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Gravinol Ameliorates High-Fructose-Induced Metabolic Syndrome through Regulation of Lipid Metabolism and Proinflammatory State in Rats

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Using a rat model with fructose-induced metabolic syndrome, the effect of gravinol was investigated. Male Wistar rats were fed a 65% fructose diet and administered 10 or 20 mg of gravinol/kg of body weight/day for 2 weeks. High-level fructose feeding led to hyperglycemia, hyperlipidemia, hypertriglyceridemia, and hypertension. On the other hand, the administration of gravinol significantly lowered serum glucose and total cholesterol levels. The tail arterial blood pressure was significantly elevated with the high-fructose diet. However, rats given gravinol showed a lower blood pressure as compared with fructose-fed control rats. In addition, the triglyceride (TG) levels in serum and lipoprotein fraction were dose-dependently reduced in rats fed gravinol. The decreases of hepatic TG and total cholesterol by gravinol were responsible for the down-regulation of hepatic sterol regulatory element binding protein (SREBP)-1. However, gravinol did not affect the protein levels of hepatic peroxisome proliferator-activated receptor-α and SREBP-2. Moreover, gravinol administration in the fructose-fed rats markedly reduced the glycosylated protein and thiobarbituric acid-reactive substance levels in the serum and hepatic mitochondria, and it inhibited the increase of the cyclooxygenase-2 protein level as a result of the down-regulation of nuclear factor kappa B (NF-κB). Furthermore, the decrease of anti-apoptotic bcl-2 protein levels and the increase of pro-apoptotic bax protein levels by the high-fructose diet were reversed by gravinol. These findings suggest that fructose-induced metabolic syndrome is attenuated by gravinol administration, which is associated with the reduction of serum lipids and protection against the proinflammatory state induced by oxidative stress.

KEYWORDS: Gravinol; high fructose; metabolic syndrome; oxidative stress; proinflammatory state

INTRODUCTION

Interest in metabolic syndrome including obesity, dyslipidemia, hypertension, and insulin resistance has grown worldwide. In particular, coronary heart disease (CHD) is the leading cause of mortality in people with metabolic syndrome (*1*). Metabolic syndrome is associated with fructose consumption, which has largely increased over the past few decades as a result of the use of high-fructose corn syrup, a substitute for sucrose. High-level fructose consumption contributes to obesity and insulin resistance, hypertension, dyslipidemia, and a decline in the level of high-density lipoprotein (HDL) cholesterol (*2–4*). Therefore, such metabolic modifications have been associated with multiple risk factors for cardiovascular diseases.

The possibility that dietary fructose facilitates metabolic derangement and induces oxidative damage is supported by recent numerous studies (*5–8*). In humans and rats, fructose is more lipogenic than glucose or starch, resulting in an increase of hepatic triglyceride (TG) synthesis and very low-density lipoprotein (VLDL) overproduction (*9, 10*). A high-fructose diet induces an increase of sterol regulatory element binding protein (SREBP)-1 and a decrease of peroxisome proliferator-activated receptor α (PPARα); thus, it can contribute to the elevation of lipogenesis and the reduction of fatty acid β-oxidation, leading to cellular lipid accumulation (*11–13*). Furthermore, the burden of high-level fructose metabolism may generate stress-activating molecules via pro-oxidant stress response pathways, and the pathological conditions of metabolic syndrome are associated with the inflammatory process (*7*). Therefore, under the conditions of metabolic syndrome, the expression of proteins related to oxidative stress and inflammatory processes, including nuclear factor kappa B (NF-κB), bcl-2, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2), is altered.

Although the positive effects of natural products on metabolic syndrome have been expected, investigations to search for phytochemicals with such protective effects have rarely been conducted. Gravinol is a proanthocyanidin from grape seeds, existing as an oligomer or polymer of polyhydroxy flavan-3-ol

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Table 1. Composition (Grams per 100 g) of the Diets

ingredient	normal diet	high-fructose diet
casein	20	20
corn starch	65	
fructose		65
corn oil	5	5
salt mixture	4	4
vitamin mixture	1	1
cellulose powder	4.5	4.5
DL-methionine	0.3	0.3
choline bitartrate	0.2	0.2

units. It plays an important role in protection from various pathological conditions including renal ischemia-reperfusion injury (*14*), ulcers and gastric mucosal injury (*15*), cancer (*16*), diabetes (*17*), and aging (*18*). However, the protective activity of gravinol against metabolic syndrome along with its related mechanisms has not yet been studied. On the basis of this evidence, the present study was focused on the protective effect of gravinol using a rat model with fructose-induced metabolic syndrome.

