdWCSP HACS

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<td>6 wk</td>
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<td>24 wk</td>
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Postprandial GIP Response

To study the underlying mechanism for the difference in the effects of CMS and HACS supplementation of the HF diet on energy consumption, we examined the postprandial response of anabolic hormones, such as insulin and GIP, after gavage of starch with fat. Unmodified starch (WCS) administration increased plasma glucose levels for 30 min, and thereafter the levels declined (Fig. 4A). Insulin levels peaked at 10 min after starch administration and reduced thereafter, reaching baseline after 30 min (Fig. 4C). When TO was administered together with WCS, the insulin level was significantly increased at 10 min, followed by a marked decline of blood glucose at 30 min (Fig. 4, A and C). The glucose response was significantly decreased after WCS plus TO compared with WCS alone (~30% decrease in glucose AUC at 120 min, Fig. 4B). The starch-induced insulin response (AUC) for 30 min was augmented by TO administration (~2-fold increase; P < 0.01) (Fig. 4D). Oral WCS increased plasma GIP levels at 10 min, and thereafter GIP levels declined, reaching baseline after 60 min. When TO was added to WCS, the GIP level was significantly increased at either 10 or 30 min compared with WCS alone (P < 0.01). The plasma GIP response for 60 min (GIP AUC at 60 min) was increased when WCS and TO were administered together.

The postprandial blood glucose response for 120 min after gavage was significantly lower in mice administered HPdWCSP or HACS than in those administered WCS (Fig. 4, A and B). The postprandial insulin response was also significantly lower in the HPdWCSP or HACS group than in the WCS group (Fig. 4, C and D). Peak GIP levels and the GIP response for 60 min (GIP AUC 60 min) after gavage were significantly lower in the HPdWCSP group compared with the HF group, but not the HACS group (Fig. 4, E and F).

Effect of GIP Treatment on Energy Consumption and Fat Utilization

We examined the effect of blood GIP level on energy consumption in mice fed the HF diet. Supplemental Fig. S4 shows the energy metabolism of mice after GIP treatment on the HF diet. Nonfasting plasma GIP levels were significantly higher in the GIP-treated than in the saline-treated group (0.313 ± 0.0948 vs. 0.868 ± 0.166 ng/ml (P < 0.05) in saline-HF and GIP-HF, respectively). Body weight gain of the mice housed in metabolic chambers was 0.49 ± 0.23 and 0.50 ± 0.16 g in saline-HF and GIP-HF groups, respectively. Although not significant, the average $\dot{V}O_2$ was lower (71.2 ± 0.6 vs. 73.8 ± 1.3 ml·kg$^{-1}$·min$^{-1}$) and the RER was higher (0.837 ± 0.003 vs. 0.827 ± 0.005) in the GIP-treated group compared with the saline-treated group. Fat utilization during the experimental period (63 h) was significantly lower and energy expenditure tended to be lower in the GIP-treated mice than in the saline-treated mice (Supplemental Fig. S4, A and B).
Intestinal Glucose and Fatty Acid Transport

We examined the effect of unmodified starch, CMS, or HACS on the transport of GIP secretagogues (glucose and fatty acids) from the lumen into the intestinal tissue. Fatty acid transport into the jejunal sacs for 10 min was significantly lower with the HPdWCSP-supplemented emulsion than the HACS-supplemented emulsion (Fig. 5B). Glucose transport into the jejunal sacs from the fat emulsion did not differ among the groups (Fig. 5A).

DISCUSSION

In this study, in vivo availability of CMS (RS4) was 59% for unmodified starch (TS, CS, and WCS) on average (HPdTSP, HPdCSP, and HPdWCSP) and equivalent to that of HACS (RS2). Therefore, energy dilution of the diet with RS is considered to be similar between RS4 and RS2. However, RS4 supplementation of the HF diet, but not RS2 supplementation, attenuated diet-induced obesity in C57BL/6J mice. To study the underlying mechanism behind the preventive effect of RS4 supplementation on diet-induced obesity, we examined fat metabolism in mice fed a HF diet supplemented with 10% RS4 or RS2 for 4 wk. RS4 supplementation increased the fatty acid oxidative capacity and its related gene expression, such as that of MCAD, ACO, ECH1, and hydroxyacyl-CoA dehydrogenase-alpha (HADHA), in the liver. The induction of hepatic fatty acid-catabolizing enzymes leads to an increased capacity for fatty acid oxidation. The increased oxidation of fatty acids in the liver is thought to drain fatty acids from the body, reduce VLDL formation, and exert antiobesity effects (14). Several studies have demonstrated that the upregulation of fatty acid catabolism by peroxisome proliferator-activated receptor (PPAR) agonists in the liver is associated with reduced fat accumulation in rodent models of high-fat diet-induced or genetic insulin resistance (7, 25, 41). These findings suggest that the upregulation of hepatic fatty acid catabolism is associated with the antiobesity effect of RS4.

