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Antidiabetic Effects of Rice Hull Smoke Extract on Glucose-Regulating Mechanism in Type 2 Diabetic Mice

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ABSTRACT: The aim of this study is to determine the protective effect of a liquid rice hull smoke extract (RHSE) against type 2 diabetes (T2D) in mice induced by a high-fat diet. As compared to the control group of mice on a high-fat diet (HFD), feeding the HFD supplemented with 0.5% or 1% RHSE for 7 weeks resulted in significantly reduced blood glucose and triglyceride and cholesterol concentrations, higher serum insulin levels, and improved glucose tolerance, as assessed by an oral glucose tolerance assay. The hypoglycemic effect of RHSE was accompanied by changes in enzyme activities and cognate gene expression assessed using RT-PCR. Among the glucose metabolism regulating genes evaluated, hepatic glucokinase (GCK), the glucose transporters GLUT2 and GLUT4, and peroxisome proliferator-activated receptor-γ (PPAR-γ) were up-regulated, whereas glucose-6-phosphatase (G6 Pase) and phosphoenolpyruvate carboxykinase (PEPCK) were down-regulated in the liver of mice with RHSE-supplementation. These changes resulted in restoration of glucose-regulating activities to normal control levels. Histopathology showed that a high-fat diet intake also induced liver necrosis and damage of the islet of Langerhans in the pancreas, whereas RHSE supplementation restored necrotic damage to normal levels. Immunohistochemistry showed that RHSE supplementation can restore the reduced insulin-producing β-cell population in islet of Langerhans associated with a high-fat diet intake to nondiabetic normal control levels in a dose-dependent manner. RHSE-supplemented food could protect insulin-producing islet cells against damage triggered by oxidative stress and local inflammation associated with diabetes.

KEYWORDS: *antidiabetic effects, rice hull smoke, mice, high-fat diet, type 2 diabetes, pancreas, liver, glucose-regulating genes, glucose-regulating enzymes, cholesterol, insulin, triglycerides*

INTRODUCTION

Type 2 diabetes (T2D) and obesity are complex human diseases of epidemic proportions caused by the under-production of insulin by the islet of Langerhans cells of the pancreas and impaired insulin resistance leading to hyperglycemia that result in impaired entry of glucose into cells, thus hindering glucose utilization. Low-carbohydrate high-fat diets increase plasma glucose in healthy men accompanied by a decrease in insulin secretion.[1]

Excess glucose can react with hemoglobin and other proteins in vivo to form so-called Amadori adducts[2] analogous to similar Maillard reactions of food proteins in vitro.[3,4] The in vivo glycations are reported to cause pro-inflammatory effects[5] and oxidative damage.[6]

The complex mechanisms that govern the molecular basis of the pathology and prevention of diabetes are the subject of vast literature.[7−10] Here, we will briefly present an overview of recent studies designed to elucidate the roles high-fat diets, reactive-oxygen species (ROS), pro-inflammatory cytokines, and glucose-regulating enzymes play in the development of diabetes that are relevant to the theme of the present study.

T2D is characterized by peripheral insulin resistance and pancreatic β-cell failure associated with mitochondrial dysfunction.[10−12] ROS produced by β-cell mitochondria activate several stress response pathways and the uncoupling protein 2 (UCP2) via peroxidation of the mitochondrial membrane phospholipids, which results in leaks of protons leading to reduced ATP synthesis in β-c-cells. These seem to be critical parameters in the regulation of glucose-stimulated insulin secretion. Elevation of ROS resulting from inflammation or excessive glucose and fatty acids may elevate antioxidant enzymes that reduce ROS and redox signaling, thus impairing β-cell function.[13] Elevated glucose levels increase the release of arachidonic and linoleic acids from phospholipids and their peroxidation to 4-hydroxy-2E-neonenal (4-HNE) in β-cells. This compound is an endogenous ligand for the peroxisome proliferator-activated receptor-δ (PPAR-δ), which amplifies insulin secretion in β-cells.[14,15] Alteration of mitochondrial function and methionine metabolism seems to contribute to increased oxidative stress in liver of obese diabetic patients, which may contribute to the development of fatty liver disease.[16] A review by Styskal et al.[17] on antioxidant mouse models suggests that oxidative stress plays a key role in the development of insulin resistance and diabetes and is the primary mechanism linking obesity and metabolic disorders.

High glucose also increases nitric oxide (NO) and inducible nitric oxide synthase (iNOS) expression and IL-1β secretion in lipopolysaccharide (LPS)-activated macrophages by enhancing protein kinase C-α/δ and NF-κB.[18] Protein kinase C-α (PKC-α) and protein kinase C-δ (PKC-δ) may be involved in diabetes-promoted inflammation. Macrophages are key medi-

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ators in obesity-induced insulin resistance, which then release cytokines such as IL-1β, IL-6, and TNF-α that create a pro-inflammatory environment that blocks adipocyte insulin action, thus contributing to the development of T2D.[19] The activation of inflammatory pathway intermediates in patients with T2D altered nuclear factor NF-κB DNA binding activity and increased phosphorylated c-Jun-N-terminal kinase (JNK) and AMP-activated kinase (AMPK) in muscle tissues. These results suggest that pathways regulating glucose uptake in skeletal muscle may be involved in the development of inflammation-associated hyperglycemia.[20] A deficiency of TNF-α improved fatty acid metabolism and protected against lipid deposition, inflammation, and fibrosis associated with HFD in the mouse liver. The reduced hepatic lipid accumulation occurs through an increase in adipose tissue storage and a decrease in fatty acid uptake and synthesis in the liver.[21]