MATERIALS AND METHODS

Materials. Gravinol was obtained from Kikkoman Corp., Noda, Chiba, Japan. The following reagents were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan: 4,6-dihydroxy-2-mercaptopyrimidine [2-thiobarbituric acid (TBA)], bovine serum albumin (BSA), 2-amino-2-hydroxymethyl-1,3-propanediol [tris(hydroxymethyl)aminomethane], Tween 20, phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail, and skim milk powder. Precision plus protein standards and the Bio-Rad protein assay kit were purchased from Bio-Rad Laboratories, Japan. Rabbit anti-human bax, mouse anti-mouse bcl-2, rabbit anti-human NF-κB p65 polyclonal antibody, rabbit anti-human inhibitor binding protein κB-α (IκB-α) polyclonal antibody, mouse anti-mouse NOS2 monoclonal antibody (primary antibody for iNOS), mouse anti-human COX-2 monoclonal antibody, goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody, and goat anti-mouse IgG HRP-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Anti-mouse β-actin antibody was purchased from Sigma-Aldrich, St. Louis, MO. ECL Western blotting detection reagents were purchased from Amersham Bioscience, Piscataway, NJ.

Animals and Treatment. The *Guidelines for Animal Experimentation* approved by the University of Toyama were followed during these experiments. Male Wistar rats, 6 weeks old, were obtained from Japan SLC Inc. (Hamamatsu, Japan). They were maintained with food and water ad libitum at a constant humidity and temperature with a light/dark cycle of 12 h. After acclimation, the animals were randomized into two groups: a normal group without supplementary fructose and a high-fructose-supplemented group (65% of the diet for 1 week; **Table 1**). After approximately 1 week on the high-fructose diet, rats were screened for the induction of hypertriglyceridemia (serum TG concentration >170 mg/dL) by obtaining blood from the tail vein. Rats were divided into three groups by the body weight and serum TG level. The three groups were supplemented with a high-fructose diet, and gravinol at 10 or 20 mg/kg of body weight/day was orally administered by daily oral gavage for 2 subsequent weeks. Gravinol was dissolved in water, and the total volume administered was adjusted to 1 mL. A normal group of rats was also included. Each experimental group contained seven or eight rats. During the experimental period, dietary consumption was kept at the same level (16 g/rat). At the end of the study, the rats were decapitated, their blood was drawn, and serum was collected by centrifuging the blood at 1000*g* for 15 min at 4 °C. The liver and epididymal fat were removed, dried on tissue paper, weighed, and stored at −80 °C until analysis.

Determination of Serum Components. The levels of serum glucose, TG, and total cholesterol were determined using a commercial reagent (Glucose CII-Test Wako, Triglyceride E-Test Wako and Cholesterol E-Test Wako obtained from Wako Pure Chemical Industries, Ltd.). Serum glycosylated protein and TBA-reactive substance levels were measured using the methods of McFarland et al. (*19*) and Naito and Yamanaka (*20*), respectively.

Isolation of Lipoproteins. Lipoproteins were isolated from serum using density-gradient ultracentrifugation, as described by Havel et al. (*21*). Lipoprotein fractions were isolated from 4 mL of serum using a Beckman Optima XL-70 ultracentrifuge and a 70.1 Ti rotor operating at 160000*g*. Serum was transferred to a tube, and the density was adjusted to 1.006, 1.019, or 1.063 g/mL with the same volume of KBr solution. Serum was divided into three lipoprotein classes by density: VLDL ($d = 1.006$); intermediate-density lipoprotein (IDL, $1.006 < d < 1.019$); low-density lipoprotein (LDL, $1.019 < d < 1.603$). The appropriate times were calculated to be 16 h for VLDL, 18 h for IDL, and 20 h for LDL isolation at 4 °C.

Measurement of Hepatic TG and Total Cholesterol Contents. The liver of each rat was homogenized, and total lipids were extracted with a mixture of chloroform and methanol (2:1, v/v) according to the method of Folch et al. (*22*), and the contents of TG and total cholesterol were determined using the Wako kit described above.

Measurement of Blood Pressure. At the end of the experiment, blood pressure was measured by the tail-cuff method using an automatic blood pressure monitoring system (UR-5000, UETA, Tokyo, Japan). The animals were kept at 37 °C for 30 min before measurement of the blood pressure. The average of five consecutive readings for accurate measurements was used for blood pressure evaluation.

Isolation of Hepatic Mitochondria and Measurement of TBA-Reactive Substance Levels. Liver was homogenized with a 9-fold volume of ice-cold 0.9% NaCl solution. Mitochondria were prepared from hepatic homogenates by differential centrifugation (800*g* and 12000*g* at 4 °C for 15 min) according to the methods of Johnson and Lardy (*23*) and Jung and Pergande (*24*), with slight modifications. Each pellet was resuspended in preparation medium, and the TBA-reactive substance concentration was determined according to the method of Buege and Aust (*25*). Briefly, a 250 μL resuspension of each pellet or working standard was added to 750 μL of TBA/TCA/HCl solution (0.4% TBA, 15% TCA, and 2.5% HCl), and it was heated at 95−100 °C for 20 min and cooled in an ice bath. Then, samples were centrifuged at 1000*g* at room temperature for 10 min to transfer supernatants from the denatured protein precipitate. The TBA-reactive substance level was determined by measuring absorbance at 532 nm. The TBA-reactive substance level was expressed in nanomoles of malondialdehyde (MDA) per milligram of protein by a calibration curve constructed from MDA (0−25 nmol/mL) in 1,1,3,3-tetramethoxypropane. The protein level was evaluated according to the method of Itzhaki and Gill (*26*) with BSA as the standard.

