Wx/ae Double-Mutant Brown Rice Prevents the Rise in Plasma Lipid and Glucose Levels in Mice

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A wx/ae double-mutant rice is generated by crossing waxy mutant and amylose-extender mutant in rice. Wx/ae brown rice contains highly beneficial nutrients for lipid and glucose metabolism, including resistant starch, dietary fiber, and γ-oryzanol, when compared to Koshihikari brown rice, the non-waxy japonica cultivar. To examine the effects of wx/ae brown rice on glucose and lipid metabolism, type 2 diabetic NSY/Hos mice were fed a high-fat diet containing 25% of wx/ae brown rice or Koshihikari brown rice for 10 weeks. The plasma total cholesterol, non-high-density lipoprotein cholesterol, triglyceride, and non-esterified fatty acid levels of the wx/ae group were significantly lower than those of the Koshihikari group. Moreover, the fasting blood glucose level and pathological score of glycosuria of the wx/ae group were also significantly lower than those of the Koshihikari group. These results indicate that wx/ae brown rice has the potential to prevent the rise in plasma lipid and glucose levels.

Key words: wx/ae brown rice; resistant starch; γ-oryzanol; lipid and glucose metabolism

Dyslipidemia and hyperglycemia are characteristic of the metabolic syndrome which appears to promote development of the atherosclerotic cardiovascular disorder as well as increasing the risk of developing type 2 diabetes.1) Food materials that are multi-effective against symptoms of the metabolic syndrome are needed to prevent and treat metabolic syndrome, and research and development of such foods are in progress throughout the world.2,3) A wx/ae double-mutant AMF18 is generated by crossing the amylose-free waxy (wx) mutant EM21 and amylose-extender (ae) mutant EM16 cultivars.4) Wx/ae rice starch lacks amylose and is composed of long-unit chains of amylpectin, making it hard to be digested in vitro and in vivo.5) Adding resistant starch (RS) to the diet improves insulin sensitivity in people with either normal glucose tolerance or impaired glucose toler-

ance.6,7) It has been reported that RS could stimulate the endogenous secretion of glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) as a possible mechanism for improving insulin sensitivity by RS.8) Lattimer and Haub have reported that the intake of rice bran reduced the plasma cholesterol level and improved the plasma cholesterol status,9) suggesting that wx/ae brown rice may also be useful in preventing both type 2 diabetes and hypercholesterolemia.

γ-Oryzanol is one of the major functional components in rice bran and has been shown to have beneficial effects on hypercholesterolemia, hyperlipidemia, and insulin resistance in animal models.10,11) It also has anti-inflammatory and antioxidative effects.12) Rice cultivars with a high γ-oryzanol content might therefore be valuable for preventing and treating various diseases.

Nagoya-Shibata-Yasuda (NSY) mice have been established as an inbred animal model of spontaneous type 2 diabetes by selective breeding for glucose intolerance from outbred ICR mice.13) NSY mice have been used in pharmaceutical and food research,3,14) since the clinical characteristics of NSY mice resemble those of the common forms of type 2 diabetes in humans such as age dependence for the onset of diabetes and insulin resistance.15)

In the present study, we compared the effects of wx/ae brown rice on lipid and glucose metabolism to those of Koshihikari brown rice, one of the most popular non-waxy japonica rice cultivars, by using high-fat diet-fed type 2 diabetic NSY/Hos mice.

Materials and Methods

Preparation of the brown rice powder. Wx/ae and Koshihikari rice plants were respectively grown in the summer of 2010 in Yamanashi and Ishikawa prefectures of Japan. Both types of brown rice were washed with water at room temperature and dried at 70 °C for 2 h with a DSI-7 electric drying machine (Shizuoka-seiki, Fukuroi, Shizuoka, Japan). Each type of rice was powdered with a WB-1 Wonder blender (Osaka Chemical, Osaka, Japan) and then used for the animal experiments. The nutritional composition of each type of brown rice was analyzed by Sunatec (Yokkaichi, Mie, Japan) as shown in

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Abbreviations: ae, amylose-extender; ALT, alanine aminotransferase; AST, aspartate aminotransferase; EF-1α, elongation factor-1α; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NEFA, non-esterified fatty acids; RS, resistant starch; TC, total cholesterol; wx, waxy
Table 1. Nutritional Constituents of Each Brown Rice