Dietary intakes did not differ between the RS4 diet-fed and corresponding normal starch diet-fed groups. However, we cannot exclude the possibility that RS4 affects the dietary or energy intake of mice, since we simply measured the dietary intake of mice on a per-cage basis. The effect of RS4 on energy intake remains to be further studied by accurate dietary measurement, e.g., the single housing of mice.

HF diet ingestion upregulated the mRNA expression of fatty acid catabolism-related enzymes such as ACO and MCAD in the liver compared with the LF diet. The expression of these genes is upregulated by fibrate, a PPAR activator, and these genes have a PPAR response element (PPRE) in their promoter region (8, 15, 16, 51). PPAR is a ligand-activated transcriptional factor belonging to the nuclear hormone receptor superfamily, which is considered to play an important role in the control of lipid metabolism (24, 29, 31, 34, 54). PPAR is abundantly expressed in the liver (13, 23). In addition, free fatty acids (FFAs) function as intrinsic ligands for PPAR (31) and transcriptionally regulate the expression of fatty acid catabolism-related enzymes (26). Keller et al. (32) investigated the effects of various fatty acids on PPAR transcriptional activity and showed that PPAR activation is increased by fatty acids. Lin et al. (36) also demonstrated that long-chain fatty acids interact with PPAR at physiological concentrations. Therefore, the increased expression of these fat catabolism-related genes in response to high-fat diet ingestion might be...
explained by the activation of PPAR by fatty acids derived from dietary fat.

Four-week ingestion of RS4, but not that of RS2, significantly reduced nonfasting plasma insulin levels in mice fed a HF diet. Insulin is known to decrease the rate of mitochondrial fat oxidation by inhibiting CPT1 activity via an elevated cytoplasmic malonyl-CoA level (50). Thus RS4 is likely to increase the mitochondrial fat oxidation rate via a lower blood insulin level.

Interestingly, RS4 ingestion stimulated the gene expression and thereby the capacity of fatty acid oxidation in the liver. To explore the underlying mechanism of the transcriptional up-regulation of hepatic fat oxidation by dietary RS4, we hypothesized an association between hepatic fat oxidation and blood insulin level and examined fatty acid oxidation in the liver of insulin-treated mice. 1) The HF diet increased hepatic gene expression of ACO, the rate-limiting enzyme involved in peroxisomal fatty acid oxidation (18), and the fatty acid oxidative capacity compared with the LF diet. 2) Insulin treatment lowered the HF diet-induced upregulation of ACO expression and fat oxidation in the liver to LF diet levels. 3) In contrast to the HF diet, insulin treatment affected neither hepatic fatty acid oxidative capacity nor ACO gene expression on the LF diet. Overall, these results suggest that a higher blood insulin level downregulates high-fat diet (possibly PPAR)-induced hepatic

Table 3. Body, liver, and WAT weights and blood glucose, insulin, TG, and NEFA in C57BL/6J mice fed 5% or 30% TG- or RS-supplemented diets for 4 wk (experiment 3)