Homogenization and Isolation of Cytosol and Nuclear Extracts. Each liver was homogenized using a Potter Elvehjem homogenizer in 4 volumes (w/v) of buffer A containing 25 mM Tris-Cl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol (DTT), and a mixture of protease inhibitors [100 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.08 mM aprotinin, 2 mM leupeptin, 5 mM bestatin, and 1 mM pepstatin A, and 1.5 mM E-64]. Homogenates were incubated for 15 min on ice, added to 10% Nonidet P-40, and then centrifuged at 4000*g* at 4 °C for 5 min. Supernatants were used for iNOS, COX-2, bax, and bcl-2 protein determinations. Nuclear extracts were isolated using the Sakurai et al. (*27*) method. Briefly, liver was homogenized by a Potter Elvehjem homogenizer in 4 volumes (w/v) of buffer A, containing 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and protease inhibitors as above. Homogenates were incubated for 15 min on ice, added to 10% Nonidet P-40, and centrifuged at 4000*g* at 4 °C for 5 min. Supernatants were used for IκB-α protein determination, and pellets were resuspended in 2 volumes of buffer B containing 20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and the protease inhibitors. Homogenates were kept for 15 min at 4 °C and then centrifuged at 14000*g* for 5 min at 4 °C. Supernatants were collected in microcentrifuge tubes and used for PPARα, SREBP-1/2, and NF-κB protein determinations. The protein concentration of homogenates and nuclear extracts was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Each sample (30 μg of protein/lane) was denatured by boiling in Laemmli sample buffer and stored at −80 °C until assaying.

Table 2. Characteristics of Experimental Animals

	normal diet	high-fructose diet[a]	gravinol, 10 mg[a]	gravinol, 20 mg[a]
body wt (g)	231.8 ± 9.2	241.0 ± 4.0a	229.0 ± 0.9f	227.2 ± 4.0f
body wt gain (g)	46.6 ± 3.7	56.9 ± 3.4a	44.7 ± 2.4f	43.5 ± 2.7f
wt of liver (g)	7.23 ± 0.21	10.52 ± 0.50c	8.70 ± 0.39cf	8.66 ± 0.30cf
rel wt of liver (g/100 g of BW)	3.13 ± 0.21	4.34 ± 0.23c	3.85 ± 0.17ce	3.77 ± 0.10cf
wt of epididymal fat pads (g)	2.32 ± 0.10	2.66 ± 0.11a	2.41 ± 0.27	2.37 ± 0.04d
rel wt of epididymal fat pads (g/100 g of BW)	1.01 ± 0.11	1.13 ± 0.04	1.06 ± 0.12	1.03 ± 0.02
fluid intake (mL/rat/day)	26.0 ± 0.8	26.9 ± 0.7	26.5 ± 0.6	26.1 ± 0.5
s-glucose (mg/dL)	171.6 ± 1.7	216.1 ± 4.2c	213.8 ± 4.7e	205.1 ± 8.4cd
s-total cholesterol (mg/dL)	60.9 ± 2.6	82.0 ± 3.5c	79.3 ± 2.4c	76.4 ± 3.8cd
s-triglycerides (mg/dL)	45.1 ± 4.1	244.2 ± 37.9c	121.3 ± 16.8bf	79.9 ± 10.3f
s-VLDL-triglycerides (mg/dL)	22.3 ± 0.7	I77.8 ± 0.2c	93.3 ± 0.5cf	71.9 ± 0.0cf
s-IDL-triglycerides (mg/dL)	6.4 ± 0.5	21.2 ± 0.0c	15.5 ± 0.5cf	10.2 ± 1.3cf
s-LDL-triglycerides (mg/dL)	0.3 ± 0.0	5.1 ± 0.3c	4.1 ± 0.3cf	3.6 ± 0.3cf
systolic blood pressure (mmHg)	114.0 ± 1.0	127.8 ± 2.2c	117.3 ± 3.7f	114.3 ± 3.8f
diastolic blood pressure (mmHg)	56.8 ± 4.6	73.3 ± 5.9b	62.6 ± 6.6d	71.1 ± 3.9b
heart rate (beats/min)	487.4 ± 8.2	488.9 ± 6.6	491.6 ± 10.8	500.9 ± 12.2

[a] Significance: a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$, vs normal rats. Significance: d, $p < 0.05$; e, $p < 0.01$; f, $p < 0.001$, vs high-fructose-diet-fed control rats.