Interventions that reduce the adverse effects of ROS on β-cell mitochondrial phospholipids may reduce the development of T2D.[11,12] For example, the antioxidants apocynin, catalase, ascorbic acid, α-lipoic acid, and α-tocopherol attenuate retinopathy in diabetes, presumably by neutralizing ROS production.[22] The drug simvastatin reduces the pro-inflammatory transcription factor NF-κB binding activity and lowers LDL-cholesterol levels in T2D.[23,24]

In previous studies, we (a) described the production and composition of a new rice hull liquid smoke with a smoky aroma and sugar-like odor prepared by pyrolysis of rice hulls followed by liquefaction of the resulting smoke;[25] (b) reported that the liquid rice hull smoke extract protected infected mice against *Salmonella* Typhimurium induced mortality;[26] and (c) found that RHSE also protected mice against alloxan-induced diabetes.[27] Because liquid smoke extracts from wood are widely used in food as flavoring and preservative agents,[28−31] to facilitate application of the newly developed RHSE to food, the main objective of the present study was to evaluate its potential to delay/prevent T2D in mice associated with a high-fat diet. The results suggest that supplementation of food with RHSE could create functional foods with antidiabetic properties.

MATERIALS AND METHODS

Materials. NADH, NADPH, hematoxylin, eosin Y, glucose-6-phosphate dehydrogenase, phosphoenolpyruvate (PEP), malic dehydrogenase, and all other reagents of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO). The AMV reverse transcriptase and dNTP mixture were obtained from Takara Bio (Kyoto, Japan). PCR primers were custom-synthesized and purified by Bioneer (Daejon, Korea).

Preparation of Rice Hull Smoke Extract. In previous publications, we described the production of RHSE, the characterization of 161 RHSE compounds by GC−MS, and beneficial activities of the liquid smoke in chemical, cell, and bacterial assays.[25−27]

Mice and Feeding Study. The method was adapted from our previous study with alloxan-treated mice.[27] Pathogen-free female ICR mice (6 weeks old) were purchased from Orient Bio (Seoul, Korea). The mice were housed in a stainless steel cage under a 12 h light/dark cycle with a temperature range of 20−22 °C and relative humidity of 50 ± 10% and fed pelletized commercial chow diet for 1 week after arrival. The mice were then randomly divided into four dietary groups (n = 10). The first and second groups were fed with normal diet and a high-fat diet, respectively, shown in Table 1. The other two groups were given a high-fat diet supplemented with either 0.5% or 1% RHSE (v/w), respectively. In a preliminary study, we found the onset of TD2 after 7 weeks on HFD. The mice were fed for 7 weeks and allowed free access to food and sterile tap water during the entire period. At the end of the experimental period, the mice were sacrificed by CO_2 inhalation following a 12 h fast. Blood samples were collected by cardiac puncture and left at 37 °C to induce blood clotting, followed by microcentrifugation at 13 000 rpm for 15 min to recover the serum. The body organs (liver and pancreas) and adipose tissues were excised, rinsed with PBS, and weighed. The organs were then stored at −70 °C until analysis. The current study protocol was approved by the Ethics Committee for Animal Care and Use, Ajou University.

Table 1. Composition of Normal and High-Fat Mouse Diets

component	normal diets (g/kg diet)	high-fat diets (g/kg diet)
casein	200.0	200.0
DL-methionine	3.0	3.0
cornstarch	150.0	150.0
sucrose	500.0	150.0
cellulose	50.0	50.0
corn oil	50.0	
salt mix #200000[a]	35.0	35.0
vitamin mix #310025[b]	10.0	10.0
choline bitartrate	2.0	2.0
beef tallow		400.0

[a]Dyets cat. no. 200000. [b]Dyets cat. no. 310025.

Blood Glucose Level and Oral Glucose Tolerance Assays. Blood samples were drawn from each mouse's tail vein at the end of the experimental period (7 weeks). The blood glucose levels were determined in tail blood samples using the Accu-Chek Active kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. An oral glucose tolerance test was performed before necropsy after 7 weeks following an overnight fast (16 h). The mice were administered glucose at a level of 0.25 g/kg body weight. The blood glucose concentrations were determined in tail blood samples before glucose administration, and at 30, 60, 90, and 120 min after glucose administration, using the same kit as described above.

Insulin Assay. The insulin content of the mouse serum was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Billerica, MA) as suggested by the manufacturer.

Serum Lipid Profile Assays. The concentrations of serum total cholesterol, triglyceride, and high-density lipoprotein (HDL) and low-density lipoprotein (LDL) were determined using a commercial kit (Asan Pharmaceuticals, Seoul, Korea) according to the manufacturer's instructions.

Glutamate Oxaloacetate (GOT) and Glutamate Pyruvate Transaminase (GPT) Assays. Serum enzyme GOT/GTP concentrations were determined using a colorimetric kit (Asan Pharmaceutical, Seoul, Korea) following the manufacturer's protocol. Briefly, diluted serum (20 μL) was added to the reaction solution. The resultant mixture was incubated at 37 °C for 30 and 60 min for GOT and GTP, respectively. The absorbance of the solution was determined at 505 nm using a microplate reader (model 550, Bio-Rad, Hercules, CA).