<table>
<thead>
<tr>
<th></th>
<th>LF</th>
<th>HF</th>
<th>HPdWCSP</th>
<th>HACS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>25.18 ± 0.33a</td>
<td>26.38 ± 0.52ab</td>
<td>26.00 ± 0.38ab</td>
<td>26.84 ± 0.52b</td>
</tr>
<tr>
<td>Total WAT, g</td>
<td>0.794 ± 0.024a</td>
<td>1.145 ± 0.109b</td>
<td>0.817 ± 0.058a</td>
<td>1.033 ± 0.111b</td>
</tr>
<tr>
<td>Liver weight, g</td>
<td>1.137 ± 0.032a</td>
<td>1.152 ± 0.027ab</td>
<td>1.225 ± 0.034c</td>
<td>1.239 ± 0.023c</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>237 ± 20a</td>
<td>265 ± 16c</td>
<td>240 ± 19a</td>
<td>271 ± 18a</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.38 ± 0.28ab</td>
<td>2.10 ± 0.44b</td>
<td>1.00 ± 0.19a</td>
<td>1.39 ± 0.44ab</td>
</tr>
<tr>
<td>TG, mg/ml</td>
<td>118 ± 12a</td>
<td>95 ± 12a</td>
<td>91 ± 14a</td>
<td>97 ± 15a</td>
</tr>
<tr>
<td>NEFA, meq/l</td>
<td>0.508 ± 0.054a</td>
<td>0.455 ± 0.042c</td>
<td>0.406 ± 0.048b</td>
<td>0.453 ± 0.029c</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; n = 10 in each group. NEFA, nonesterified fatty acid; HF, high fat. Statistical analysis was conducted with 1-way ANOVA and, subsequently, Fisher’s PLSD multiple comparison. Means not sharing a given superscript letter differ significantly (P < 0.05).
gene expression, and thereby reduces the capacity for fat oxidation. Higher-level hepatic fat oxidation and its related gene expression levels induced by dietary RS4 are likely to be mediated by a lower blood insulin level.

The molecular mechanism leading to the upregulation of ACO mRNA expression induced by a decreased blood insulin level on a high-fat diet still remains to be clarified. Hirota et al. (27) showed that insulin promotes the phosphorylation of FKHR followed by its dissociation from hepatic nuclear factor-4 (HNF-4) in HepG2 cells, which facilitates the access of HNF-4 to its DNA-binding element. HNF-4 binds to the DR1 motif in the PPRE of the ACO gene with high affinity but fails to activate the ACO promoter, whereas the binding of the PPARα-retinoid X receptor (RXRα) heterodimer to PPRE stimulates the transcriptional response of ACO gene (42, 44).

In our preliminary experiments, insulin promoted the phosphorylation of FKHR, followed by its redistribution from the nucleus to the cytoplasm, and significantly reduced the PPAR-modulated gene expression of ACO in the primary culture of murine hepatocytes (unpublished data). These findings suggest that a lower blood insulin level stimulates the PPAR-promoted transcriptional response of fatty acid catabolism-related genes through the possible inactivation of HNF-4 in the liver.

Under the HF diet-fed condition, energy expenditure and fat utilization were higher in the RS4-fed mice than in the RS2-fed mice. To study the underlying mechanism of the difference between RS4 and RS2 supplementation of the HF diet in energy consumption, we examined the postprandial response of anabolic hormones, such as insulin and GIP, after the gavage of starch with fat. Recently, we showed (55) that the coinges...
RS4 PREVENTS DIET-INDUCED OBESITY IN MICE

E660

A

![Glucose uptake](image)

B

![Oleate uptake](image)

Fig. 5. Nutrient transport from emulsion to jejunal sacs. ([14C]glucose (A) and [3H]oleate (B) transported from emulsion containing WCS, HPdWCSP, or HACS were determined from radioactivity accumulated in the jejunal tissue. Data are expressed as means ± SE; n = 10 in each group. Statistical analysis of the AUC was conducted with 1-way ANOVA and, subsequently, Fisher’s PLSD multiple comparison. Means not sharing a given superscript letter differ significantly (P < 0.05).

The ingestion of fat augments glucose-induced insulinemia via gut-derived GIP and thereby influences postprandial nutrient metabolism in mice. In this study, postprandial insulin and GIP responses were significantly increased after the coingestion of unmodified starch and fat compared with starch alone. The starch-induced glycemic response was decreased on coingestion with fat.

When coingested with fat, RS4 but not RS2 induced a significantly lower postprandial GIP response compared with unmodified starch. To study the association between physiological blood GIP level and energy and fat consumption, we examined the effect of continuous GIP administration on energy consumption in mice fed a HF diet. Nonfasting plasma GIP levels were significantly higher in the GIP-administered group than in the saline-administered group. GIP treatment significantly decreased fat utilization under the fed condition. Previous studies have shown that the inhibition of GIP signaling increases fat oxidation and prevents the onset of obesity and consequent insulin resistance induced by a high-fat diet (38). Recently, Altthage et al. (3) reported that targeted ablation of GIP-producing cells in transgenic mice enhanced energy expenditure and reduced high-fat diet-induced obesity. Our results are consistent with the above studies and suggest that a lower blood GIP level enhanced fat oxidation, and thereby prevented diet-induced obesity.