Western Blot Analysis. Western blot analysis was carried out using 30 μg of protein for iNOS, COX-2, bcl-2, and bax, cytosol extract for IκB-α, and crude nuclear extract for PPARα, SREBP-1/2, and NF-κB from the liver. The proteins were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (10% w/v), and the separated proteins were blotted onto nitrocellulose (Bio-Rad, Hercules, CA). Blots were blocked overnight at 4 °C with 5% nonfat dry milk in TBS-T [25 mM Tris-Cl (pH 8.3), 140 mM NaCl, 2 mM KCl, and 0.1% Tween 20]. Membranes were then incubated for 3 h at 4 °C with the primary polyclonal antibody raised against NF-κB, IκB-α, bax, PPARα, and SREBP-1/2 (dilution, 1:1000) and monoclonal antibodies against iNOS, COX-2, bcl-2 (dilution, 1:1000), and β-actin (1:5000) (antibodies from Santa Cruz Biotechnology). After extensive washing, incubation with the secondary antibody (rabbit polyclonal or mouse monoclonal antibody) at a dilution of 1:1000 (Santa Cruz Biotechnology) was also performed for 40 min at room temperature. Specific protein was detected by enhanced chemiluminescence (ECL, Amersham International), evaluated by densitometry (Molecular Dynamics), and quantified by a Phosphor Imager (Bio-Rad Laboratories).

Statistical Analysis. Results are expressed as means ± SE. The effect on each parameter was examined using one-way analysis of variance. Individual differences between groups were evaluated using Dunnett's test, and those at $p < 0.05$ were considered to be significant.

RESULTS

Characteristics of Experimental Animals. As shown in **Table 2**, the body weight in the high-fructose-diet-fed control rats was significantly increased as compared to normal-diet-fed rats, from 231.8 to 241.0 g, but it was significantly decreased by the oral administration of gravinol. The body weight gain also showed similar variations. In addition, compared with the normal-diet-fed rats, the liver weight was significantly heavier in the high-fructose-diet-fed control rats, whereas gravinol suppressed the fructose-induced increase in liver weight. The increase in weight of the epididymal fat pads was significantly attenuated by gravinol compared with high-fructose-diet-fed control rats. Daily fluid intake was not affected by the high-fructose diet. In addition, the high-fructose diet given to rats significantly increased serum levels of glucose by 25.9% and total cholesterol by 34.6%, but the group fed 20 mg of gravinol showed significant decreases in these levels. Moreover, TG levels in serum and VLDL, IDL, and LDL fractions markedly increased on the high-fructose diet. Such elevated levels were reversed significantly and dose-dependently by gravinol administration. The systolic and diastolic blood pressures were significantly higher in the high-fructose-diet-fed rats than in the normal rats, but it was effectively controlled by gravinol. The heart rate was not significantly different among all groups.

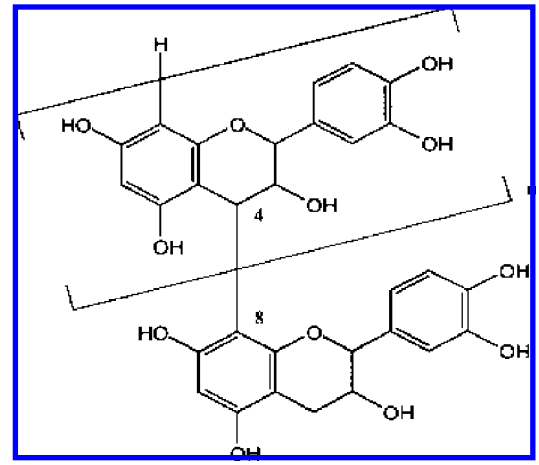

Figure 1. Structure of gravinol.

Lipid Contents and Protein Levels in Liver (PPARα and SREBP-1/2). The TG and total cholesterol contents in liver are summarized in **Figure 2**. Hepatic TG contents in fructose-fed control rats increased by 1.8-fold compared with the normal rats (109.9 vs 60.0 mg/liver/100 g of body weight). Hepatic TG contents were significantly lower in rats fed gravinol at an oral dose of 10 and 20 mg by 26.8 and 32.4%, respectively, than in the high-fructose-diet-fed control rats. In addition, hepatic total cholesterol in the fructose-fed control rats was increased compared with the normal rats from 28.4 to 44.5 mg/liver/100 g of body weight. However, the rats administered 10 and 20 mg of gravinol showed a significant decrease in the hepatic total cholesterol contents by 20.0 and 23.7%, respectively. Furthermore, the effect of gravinol on protein levels involved in lipid metabolism in the nuclei of liver cells was investigated by Western blot analysis (**Figure 3**). Whereas the SREBP-1 level of high-fructose-diet-fed control rats was significantly elevated by 1.9-fold compared with normal rats, that of rats fed gravinol at 10 and 20 mg was significantly decreased by 36.5 and 45.0%, respectively. On the other hand, there was no significant difference in the protein levels of PPARα and SREBP-2 in the nuclei of liver cells among the experimental groups.