Pro-inflammatory Cytokine Assays. The published method for the preparation of skin tissue extracts[25] was used for cytokine determination of adipose tissue extracts. Briefly, adipose tissues were homogenized in a phosphate buffer (pH 7.0) containing 0.4 M NaCl, 0.05% Tween-20, 0.5% BSA, 0.1 mM PMSF, and 10 mM EDTA. The homogenates were microcentrifuged at 14 000g for 15 min at 4 °C to recover the supernatant. Cytokines TNF-α, IL-1β, and IL-6 in the supernatants and serum were determined by ELISA kits (Biosource International, Camarillo, CA) as suggested by the manufacturer. The absorbance of final solution at 420 nm was measured in the microplate reader.

Hepatic Glucose-Regulating Enzyme Activities. The hepatic enzyme source was prepared as previously described.[32] The glucokinase (GCK) activity was determined as previously described,[33] with slight modifications. The reaction mixture (98 μL) containing the following ingredients was preincubated at 37 °C for 10 min: Hepes-NaOH (50 mM, pH 7.4), KCl (100 mM), $MgCl_2$ (7.5 mM),

dithioerythritol (2.5 mM), albumin (10 mg/mL), glucose (10 mM), glucose-6-phospate (G6 Pase) dehydrogenase (4 units), NADH (50 mM), and cytosol (10 μL). The reaction was initiated with the addition of ATP (10 μL of 5 mM), followed by incubation at 37 °C for 10 min. The change in absorbance at 340 nm was recorded.

The G6 Pase activity was measured following the method of Alegre et al.[34] The reaction mixture contained Hepes-NaOH (765 μL of 131.58 mM; pH 6.5), EDTA (100 μL of 18 mM; pH 6.5), G6 Pase (100 μL of 265 mM), NADPH (10 μL of 0.2 M), mutarotase (0.6 IU/mL), and glucose dehydrogenase (0.6 IU/mL). After preincubation at 37 °C for 3 min, the mixture was added to microsome (5 μL) and incubated at 37 °C for 4 min. The change in absorbance at 340 nm was also recorded. The phosphoenolpyruvate kinase (PEPCK) activity was determined following the method of Bentle and Lardy.[35] Briefly, the reaction mixture (1 mL) containing the following ingredients was incubated at 25 °C for 1 h: sodium Hepes (72.92 mM; pH 7.0), dithiothreitol (10 mM), $NaHCO_3$ (500 mM), $MnCl_2$ (10 mM), NADH (25 mM), IDP (100 mM), PEP (200 mM), malic dehydrogenase (7.2 units), and cytosol (10 μL). Enzyme activity was based on the decrease in absorbance at 350 nm at 25 °C.

Histopathology of Liver and Pancreas Tissues and Immunohistochemistry of Pancreas Tissues. For histological analysis, the liver and pancreas tissues were fixed with 4% paraformaldehyde in 0.5 M phosphate buffer (pH 7.4). The tissues were then rinsed with water, dehydrated with ethanol, and embedded in paraffin. The samples were sectioned into 4 μm and mounted onto glass slides. The sections were dewaxed using xylene and ethanol and then stained with hematoxylin and eosin Y (H&E) to reveal the necrosis in the liver or integrity of the islet of Langerhans of the pancreas.

For immunohistochemical analysis, the deparaffinized pancreas sections were treated with 3% H_2O_2 to block the undesirable effects of endogenous peroxidase. Following incubation with 10% fetal calf serum in PBS to reduce background staining and nonspecific antibody binding, the samples were incubated with antimouse insulin antibody (Biogenex, Fremont, CA) and then labeled with horseradish peroxidase (HRP)-conjugated anti-IgG antibody (Abcam, Cambridge, MA). After being washed, the tissue sections were incubated in diaminobenzidine (DAB) solution, followed by counter-staining with 0.2% modified Harris hematoxylin solution (Sigma-Aldrich). For histopathological and immunohistochemical examination, representative samples (n = 3) were selected from the tissue samples (n = 10), and observations was carried out under the microscope at 100× magnification.

Reverse Transcription (RT) PCR of Cellular RNA. Total cellular RNA was prepared from liver and adipocyte tissues following the acid phenol guanidium thiocyanate-chloroform extraction method of Chomczynski et al.[36] For reverse transcription, total RNA (1 μg) was incubated with AMV reverse transcriptase (5 U) and oligo (dT18) as primer (100 ng), except that a 6-mer random primer (IDT, Coralville, IA) was used for the reverse transcription of PEPCK gene. DNA amplification was then primed in a reaction mixture containing dNTP mix (400 μM), *Taq* polymerase (2.5 U), and primer sets (20 μM each) representing target genes (Table 2). PCR was conducted using a thermocycler (model PTC-200, MJ Research Inc., Reno, NV) with one cycle for 5 min at 94 °C, followed by 30 cycles for 30 s at 94 °C, 45 s at 58 °C, and 45 s at 72 °C, and finally one cycle for 5 min at 72 °C. All amplified PCR products were subjected to 1.5% agarose gel electrophoresis and visualized with a UV illuminator. The intensity of separated bands under DNA was quantified using a gel documentation system (model LAS-1000CH, Fuji Photo Film Co., Tokyo, Japan).

Statistical Analysis. Results are expressed as the mean ± SD of three separate experiments. Significant differences between means were determined by ANOVA test using the Statistical Analysis Software package SAS (Cary, NC). $p < 0.05$ is regarded as statistically significant.