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Koshihikari brown rice</th>
<th>Wx/ae brown rice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%)</td>
<td>6.7</td>
<td>7.4</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>3.5</td>
<td>5.3</td>
</tr>
<tr>
<td>NFE* (%)</td>
<td>74.1</td>
<td>67.7</td>
</tr>
<tr>
<td>Digestible starch (%)</td>
<td>66.9</td>
<td>26.5</td>
</tr>
<tr>
<td>Resistant starch (%)</td>
<td>&lt;2.0</td>
<td>27.8</td>
</tr>
<tr>
<td>Dietary fiber (%)</td>
<td>4.9</td>
<td>9.0</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>1.3</td>
<td>1.6</td>
</tr>
<tr>
<td>Moisture (%)</td>
<td>9.5</td>
<td>9.0</td>
</tr>
<tr>
<td>α-Oryzanol (µg/g)</td>
<td>268</td>
<td>514</td>
</tr>
<tr>
<td>Energy (kcal/100g)</td>
<td>364.5</td>
<td>366.1</td>
</tr>
</tbody>
</table>

*NFE, nitrogen free extract (carbohydrate and others)

Table 1. Dietary fiber was measured by using a total dietary fiber assay kit (Megazyme, Wicklow, Ireland) based on AACC method 32-05.

Measurement of resistant starch and digestible starch. RS and digestible starch (DS) were measured by using an RS assay kit (K-RSTAR, Megazyme; AOAC Method 2002.02, AACC Method 32-40). This method can give precise results for over a 2% RS content. Four milliliters of pancreatic α-amylase (10 mg/mL) containing amylglucosidase (AMG, 3 U/mL) was added to 100 mg of brown rice powder samples, and then the samples were incubated at 37°C while continuously shaking for 16 h. After adding EtOH, each sample was centrifuged to separate DS and RS. The resulting supernatant was collected as a DS solution. A 2-mL amount of 2N KOH was subsequently added to the precipitate, and the dissolved sample was hydrolyzed at 50°C for 30 min by adding an AMG solution (3000 U/mL). After centrifugation, the supernatant was collected as an RS solution. The glucose concentrations in the DS and RS solutions were determined by the glucose oxidase-peroxidase method and converted to starch contents by multiplying by 0.9. Each sample was analyzed three times.

Determination of the α-oryzanol content. One gram of each type of brown rice powder was soaked in 10 g of a soaking solution (methanol/acetonitrile/acetic acid, 52/45/3 (v/v/v)) while gently stirring for 2 h. The extracted α-oryzanol solution was collected on a no. 2 filter paper (Advantec, Tokyo, Japan). The α-oryzanol content of each extracted solution was determined by high-performance liquid chromatography (HPLC). The reverse-phase HPLC system consisted of a PU-2089 pump (Jasco, Tokyo, Japan) equipped with a Cadenza CD-C18 column (250 mm × 4.6 mm I.D.; Intakt, Kyoto, Japan), a CO-8010 column oven (Tosoh, Tokyo, Japan), and an SPD-10A UV-VIS detector (Shimadzu, Kyoto, Japan). The conditions used were as follows: flow rate, 0.8 mL/min; oven temperature, 30°C; injection volume, 5 µL; detection, UV at 320 nm; and mobile phase, methanol/acetonitrile/acetic acid (52/45/3 v/v/v).

The data were produced in triplicate and with reference to the peak area-concentration calibration curve from authentic α-oryzanol standards (Tokyo Chemical Industry, Tokyo, Japan). We confirmed by the same method that each extracted residue did not contain γ-oryzanol.

Diet and animals. Six-week-old male type 2 diabetic mice (NSY/Hos strain) were purchased from Hoshino Laboratory Animals (Bando, Ibaraki, Japan). After 2 weeks on a AIN-76-modified high-fat diet (milk casein, 28.2%; corn oil, 6.0%; beef tallow, 10.0%; sucrose, 22.0%; cellulose, 3.8%; st-methionine, 0.3%; choline bitartrate, 0.2%; AIN-76 vitamin mix, 1.0%; AIN-76 mineral mix, 3.5%; α-cornstarch, 25.0%; energy, 438.1 kcal/100 g), the mice were individually housed and fed for 10 weeks with the AIN-76-modified high-fat diet containing each type of brown rice powder (25.0% w/w) instead of α-cornstarch. The body weight and food intake were measured every week. The fasting blood glucose level was measured biweekly from the tail vein after 8 h of fasting by the glucose oxidase method, using a G-checker (Gunce, Kyoto, Japan). The mice were fasted for 14 h before euthanasia was applied with CO2 gas. Blood samples and organ samples were harvested and subsequently dissected for analysis. All experiments were conducted in accordance with the guidelines for the proper conduct of animal experiments by the Science Council of Japan (2006).