Glycosylated Protein and TBA-Reactive Substance Levels in Serum and Hepatic Mitochondria. As shown in **Figure 4**, the serum glycosylated protein level of the high-fructose-diet-fed control rats was significantly increased as compared with that of the normal-diet-fed rats, from 34.5 to 38.3 nmol/mg of protein. However, it was lowered to 33.3 nmol/mg of protein

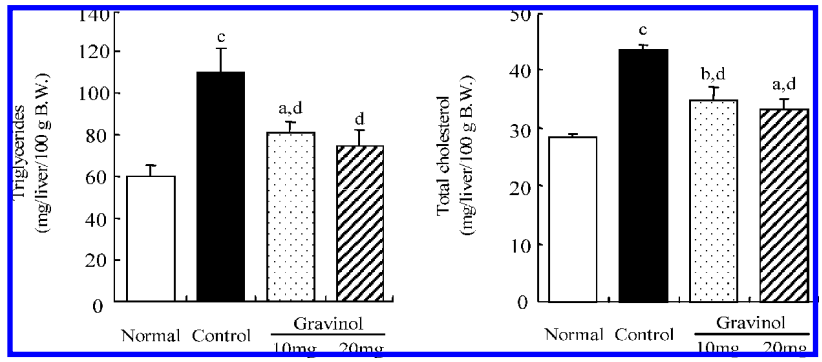

Figure 2. Hepatic TG and total cholesterol contents. Significance: a,$p < 0.05$; b, $p < 0.01$; c, $p < 0.001$, versus normal rats; d, $p < 0.001$, versus high-fructose-diet-fed control rats.

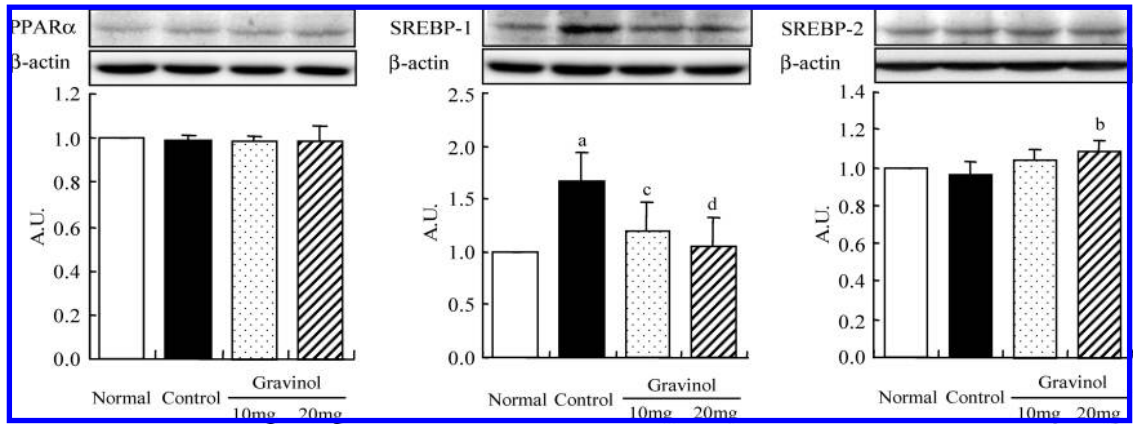

Figure 3. Western blot analysis of expressions of PPARα, SREBP-1, and SREBP-2 in the hepatic homogenate. Significance: a, $p < 0.001$, versus normal rats; b, $p < 0.05$; c, $p < 0.01$; d, $p < 0.001$, versus high-fructose-diet-fed control rats.