Table 2. Primer Sets Representing Target Genes and the Internal Control β-Actin Gene

primer	sequence
tumor necrosis factor-α (TNF-α) sense	5′-TACTGAACTTCGGGGTGATCGGTCC-3′
TNF-α antisense	5′-CAGCCTTGTCCCTTGAAGAGAACC-3′
interleukin-1β (IL-1β) sense	5′-GTAGCCCACGTCGTAGCAAA-3′
IL-1β antisense	5′-CCCTTCTCCAGCTGGGAGAC-3′
interleukin-6 (IL-6) sense	5′-GAAATGATGGATGCTTCCAAACTGG-3′
IL-6 antisense	5′-GGATATATTTTCTGACCACAGTGAGG-3′
peroxisome proliferator-activated receptor (PPAR)-γ sense	5′-CGCTGATGCACTGCCTATGA-3′
PPAR-γ antisense	5′-AGAGGTCCACAGAGCTGATTCC-3′
glucose transporter 2 (GLUT-2) sense	5′-GGCTAATTTCAGGACTGGTTC-3′
GLUT-2 antisense	5′-TTTCTTTGCCCTGACTTCCT-3′
glucose transporter 4 (GLUT-4) sense	5′-CCTGCCCGAAAGAGTCTAAAGC-3′
GLUT-4 antisense	5′-ACTAAGAGCACCGAGACCAACG-3′
glucose-6-phosphatase (G6 Pase) sense	5′-AAGACTCCCAGGACTGGTTCATCC-3′
G6 Pase antisense	5′-TAGCAGGTAGAATCCAAGCGCG-3′
glucokinase (GCK) sense	5′-TTCACCTTCTCCTTCCCTGTAAGTCG-3′
GCK antisense	5′-TACCAGCTTGAGCAGCACAAGTCG-3′
phosphoenolpyruvate carboxykinase (PEPCK) sense	5′-TGCTGATCCTGGGCATAACTAACC-3′
PEPCK antisense	5′-TGGGTACTCCTTCTGGAGATTCCC-3′
B-actin sense	5′-GTGGGGCGCCCCAGGCACCA-3′
B-actin antisense	5′-GTCCTTAATGTCACGCACGATTTC-3′

RESULTS AND DISCUSSION

Effects of RHSE on Body and Organ Weight Gain. The initial body weights of mice, prior to feeding with experimental diets, did not significantly differ, and daily food consumption was also similar among the mice on control, high-fat diet, and RHSE-supplemented high-fat diets (Table 3). At the end of the experimental period, however, a significant increase in the body of high-fat control mice was observed as compared to normal control mice (14% increase). On the other hand, the mice fed a high-fat diet supplemented with 1% RHSE had a lower body weight comparable to the level of normal control group. These results indicate that diets supplemented with RHSE suppressed body weight gain induced by a high-fat diet.

The liver weight was 44% higher in HFD control mice as compared to that of normal control mice. Although the weight of white adipose tissue was lowest in the control mice, a considerable increase in the body fat was observed in HFD control mice (about a 3-fold increase). However, supplementation with 0.5% and 1% RHSE reduced the amount of adipose tissue by 50% and 68%, respectively, as compared to that of HFD control mice. The cited data suggest that RHSE has the potential to control obesity.

Effect of RHSE on Blood Glucose Level, Oral Glucose Tolerance, and Serum Insulin Concentration. The blood glucose value of HFD control mice was 71% higher than that of the normal control mice (Table 4). Supplementation with 0.5% and 1% RHSE, however, lowered blood glucose levels by 33% and 38%, respectively, as compared to that of the HFD control.

Table 3. Body Weight Gain, Weights of Liver, and Adipose Tissue in Mice Fed with High-Fat Diet Supplemented with Rice Hull Smoke Extract (RHSE)[a]

experiment	initial weights (g)	final weights (g)	liver weights (g)	white adipose tissue weights (g)	feed intakes (g/day)
normal control (PBS)	24.42 ± 2.30 a	31.30 ± 1.85 b	1.87 ± 0.25 b	0.43 ± 0.06 c	3.23 ± 0.23 a
HFD-control	25.06 ± 0.52 a	35.72 ± 2.46 a	2.70 ± 0.17 a	1.80 ± 0.27 a	3.16 ± 0.06 a
HFD-0.5% RHSE	25.52 ± 0.67 a	33.00 ± 1.70 ab	2.00 ± 0.20 b	0.90 ± 0.10 b	3.02 ± 0.23 a
HFD-1% RHSE	25.36 ± 1.33 a	30.00 ± 3.66 b	1.97 ± 0.23 b	0.57 ± 0.21 bc	3.02 ± 0.25 a

[a]Data are expressed as the mean ± SD (n = 10). Values in each column with the same letter are not significantly different between groups at $p < 0.05$. RHSE indicated rice hull smoke extract. HFD indicates high-fat diet.

Table 4. Blood Glucose and Serum Insulin Level in Mice on a High-Fat Diet Supplemented with Rice Hull Smoke Extract (RHSE)[a]

experiment	blood glucose (mg/dL)	serum insulin (ng/mL)
normal control (PBS)	117.667 ± 4.163 c	0.832 ± 0.051 a
HFD control	201.333 ± 3.512 a	0.401 ± 0.023 d
HFD-0.5% RHSE	135.667 ± 4.041 b	0.527 ± 0.048 c
HFD-1% RHSE	124.333 ± 6.658 c	0.673 ± 0.052 b

[a]Data are expressed as the mean ± SD (n = 10). Values in each column with the same letter are not significantly different between groups at $p < 0.05$.