Blood chemistry. Plasma total cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, non-esterified fatty acids (NEFA), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were respectively analyzed by cholesterol E, HDL cholesterol E, Triglyceride E, NEFA-C, and Transaminase CII kits (Wako Pure Chemical Industries, Osaka, Japan). Plasma non-HDL cholesterol, including chylomicron, very-low-density lipoprotein, and low-density lipoprotein cholesterol, was calculated from the total cholesterol and HDL cholesterol levels. Plasma insulin was measured by using an insulin assay kit (Shibayagi, Shibukawa, Gunma, Japan), and plasma adiponectin was measured by using an Adiponectin DuoSet kit (R&D Systems, Minneapolis, MN, USA). Plasma lipid peroxidation was analyzed by measuring thiobarbituric acid reactive substances (TBARS) with a TBARS assay kit (Cayman Chemical, Ann Arbor, MI, USA).

Hepatic cholesterol and triglyceride. Total lipids were extracted from 30–40 mg of liver tissue by the method described by Bligh and Dyer,12 and then dissolved in isopropanol. Hepatic total cholesterol and triglyceride were respectively analyzed by using cholesterol E and triglyceride E kits.

Measurement of glycosuria. Fresh urine samples were collected at 10 weeks, and the glycosuria level was analyzed by the glucose oxidase method, using a urine glucose test strip (Terumo, Tokyo, Japan). The pathological scores for glycosuria are defined as follow: 0, <30 mg/dL; 1, 50–100 mg/dL; 2, 100–500 mg/dL.

Analysis of feces. Feces were collected for 48 h at 10 weeks. The collected feces were lyophilized, homogenized, and then re-lyophilized. Fecal cholesterol and bile acid were extracted at 55°C for 4 h with 90% ethanol. Fecal triglyceride was extracted at 60°C for 90 min with a buffer containing 100 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% (v/v) TritonX-100, and 80% isopropanol. The cholesterol, bile acid, and triglyceride concentrations were respectively analyzed by using cholesterol E, total bile acid (Wako Pure Chemical Industries), and triglyceride E kits.

Quantitative real-time RT-PCR. Total RNA samples of the liver tissues were isolated by using a QuickPrep total RNA extraction kit (GE Healthcare, Piscataway, NJ, USA); cDNA was prepared from 2 μg of each total RNA sample which was reverse-transcribed by using Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo (dT) primers (Invitrogen). Each prepared cDNA sample was purified by using a PCR purification kit (Qiagen, Valencia, CA, USA). Quantitative real-time RT-PCR was performed by a StepOne real-time PCR system (Life Technologies Japan, Tokyo, Japan), using a Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The primers used for each gene are listed in Table 2. The PCR products were evaluated by an analysis of their melting curves (data not shown), all experiments being performed in duplicate.

Statistics. Each result is expressed as the mean ± standard deviation. Independent sample r-tests or two-way repeated-measures ANOVA with a post hoc analysis by Dunnett’s test were carried out by using Ekusou-Toukei software version 2010 (SSRI, Tokyo, Japan). Differences were considered significant at p < 0.05.
Results

Prevention of the rise in plasma lipid level by wx/ae brown rice

Table 1 shows that wx/ae brown rice contained 27.8% RS, 9.0% dietary fiber, and 514 μg/g of γ-oryzanol, while Koshihikari brown rice contained <2.0% RS, 4.9% dietary fiber and 268 μg/g of γ-oryzanol. These nutritional constituents are known to have beneficial effects on lipid and glucose metabolism.3,14) suggesting that an intake of wx/ae brown rice would prevent the development of dyslipidemia and hyperglycemia more effectively than an intake of Koshihikari brown rice. We demonstrated this hypothesis by examining the effect of wx/ae brown rice on lipid and glucose metabolism by using a high-fat diet-fed type 2 diabetic mouse model which developed both dyslipidemia and hyperglycemia.3,14) We fed AIN-76-modified high-fat diets containing 25% Koshihikari brown rice or wx/ae brown rice to type 2 diabetic NSY/Hos mice for 10 weeks. All animals were in good health throughout the experimental period, and no such side effect as diarrhea was apparent (data not shown).