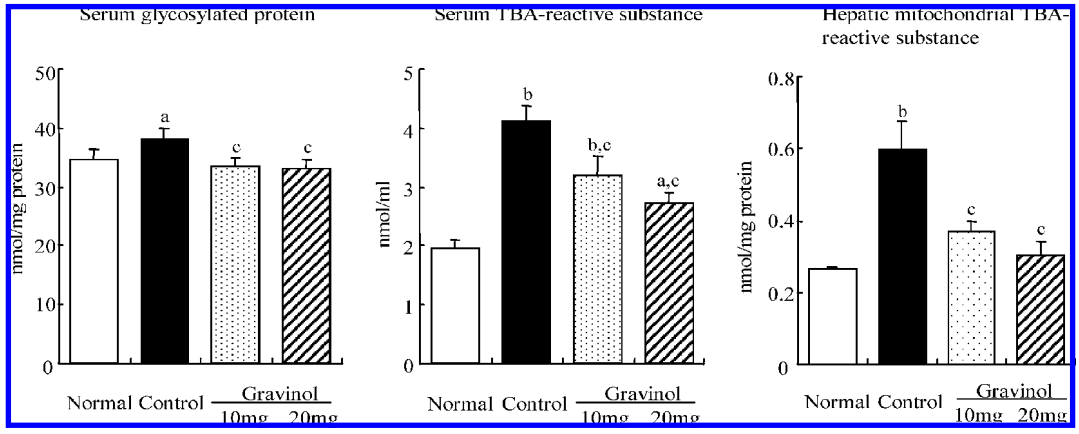

Figure 4. Glycosylated protein and TBA-reactive substance in the serum and hepatic homogenate. Significance: a, $p < 0.05$; b, $p < 0.001$, versus normal rats; c, $p < 0.001$, versus high-fructose-diet-fed rats.

(13.1% decrease) and 33.0 nmol/mg of protein (13.8% decrease) with the administration of gravinol at 10 and 20 mg, respectively. Furthermore, the TBA-reactive substance level of serum was markedly higher in the high-fructose-diet-fed control rats compared with the rats fed a normal diet (4.1 vs 1.9 nmol/mL, respectively). However, the administration of gravinol at an oral dose of 10 and 20 mg/kg significantly lowered the level of serum by 22.1 and 34.2%, respectively, compared to fructose-fed control rats. In addition, the TBA-reactive substance level of hepatic mitochondria was significantly decreased by the oral administration of gravinol.

Protein Levels Involved in Proinflammatory State of Liver. The effects of gravinol on protein levels involved in the proinflammatory state of the liver in high-fructose-diet-fed rats were examined by Western blot analysis (**Figure 5**). Hepatic protein levels of NF-κB showed a significant decrease in groups fed gravinol at 10 and 20 mg compared with those of high-fructose-diet-fed control rats. However, there was no significant difference between the groups in terms of the hepatic IκB-α protein level. The protein level of COX-2 in the liver was decreased in groups fed gravinol at 10 and 20 mg by 24.4 and 26.0%, respectively, compared with the high-fructose-diet-fed control rats. However, the protein level of iNOS was not different between normal and control rats.

Protein Levels Involved in Apoptosis. Figure 6 shows the effect of gravinol on apoptosis under high-fructose diet. Bax protein, the pro-apoptosis factor, in the high-fructose-diet-fed rats was significantly elevated compared with normal-diet-fed rats, whereas the oral administration of gravinol led to significant decreases by 8.6 and 18.0% at doses of 10 and 20 mg/kg of

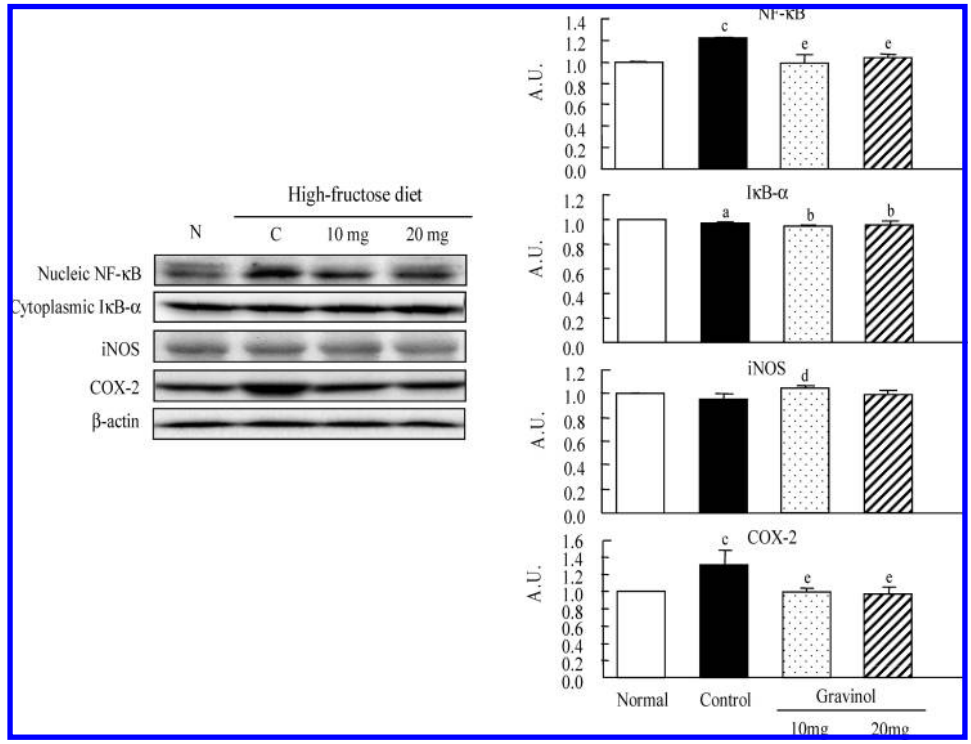

Figure 5. Western blot analysis of expressions of NF-κB, IκB-α, iNOS, and COX-2 in the hepatic nucleus and cytoplasm. Significance: a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$, versus normal rats; d, $p < 0.01$; e, $p < 0.001$, versus high-fructose-diet-fed control rats.