By contrast, serum insulin levels showed a significant increase in the mice groups fed with 0.5% and 1% RHSE-supplemented diet (39% and 111% increases, respectively, as compared to the HFD control mice). The dietary supplementation with RHSE also improved glucose tolerance in mice (Figure 1). Blood glucose levels at 30, 60, 90, and 120 min after oral glucose loading were significantly lower in the mice groups fed a diet supplemented with 0.5% and 1% RHSE ($p < 0.05$ or $p < 0.01$) as compared to that of HFD control mice. These results suggest that RHSE has the potential to reduce serum glucose levels.

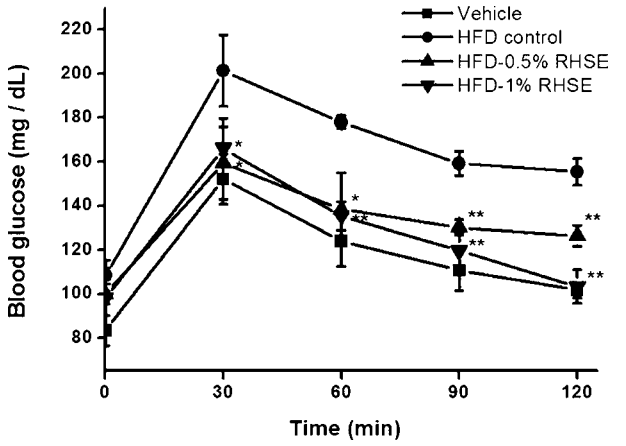

Figure 1. Effect of 0.5% and 1% RHSE on glucose tolerance in mice fed a high-fat diet. Values are means for 10 mice with SD represented by vertical bars. The mean value was significantly different from that of the HFD control group at *$p < 0.05$ or **$p < 0.01$.

Effects of RHSE on Serum Lipids Levels. Feeding mice a high-fat diet resulted in a significant increase in their serum triglyceride and total cholesterol levels (Table 5). The serum triglyceride concentrations were lower in the mice groups fed 0.5% and 1% RHSE-supplemented diets than in the HFD control group (15% and 33% decreases, respectively). Similarly, total cholesterol levels were decreased by the dietary supplementation with RHSE in a dose-dependent manner (7% and 24% decreases in the mice fed with 0.5% and 1% RHSE-supplemented diets, respectively). As compared to the HFD control group, RHSE-supplemented diets induced dose-dependent increases in so-called good HDL and decreases in bad LDL cholesterol concentrations. These results indicate that RHSE has the potential to increase good HDL and reduce bad LDL cholesterol levels associated with obesity.

Effects of RHSE on High-Fat Diet-Induced Liver Injury. Figure 2 shows that the HFD control group exhibited markedly increased concentrations of GOT and GTP, indicating the induction of severe liver damage. Feeding a RHSE-supplemented diet markedly reduced serum levels of these enzymes. With 1% RHSE, GOT and GTP levels were restored to almost the same level as that of the normal control group. Histopathology revealed necrotic liver injuries in HFD diabetic control mice. Minimal liver damage was observed, however, in mice fed 0.5% and 1% RHSE. The cited results suggest the RHSE has the potential to protect against liver necrosis associated with obesity.

Effects of RHSE on Glucose-Regulating Enzyme Activities. Table 6 shows that the hepatic GCK activity was 67% and 132% higher in mice fed 0.5% and 1% RHSE, respectively, relative to that of HFD control mice. As compared to the normal control diets supplemented with PBS buffer (vehicle), the diabetic HFD control group showed an approximately 2-fold increase in G6 Pase activity as compared to the normal control. PBS was used to supplement the control diet because it was used to dilute evaluated RHSE concentrations. By contrast, the dietary supplementation with 1% RHSE suppressed the rise of the enzyme activity to the

Table 5. Lipid Profile in Sera from Mice Fed on a High-Fat Diet Supplemented with Rice Hull Smoke Extract (RHSE)[a]

experiment	triglyceride (mg/dL)	total cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)
normal control (PBS)	125.67 ± 11.59 c	146.67 ± 7.02 bc	86.00 ± 10.54 a	30.00 ± 1.00 ab
HFD control	170.00 ± 11.53 a	176.33 ± 9.29 a	60.00 ± 7.94 c	36.33 ± 3.06 a
HFD-0.5% RHSE	144.67 ± 11.24 b	163.67 ± 16.92 ab	63.00 ± 7.81 bc	32.20 ± 1.13 ab
HFD-1% RHSE	113.33 ± 7.10 c	134.67 ± 10.50 c	77.33 ± 8.62 ab	24.33 ± 3.51 b

[a]Data are expressed as the mean ± SD (n = 10). Values in each column with the same letter are not significantly different between groups at $p < 0.05$.