There were no differences between the two groups in daily intake, total energy intake, body weight gain, or organ weight (Table 3). Figure 1 tracks the body weight of the mice during the 10-week study period. The body weight of the wx/ae group was slightly lower than that of the Koshihikari group, but there was no statistically significant difference between the two groups (p = 0.1144). In addition, no difference in the weight of the epididymal white adipose tissue between the two groups was apparent (Table 3), suggesting that wx/ae brown rice had no anti-obese effect. On the other hand, the plasma total cholesterol (p < 0.001), non-HDL cholesterol (p < 0.01), triglyceride (p < 0.05), and NEFA (p < 0.001) levels were significantly lower in the wx/ae group. The total cholesterol/HDL cholesterol ratio, a predictor of cardiovascular disease,20) was also lower in the wx/ae group than in the Koshihikari group (p < 0.05).

No significant differences were apparent in the plasma HDL cholesterol, insulin, adiponectin, TBARS, or hepatic lipid levels, while AST (p < 0.05) and ALT (p < 0.001), markers for the liver conditions, were lower in the wx/ae group than in the Koshihikari group, suggesting that wx/ae brown rice might have exerted a hepatoprotective effect against high-fat diet-induced liver damage.

Effect on fecal lipid excretion

Inhibiting dietary lipid absorption can induce favorable change in the plasma lipid content.21) We analyzed the fecal lipid content to examine the effect of wx/ae brown rice on dietary lipid absorption (Table 3). The wx/ae group mice excreted more feces (p < 0.05), although the daily fecal excretion of cholesterol and bile acid were almost equal in both groups. Interestingly, the fecal triglyceride level in the wx/ae group was five times higher than that in the Koshihikari group (p < 0.001), indicating that dietary triglyceride absorption had been inhibited in the wx/ae group.

Effects on glycemic status

An intake of RS helps to prevent diabetes and insulin resistance.5,6) We analyzed the fasting blood glucose level throughout the experimental period and the glycemia level at week 10 to identify the effects of wx/ae brown rice on the glycemic status (Fig. 2). The fasting blood glucose level was significantly lower in the wx/ae group than in the Koshihikari group (p < 0.005; Fig. 2A). On the other hand, the pathological score for glycemia at 10 weeks was significantly better in the wx/ae group than in the Koshihikari group (p < 0.05; Fig. 2B). Accordingly, the glycemic status of the wx/ae group is considered to have been superior to that in the Koshihikari group.

Gene expression analysis in the liver

Treatment with a hypolipidemic agent can influence the expression of hepatic genes related to lipid metabolism.3,14) We performed a SYBR green-based real-time RT-PCR analysis of the liver to compare the expression levels of lipid metabolism-related genes between the two groups (Fig. 3). Among the 12 genes examined, the sterol regulatory element-binding protein (SREBP)-1, SREBP-2, fatty acid synthetase (FAS), low-density lipoprotein receptor (LDL-R), and cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP2R1) genes were significantly upregulated in the wx/ae group.
Discussion