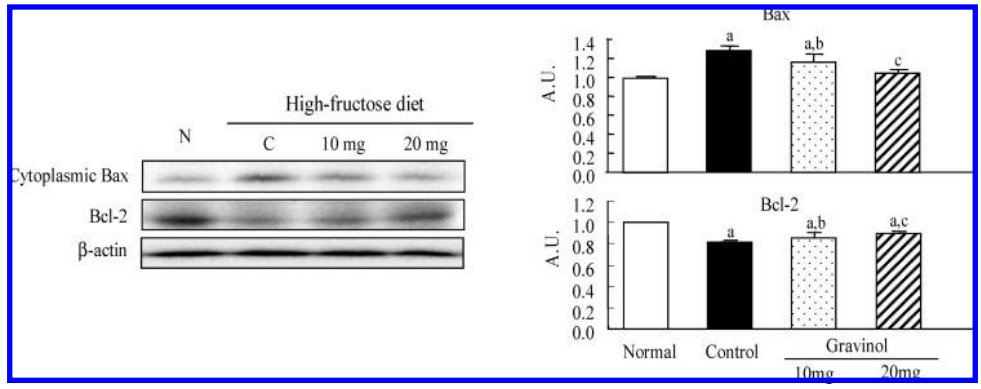

Figure 6. Western blot analysis of expressions of bax and bcl-2 in the hepatic cytoplasm. Significance: a, $p < 0.001$, versus normal rats; b, $p < 0.01$; c, $p < 0.001$, versus high-fructose-diet-fed control rats.

body weight/day, respectively. On the other hand, gravinol attenuated the decrease in bcl-2 protein expression, the anti-apoptosis factor, brought about by the high-fructose diet.

DISCUSSION

A high-fructose diet induces well-characterized metabolic syndrome, typically resulting in hyperinsulinemia, insulin resistance, hypertension, hypertriglyceridemia, and decreased HDL cholesterol (*6–8*). Due to hepatic stress as a result of the burden of fructose metabolism, high-fructose-diet-fed animals exhibit an alteration of lipid metabolism (*7*). Recently, antioxidants derived from natural sources have been reported to show potential effects in the treatment and prevention of metabolic syndrome induced by a high-fructose diet (*28, 29*). Several studies demonstrated the protective effect of gravinol against pathological conditions including cancer, diabetes, and renal injury (*14, 16, 17*). Gravinol is expected to play a promising role in protection against metabolic syndrome; thus, the present study was focused on it, along with its related mechanisms.

In agreement with other studies (*6–8*), the present study also showed that a high-fructose diet induced hyperglycemia, dyslipidemia, and hypertension. In addition, the body, liver, and adipose tissue weights were higher in rats fed a high-fructose diet, whereas the oral administration of gravinol attenuated these changes. The increase of body weight by high-fructose diet was related with stimulation of glycolysis and elevation in synthesis of TG. However, it was attenuated by gravinol administration. It probably resulted from a decrease in lipid synthesis with regulation of the glycolytic pathway. An elevation of blood pressure was also observed in rats fed a high-fructose diet, although the mechanisms underlying fructose-induced hypertension are unknown. It could be related to the levels of endothelin-1, nitric oxide, angiotensin II, and insulin sensitivity (*30–33*). Several studies suggest that, in a model of metabolic syndrome induced by fructose feeding, polyphenols reduce the overproduction of O_2^- by the aorta, indicating that the prevention of high blood pressure by polyphenols could be linked to the prevention of endothelial dysfunction (*28, 29*). The present

results also showed that gravinol regulated the increase of blood pressure by a high-fructose diet (**Table 2**), although its related mechanisms are unclear.

Carbohydrate feeding in rats significantly increases de novo lipogenesis, and fructose has a stronger effect than glucose (*34*). Of key importance is the ability of fructose to bypass the main regulatory step of glycolysis, the conversion of glucose-6-phosphate to fructose 1,6-diphosphate, controlled by phosphofructokinase. Whereas glucose metabolism is negatively regulated by phosphofructokinase, fructose can continuously enter the glycolytic pathway. Therefore, fructose can uncontrollably produce glucose, glycogen, lactate, and pyruvate, providing both the glycerol and acyl portions of acyl-glycerol molecules. These particular substrates and the resultant excess energy flux due to unregulated fructose metabolism will promote hepatic TG synthesis and the overproduction of VLDL (*9*). Moreover, high rates of lipolysis in visceral adipose tissue can increase the availability of free fatty acids and promote hepatic TG synthesis (*10*). Hypertriglyceridemia and TG-rich lipoproteins are part of the metabolic syndrome frequently encountered in individuals with early-onset CHD (*1*). Accordingly, high-level fructose feeding could result in a higher occurrence of aortic atherosclerotic plaques. In the present study, we observed a significant elevation in the serum TG level in fructose-fed rats, suggesting that an increased flux of free fatty acid into the liver may contribute to VLDL overproduction. Gravinol attenuated the increase of hepatic TG, lipoprotein, and total cholesterol contents (**Table 2** and **Figure 2**). These results suggest that gravinol may play a protective role against CHD through attenuation of hypertriglyceridemia and hyperlipidemia induced by a high-fructose diet.