Dietary carbohydrates and fats stimulate a postprandial increase in blood GIP level (21, 37). GIP secretion from GIP-producing K cells is triggered by glucose and FFAs (46). Thus a lower GIP response after the ingestion of RS4 could be explained by its decreased availability (glucose release by digestion) compared with unmodified starch. However, we cannot explain why RS4, but not RS2, lowers the postprandial GIP response after coingestion with fat, since the in vivo availability of RS4 is equivalent to that of RS2. There are several possible factors that could have contributed to the difference in GIP response between RS2 and RS4. Although we cannot exclude the possibility that RS4 ingestion may alter gastric emptying, the most likely explanation is that RS4 lowers nutrient transport from the lumen to the intestinal epithelium, and thereby stimulates lower GIP secretion from K cells located in the intestinal epithelium. Consistent with this, fatty acid transport from the emulsion into the jejunal sacs was significantly lower when diet was supplemented with RS4 compared with RS2. Despite the lack of apparent cellular or molecular mechanisms, the results suggest that the decreased transport of fatty acids (GIP secretagogue) from the lumen to the jejunum might be attributed to a lower GIP release after RS4 ingestion together with fat compared with RS2.

Although not detracting from the significance of the present findings, limitations of the present study should be considered. Similarly to the liver, the skeletal muscle is a major insulin target and a key fat-metabolizing organ. We cannot exclude the possibility that RS4 also positively affects the skeletal muscle. A high-fat diet induces glucose intolerance (1) and insulin resistance in skeletal muscle (58). Increasing evidence indicates that some adipose tissue-derived molecules, e.g., adiponectin (9), are involved in the pathophysiology of obesity-related insulin resistance. Naitoh et al. (40) showed that adiponectin expression in WAT and its serum level were decreased in high-fat diet-fed C57BL/6J mice. They also found that the inhibition of GIP signaling increased adiponectin levels in WAT and blood, and thereby upregulated PPARα and downregulated acetyl-CoA carboxylase (ACC) expression, both of which indicate increased fat oxidation in skeletal muscle (40). The beneficial effects of RS4, which stimulates less GIP and insulin compared with normal starch, on skeletal muscle still remain to be elucidated.

Moreover, skeletal muscle plays an important role in glucose homeostasis. In this study, the 4-wk ingestion of a HF diet elevated blood insulin levels, whereas the blood glucose levels were similar compared with those of LF diet-fed mice, indicative of insulin resistance. RS4 ingestion attenuated the diet-induced hyperinsulinemia without elevating blood glucose levels, which implies that RS4 improved the insulin sensitivity in HF diet-fed mice. Thus it will be of interest to explore whether RS4 improves high-fat diet-induced glucose intolerance and insulin resistance. Bonnard et al. (12) showed that high-fat diet-fed C57BL/6J mice were glucose intolerant after 4 wk and after 16 wk became markedly diabetic, that is, hyperinsulinemic and insulin resistant. They also showed that adiponectin receptor expression and AMP-activated protein kinase (AMPK) activity were downregulated in the skeletal muscle of diabetic mice, which suggests that a decreased adiponectin response in skeletal muscle could be associated with high-fat diet-induced diabetes. Further investigations, such as glucose and/or insulin tolerance tests involving high-fat diet-fed mice, will improve our understanding of the beneficial effect of RS4-type starch on skeletal muscle and glucose homeostasis.

In this study, the ingestion of RS4-type resistant starch reduced fat in diet-induced obesity-prone C57BL/6J mice as-
sociated with stimulating fatty acid catabolism in the liver. RS4 also lowered postprandial GIP release and increased fat utilization when coingested with fat. These findings might provide clues to understanding the effects of RS4 on energy metabolism and help clarify the role of postprandial anabolic hormone (GIP) release in the control of obesity.

DISCLOSURES
The authors are not aware of financial conflict(s) with the subject matter or materials discussed in this manuscript with any of the authors, or any of the authors’ academic institutions or employers.

REFERENCES