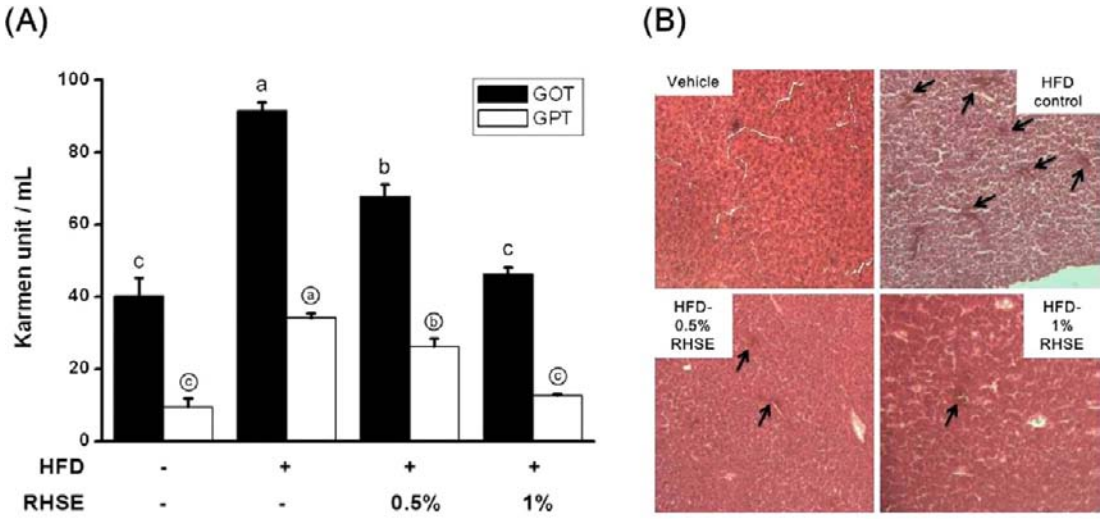

Figure 2. Modulation of liver injury in mice fed a high-fat diet by RHSE. (A) Dietary RHSE suppresses serum glutamic oxaloacetic transaminase/glutamic pyruvic transaminase (GOT/GPT) activities. Data are expressed as the mean ± SD (n = 10). Bars sharing a common letter are not significantly different at $p < 0.05$. (B) RHSE ameliorates liver damage in a dose-dependent manner. Each liver specimen was fixed with 4% paraformaldehyde, and sections were stained with hematoxylin and eosin (H&E). Magnification, ×100. Arrows indicate necrotic lesions in the liver. Figures represent results from at least three separate experiments.

Table 6. Hepatic Glucose-6-Phosphates (G6 Pase), Hepatic Glucokinase (GCK), and Phosphoenolpyruvate Carboxykinase (PEPCK) Enzyme Activities in Mice Fed a High-Fat Diet Supplemented with Rice Hull Smoke Extract (RHSE)[a]

	enzyme activity (nmol/min/mg protein)		
experiment	GCK	PEPCK	G6 Pase
normal control (PBS)	13.58 ± 0.99 a	28.49 ± 3.52 c	65.72 ± 2.49 d
HFD control	5.65 ± 0.66 c	45.93 ± 2.45 a	198.25 ± 3.99 a
HFD-0.5% RHSE	9.35 ± 0.55 b	39.44 ± 1.58 b	118.36 ± 5.36 b
HFD-1% RHSE	12.99 ± 0.79 a	29.79 ± 3.25 c	78.65 ± 4.21 c

[a]Data expressed as the mean ± SD (n = 5). Values in each column with the same letter are not significantly different between groups at $p < 0.05$.

normal level. Similarly, the 1% RHSE-supplemented diet also suppressed the HFD-induced rise of PEPCK activity to the normal control level. RT-PCR analysis indicates that an alteration in these hepatic glucose-regulating enzyme activities was closely associated with the extent of expression of associated genes (Figure 3A). The cited data suggest that RHSE has the potential to suppress genes and glucose-regulating enzymes associated with diabetes.

Effects of RHSE on Expression of Glucose Transporters and PPAR-γ. Changes in glucose transporters and PPAR-γ in liver and adipose tissues were evaluated by RT-PCR. The mRNA expression of hepatic GLUT2 and PPAR-γ was 49% and 25% greater in the HFD-1% RHSE group than in the HFD control group (Figure 3B). Similarly, the expression of adipose mRNA expression of GLUT4 and PPAR-γ was increased more than 2-fold higher in HFD-1% RHSE group as compared to the HFD control group (Figure 3C). The augmented expression of glucose transporters in liver and adipose tissues might have resulted from the increased expression of PPAR-γ in both tissues.[37]

Effects of RHSE on Pro-inflammatory Cytokine Expression. To find out whether inflammatory damage of β-cells could be ameliorated by the dietary supplementation with RHSE, changes in the expression profiles of the pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 were assessed by ELISA in serum and adipose tissue extracts. Table 7 shows that, as compared to the HFD control, the 1% RHSE treatment suppressed in a dose-dependent manner TNF-α, IL-1β, and IL-6 by about 50%, 80%, and 55%, respectively, in serum, or by about 67%, 57%, and 57%, respectively, in adipose tissue. These results suggest a possible mechanism by which RHSE changes the expression of pro-inflammatory cytokine biomarkers associated with diabetes.

Effects of RHSE on β-Cell Integrity in the Pancreatic Islet of Langerhans. The histopathology of tissues showed normal acini and normal cellular populations in the islet of Langerhans of the pancreas of normal control mice (Figure 4A). By contrast, feeding a HFD induced a reduction in the cell population in the islet of Langerhans. The reduction was determined by approximate cell counting in the stained tissue section under a microscope. The cell populations, including β-cells, were restored to normal levels following RHSE treatment. To clarify the effect of RHSE supplementation on the insulin-producing β-cell population, immunohistochemistry using an antimouse insulin antibody was performed. The results show a dense staining intensity in the islet of Langerhans of normal control mice, indicating integrity of the insulin-producing β-cell population (Figure 4B). By contrast, feeding a HFD induced a reduction in the β-cell population together with a low level of insulin production. The β-cell populations were, however, restored to normal levels following RHSE supplementation in a dose-dependent manner. These results provide direct evidence for the observed increased insulin production induced by RHSE in the pancreas.