Fatty acid availability for TG synthesis depends not only on the free fatty acid supply to the liver from plasma but also on de novo fatty acid synthesis and β-oxidation. It is well-known that the β-oxidation of fatty acids and the synthesis of fatty acids and TG in the liver are regulated by the nuclear receptor PPARα and SREBP-1, respectively (*11–13*). In addition, SREBP-2 preferentially activates cholesterol synthesis. PPARα plays an important role in the metabolic homeostasis of fatty acids through the regulation of target genes encoding enzymes for fatty acid oxidation and fatty acid transporters (*35, 36*). In the present study, although the increase of serum and hepatic TG levels by fructose feeding corresponded with the increase in hepatic SREBP-1 levels, the levels of PPARα and SREBP-2 were not affected (**Figure 3**). Miyazaki et al. (*37*) also reported that fructose is a stronger inducer of hepatic SREBP-1c and lipogenic gene expression including fatty acid synthase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase than glucose, but the levels of the mature form of SREBP-2 were not altered. However, the oral administration of gravinol resulted in decreases of serum and hepatic TG levels through a signaling pathway that regulates hepatic TG synthesis via a decrease in hepatic SREBP-1 without an alteration in fatty acid β-oxidation by PPARα.

The induction of hyperglycemia by a high-fructose diet has been well established (*6, 8*), suggesting a decrease in insulin sensitivity of the liver and skeletal muscle, which are primarily involved in the effects of insulin on carbohydrate metabolism. Insulin resistance is positively correlated with hyperglycemia and insulinemia. Furthermore, rats fed a high-fructose diet provide an animal model of insulin resistance associated with hyperinsulinemia, hypertriglyceridemia, and hypertension (*38, 39*). Fructose-induced hyperglycemia and insulin resistance could be important factors that may induce reactive oxygen species (ROS) production and glucose oxidation and alter the antioxidant defense status (*28, 40–42*). Furthermore, fructose is a more potent reducing sugar than glucose with regard to the formation of advanced glycation end-products, which may account for several complications of diabetes mellitus, accelerated aging, and the development of atherosclerosis (*5*). The present study also indicated that glucose and glycosylated protein levels of serum in the fructose-fed rats were elevated, consistent with other numerous studies. However, these changes were significantly reversed by gravinol (**Table 2** and **Figure 4**). The alteration of hepatic metabolism due to fructose under conditions of metabolic syndrome may generate stress-activating molecules directly. This increase in the glycosylated protein level of serum in high-fructose-diet-fed rats contributes to the increase in lipid and protein oxidative products such as TBA-reactive substance. The present results demonstrated that gravinol attenuated oxidative stress, as shown by a decline in the TBA-reactive substance level in the serum and hepatic mitochondria (**Figure 4**).

Recent compelling evidence has demonstrated that metabolic syndrome is associated with a proinflammatory state and results in the activation of inflammatory signals by the generation of ROS and reduction of the antioxidative status (*43–45*). Therefore, we focused on its effects on protein levels involved in inflammation by oxidative stress in the liver of high-fructose-diet-fed rats. The present data showed that gravinol attenuated the increase of hepatic COX-2 protein brought about by the fructose diet through the down-regulation of the NF-κB signaling pathway (**Figure 5**). Thus, the inhibitory effect of gravinol on NF-κB activation could be attributed to its strong ability to scavenge ROS. In addition, our results suggest that gravinol led to the down-regulation of bax, the pro-apoptosis factor, and up-regulation of bcl-2, the anti-apoptosis factor (**Figure 6**). Gravinol played a role in the apoptosis process with regulation of related protein expression. It was associated with a reduced severity of hepatic inflammation and liver cell injury, indicating that the recruitment of an inflammatory reaction and the consequent injury to liver cells, is, at least partly, driven by NF-κB, which regulates proinflammatory genes (*45*).

In conclusion, our results indicate that gravinol significantly improved fructose-induced metabolic derangements such as hyperglyceridemia, hyperlipidemia, hypertension, and fat accumulation in the liver. These findings suggest that gravinol ameliorates the pathological conditions of metabolic syndrome in high-fructose-diet-fed rats, which is associated with a reduction of the TG concentration by the down-regulation of SREBP-1 expression and decrease of oxidative stress via regulation of the NF-κB signaling pathway.

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