A wx/ae double-mutant rice generated by crossing wx mutant line EM21 and ae mutant line EM16 lacks amylose and has amylopectin with relatively long-unit chains. Its starch resists digestion in vitro and in vivo. The RS content of wx/ae brown rice is 27.8% (Table 1), while that of Koshihikari brown rice is under 2%. In addition, wx/ae brown rice has more dietary fiber and γ-oryzanol than Koshihikari brown rice. These three nutrients have beneficial effects on dyslipidemia and
insulin resistance. The consumption of RS and dietary fiber reduces the risk of type 2 diabetes.6,7,9 γ-Oryzanol has beneficial effects on hypercholesterolemia, hyperlipidemia, and insulin resistance in animal models,10,11 and has been approved for the treatment of dyslipidemia in Japan. The results of the present study, as expected from the data on nutritional information, show that the intake of wx/ae brown rice resulted in a better plasma lipid level and glycemic status than the intake of Koshihikari brown rice by high-fat diet-fed NSY/Hos mice, type 2 diabetic mice with clinical characteristics resembling the common forms of type 2 diabetes in humans.15 The plasma total cholesterol, non-HDL cholesterol, triglyceride and NEFA levels, and the total cholesterol/HDL cholesterol ratio were significantly lower in the wx/ae group than in the Koshihikari group. The fasting blood glucose level throughout the experimental period and the pathological score for glycosuria at 10 weeks were significantly lower in the wx/ae group. We also observed a protective effect on high-fat diet-induced liver damage. AST and ALT, both markers for liver conditions, were lower in the wx/ae group. These results indicate that wx/ae brown rice was more effective than Koshihikari brown rice in preventing dyslipidemia, cardiovascular disease, and hyperglycemia.

Inhibiting dietary lipid absorption induces a favorable change in the plasma lipid content.21 Gastrointestinal lipase inhibition prevents dyslipidemia,24 and inhibition of bile acid reabsorption ameliorates hypercholesterolemia.3,14 We observed in this study lower levels of plasma triglyceride, NEFA, and fasting glucose in the wx/ae group, as well as a five-fold increase in the fecal triglyceride level. These results indicate that wx/ae inhibited the absorption of dietary triglycerides, resulting in decreased plasma triglyceride and NEFA levels. In addition, gastrointestinal lipase inhibition has been shown to decrease glycemia, because of the close relationship between glucose and lipid for energy metabolism,24 indicating that the increased excretion of fecal triglyceride may be involved in the prevention of hyperglycemia by wx/ae brown rice. Tsujita and colleagues have reported that basic polysaccharides of RS and dietary RS has increased secretion of the endogenous gut-secreted peptide hormones, glucagon-like peptide-1 (GLP-1) and peptide YY (PYY), by rodents.6 GLP-1 and PYY are produced by the L cells of the gut and suppress the appetite.20 Moreover, GLP-1 induces glucose-dependent insulin secretion. Therefore, maintaining substantially high plasma levels of GLP-1 and PYY are being sought in the treatment of type 2 diabetes and obesity. Wx/ae brown rice contains much more RS than Koshihikari brown rice, and this may explain the favorable glycemic status of the wx/ae group.

Treating with hypolipidemic agents has influenced the expression of genes related to lipid metabolism in the liver.1,4,14 This present study also shows that wx/ae brown rice upregulated the genes related to fat synthesis and cholesterol synthesis in association with a reduced plasma lipid level. SREBP-1 has regulated the expression of such genes related to fatty acid metabolism as FAS, while SREBP-2 has regulated such cholesterol- androgenic enzyme genes as HMG-CoAR and LDL-R.29 The expression of the SREBP-1, FAS, SREBP-2, and LDL-R genes was significantly higher in the wx/ae group than in the Koshihikari group, while the expression of the HMG-CoAR gene also tended to be higher in the wx/ae group. These results indicate that lipogenesis in the liver of the wx/ae group mice was upregulated in comparison with the Koshihikari group mice, although no difference in the triglyceride or cholesterol content in the liver was apparent, suggesting that reduced plasma lipids induced hepatic lipogenesis in the wx/ae group.

CYP2R1 is the key enzyme required for 25 hydroxylation of vitamin D and plays an important role in regulating the concentration of serum 25-hydroxy vitamin D, the best indicator of vitamin D status.29,30 Mathieu et al. have reported that vitamin D deficiency may be related to the pathogenesis of type 1 and 2 diabetes.32 The gene expression level of CYP2R1 in the liver in this present study was significantly higher in the wx/ae group than in the Koshihikari group, suggesting that the favorable glycemic status of the wx/ae group may have resulted in upregulation of the CYP2R1 gene.

In summary, an intake of wx/ae brown rice showed more beneficial effects to both the plasma lipid level and glycemic status than an intake of Koshihikari brown rice by high-fat diet-fed NSY mice. Although the conclusions need to be confirmed by further studies, including a dose-dependent study on animals and a human intervention study, wx/ae brown rice has the potential for preventing the rise in plasma lipid and glucose levels.

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