Dietary Significance. The results of the present study suggest that RHSE suppressed HFD-induced T2D and associated biomarkers. Taken together, the described findings from oral feeding studies on pro-inflammatory biomarkers, cytokine gene expression, glucose-metabolizing enzymes,

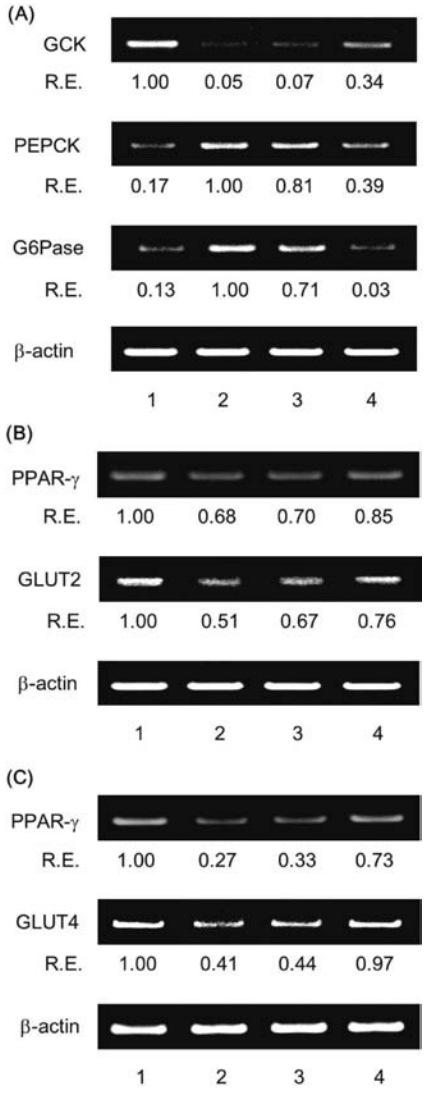

Figure 3. RT-PCR analysis of RHSE-modulated gene expressions for the synthesis of representative molecules involved in glucose metabolism in mice fed a high-fat diet. (A) Expression of the genes for enzymes regulating hepatic glucose metabolism. (B and C) Expression of the genes for the transcriptional regulator and glucose transport in liver and adipose tissue, respectively. The relative ratio of expression of each gene is expressed as a relative expression (R.E.) value calculated from target gene/β-actin gene expression. GCK, PEPCK, G6 Pase, PPAR-γ, and GLUT indicate glucokinase, phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, peroxisome proliferator-activated receptor-γ, and glucose transporter, respectively. Lane 1, vehicle; lane 2, HFD control; lane 3, HFD-0.5% RHSE; lane 4, HFD-1% RHSE. Figures represent results from at least three separate experiments.

glucose transporters, histology, cytochemistry, immunohistochemistry, and plasma insulin, cholesterol, and triglyceride levels indicate that the protective effect of RHSE against type 2 diabetes in the mouse can be attributed to the blockage of oxidative stress-induced damage of islet of Langerhans β-cells of the pancreas and improved metabolism of glucose in the liver.

On the basis of the description of biomarkers associated with causes and prevention of diabetes in the Introduction, the strong antioxidative effect of RHSE might inhibit oxidative stress induced by HFD, resulting in the observed reduction of fat accumulation and tissue damage, and in an increase in glucose uptake and corresponding decrease in blood glucose levels. The anti-inflammatory effect of RHSE observed in the present study is reinforced by our previous finding that dietary RHSE inhibited skin edema induced by the 12-*O*-tetradecanolylphorbol-13-acetate (TPA).[25] The data also suggest that RHSE reduces glucose levels via both decreased insulin resistance and increased insulin production. Improvement in insulin resistance could explain the observed increase in HDL cholesterol and decreases in LDL cholesterol and triglyceride levels.

Although we did not measure oxidative stress directly, indirect evidence for oxidative stress includes the observed elevation of GOT and GTP enzyme levels in the liver and adverse effects on the islet of Langerhans tissues in the mice fed a high-fat diet. Because chronic inflammation induced by a high-fat diet adversely affects pancreatic as well adipose, brain, liver, and other tissues, the observed decreases in pro-inflammatory cytokines induced by RHSE treatment may also reduce injury to multiple organs.[38]

The results suggest that functional food supplemented with rice hull extract might contribute to the prevention and management of diabetes. Because the previous and the present studies show that the rice hull smoke extract has antimicrobial and anti-inflammatory properties, functional food treated with rice hull smoke could have anti-inflammatory and antimicrobial effects as well. We do not know which of the 161 characterized compound in RHSE[25] are responsible for the observed multiple anti-inflammatory, antimicrobial, and antidiabetic beneficial effects. Finally, based on the limited observations in the previous and present studies, RHSE seems to be "safe" for mice. However, the safety aspects merit further study.

The >100 million tons of rice hulls produced worldwide provides a new source of bioactive compounds derived from an agricultural byproduct that merit further evaluation for their potential to impart health-promoting effects to food. Whether RHSE can inhibit the formation hemoglobin−glucose adducts in vivo and protect/prevent against human diabetes merits study.

Table 7. Pro-inflammatory Cytokines Concentrations in Serum and Adipose Tissue in Mice Fed a High-Fat Diet Supplemented with Rice Hull Smoke Extract (RHSE)[a]

	serum			adipose tissue		
experiment	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)
normal control (PBS)	3.11 ± 0.81 c	15.57 ± 0.36 d	17.63 ± 1.44 d	28.11 ± 0.14 c	61.30 ± 4.11 c	82.21 ± 2.61 d
HFD control	16.35 ± 2.70 a	102.54 ± 0.89 a	56.77 ± 3.31 a	76.76 ± 1.49 a	164.74 ± 10.76 a	239.82 ± 9.61 a
HFD-0.5% RHSE	10.41 ± 0.27 b	63.79 ± 1.07 b	42.72 ± 1.24 b	49.32 ± 4.87 b	138.36 ± 1.61 b	158.77 ± 4.45 b
HFD-1% RHSE	4.87 ± 0.68 bc	20.21 ± 1.79 c	25.34 ± 1.11 c	25.41 ± 1.22 c	70.19 ± 2.10 c	102.88 ± 3.60 c

[a]Data expressed as the mean ± SD (n = 5). Values in each column with the same letter are not significantly different between groups at $p < 0.05$.

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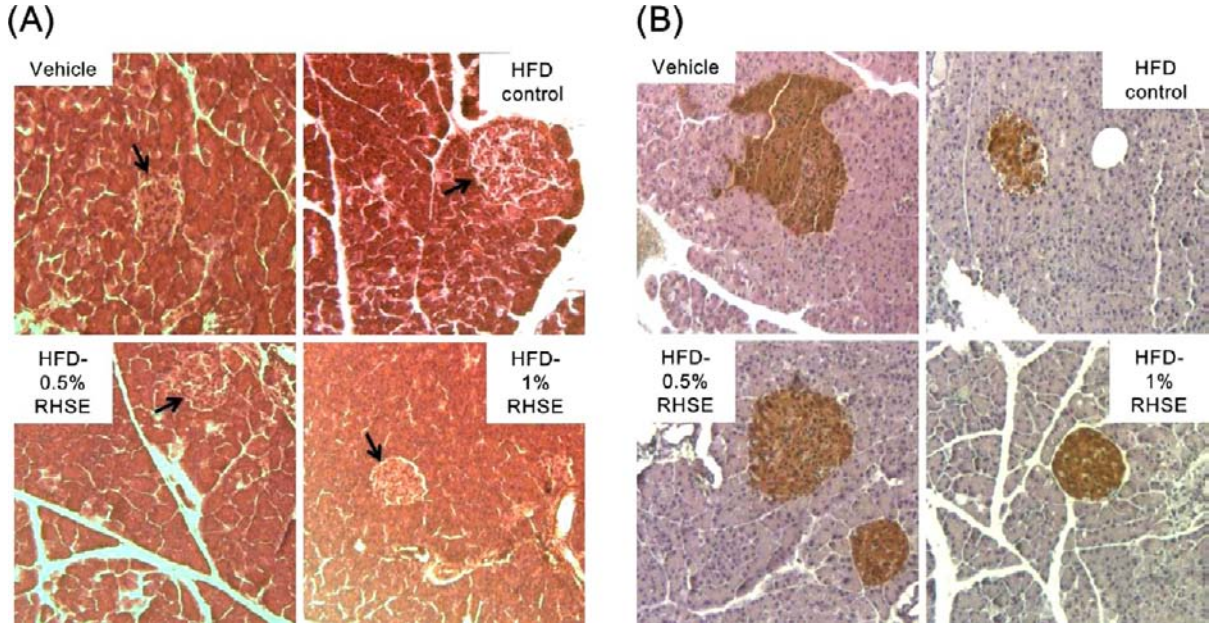

Figure 4. Inhibitory effect of RHSE on atrophy of islet of Langerhans of the pancreas. (A) To observe morphology of islet of Langerhans, paraformaldehyde-fixed and paraffin-embedded pancreas sections were stained with hematoxylin and eosin Y (H&E). (B) The observation of dose-dependent restoration of insulin production in islet of Langerhans by immunostaining with anti-insulin antibody. Magnification, ×100. Arrows indicate islet of Langerhans. Figures represent results from at least three separate experiments.

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Notes

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ABBREVIATIONS USED

AMPK, AMP-activated protein kinase; ELISA, enzyme-linked immunosorbent assay; GCK, hepatic glucokinase; GOT, glutamate oxoloacetate transaminase; GPT, glutamic pyruvic transaminase; G6 Pase, glucose-6-phosphatase; GLUT-2, glucose transporter-2; GLUT-4, glucose transporter-4; HDL, high-density lipoprotein; HFD, high-fat diet; 4-HNE, 4-hydroxy-2E-neonenal; HRP, horse radish peroxidase; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; JNK, c-jun-N-terminal kinase; LDL, low-density lipoprotein; LPS, likpopolysaccharide; NO, nitric oxide; PBS, phosphate-buffered saline; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase; PI3K, phosphatidylinositol 3-kinases; PKC-α, protein kinases C-α; PPAR-γ, peroxisome proliferator-activated receptor-γ; R.E., relative expression of genes and proteins; RHSE, rice hull smoke extract; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α; UCP 2, uncoupling protein 2

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