The Beneficial Effects of Morroniside on the Inflammatory Response and Lipid Metabolism in the Liver of db/db Mice

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Diabetes mellitus is a major cause of mortality and morbidity worldwide, and its prevalence is increasing at an alarming rate. Hyperglycemia and dyslipidemia lead to complications associated with diabetes mellitus, and, also, the attenuation of oxidative stress and regulation of stress-sensitive signaling pathways have been considered as ways to alleviate diabetes.1–4)

Hyperglycemia causes oxidative stress by two mechanisms: first, by decreasing the regeneration of the important cellular antioxidant, reduced glutathione (GSH) from oxidized glutathione (GSSG), and second, by decreasing the availability of reduced nicotinamide adenine dinucleotide phosphate (NADPH). Furthermore, hyperglycemia-induced reactive oxygen species (ROS) stimulate the activation of protein kinase C (PKC), formation of advanced glycation endproducts (AGE), and sorbitol accumulation. Also, increased ROS lead to the activation of nuclear factor-kappa B (NF-kB), and activated NF-kB can enhance the expression of proinflammatory cytokines, chemokines, adhesion molecules, inflammatory receptors, and inflammatory enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).5–7)

Dyslipidemia is the abnormal state of elevated cholesterol, triglyceride, and low-density lipoprotein or decreased high-density lipoprotein in the plasma level that contribute to the development of atherosclerosis. Dyslipidemia is a risk factor for coronary artery disease, a leading cause of mortality in patients with diabetes mellitus.8–10) Triglyceride is involved in the ectopic accumulation of lipid stores in the liver and are associated with a number of diseases such as metabolic syndrome and type 2 diabetes. Critical players in potentiating associated with a number of diseases such as metabolic syndrome and type 2 diabetes. Critical players in potentiating the promotion effect of hyperinsulinemia on hepatic lipid accumulation are the anabolic transcription factor peroxisome proliferator activated receptor α (PPARα) and sterol regulatory element binding proteins (SREBPs), which upregulate genes such as those for fatty acid synthase and cholesterol levels.11–13)

Corni Fructus (Cornus officinalis Sieb. et Zucc.) is a rich source of iridoid glycosides in traditional medicine.14) Iridoids are a group of natural products belonging to the terpenoids, which are ubiquitous in plants. However, only a limited number of taxa possess the enzymes that give rise to the cyclopentane ring that is characteristic of the carbocyclic iridoids.15) Morroniside is a carbocyclic iridoid glycoside which has been reported to exhibit marked antioxidant activity.16) In our previous studies, we reported the beneficial effect of morroniside on streptozotocin-induced type 1 diabetic renal damage. The administration of morroniside could ameliorate hyperglycemia, renal oxidative stress, and inflammation in diabetic rats.17) However, the effect of morroniside on the type 2 diabetic liver, especially in dyslipidemia, has not yet been determined. The liver is an important organ for maintaining glucose metabolism and lipid homeostasis, since it can store (via glycogen synthesis and lipogenesis) or release (via glycogenolysis and gluconeogenesis) glucose and release lipid fuels (as lipoproteins).11,12) Therefore, we investigated the effect of morroniside on hepatic glucose production, lipid synthesis, and inflammation based on the hyperglycemia response in type 2 diabetes mellitus using db/db mice to identify the roles of morroniside in dyslipidemia.

MATERIALS AND METHODS

Materials Morroniside (Fig. 1) was isolated from Corni Fructus as described previously.17) Protease inhibitor mixture dimethyl sulfoxide (DMSO) solution, 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid, TBA), oxalic acid, GSH, and GSSG were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes.

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The Bio-Rad protein assay kit and pure nitrocellulose membrane were purchased from Bio-Rad Laboratories (Tokyo, Japan). β-Actin, o-phthalaldehyde, and N-ethylmaleimide (NEM) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rabbit polyclonal antibodies against NF-xBp65, SREBP-1, SREBP-2, and PPARα, and mouse monoclonal antibody against COX-2 and iNOS were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Goat anti-rabbit and goat antiamouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). ECL Western Blotting Detection Reagents were purchased from Amersham Bioscience (Piscataway, NJ, U.S.A.).

**Experimental Protocol** All experiments were performed according to the animal care guidelines of the University of Toyama. Five-week-old male C57BLKS/J db/db and m/m mice were purchased from Japan SLIC Inc. (Hamamatsu, Japan). C57BLKS/J m/m mice were used as a normal control in the experiment. Mice were maintained under a 12-h light/dark cycle and fed a standard laboratory pellet chow containing 24.0% protein, 3.5% lipids, and 60.5% carbohydrate) and water (CLEA Japan Inc., Tokyo, Japan, comprising 24.0% protein, 3.5% lipids, and 60.5% carbohydrate) and water ad libitum. Morroniside (20 or 100 mg/kg bodyweight) was orally administered using gavage to db/db mice (n = 10, respectively), while vehicle db/db (n = 10) and non-diabetic control m/m (n = 6) mice received water for 8 weeks. The doses of morroniside were the same as our previous study using streptozotocin-induced diabetic rats, which were determined according to our studies of Corni Fructus and its fractions. The body weight, food intake, and water intake were measured every day during the treatment period. After 8 weeks of morroniside treatment, blood samples were collected by the cardiac puncture of anesthetized mice. The serum was separated immediately from each mouse, and the livers were harvested, plunged into liquid nitrogen, and stored at −80 °C until analysis.

**Measurement of Serum Parameters** Serum glucose, triglyceride, total cholesterol, and alanine aminotransferase levels were measured by commercial kits (Glucose CII-Test, Triglyceride E-Test, Total Cholesterol E-Test, and Transaminase CII-Test obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**Measurement of Hepatic Glucose, Triglyceride, and Total Cholesterol Contents** The hepatic glucose level was determined by employing the method of Momose et al. with minor modifications. Hepatic tissue was homogenized with ice-cold 0.9% NaCl buffer, and then the homogenate was deproteinized with 0.15 M Ba(OH)₂ and 5% ZnSO₄. The supernatant was obtained by centrifugation at 1670 × g for 15 min, and then the glucose level was determined using the Wako kit after incubation for 30 min at 37 °C. Also, total lipids of the liver homogenates were extracted with a mixture of chloroform and methanol (2:1, v/v) according to the method of Folch et al., and the amounts of triglyceride and total cholesterol were determined using the Wako kit, as described by the manufacturer.

**Assessment of ROS Generation and TBA-Reactive Substance Levels** ROS generation was measured by the method of Ali et al. Hepatic tissue was homogenized on ice with 1 mM ethylenediaminetetraacetic acid (EDTA)–50 mM sodium phosphate buffer (pH 7.4), and then 25 mM DCFH-DA was added to homogenates. After incubation for 30 min, the changes in fluorescence values were determined at an excitation wavelength of 486 nm and emission wavelength of 530 nm. The hepatic TBA-reactive substance content was determined using the method of Mihara and Uchiyama.

**Determination of GSH and GSSG Levels** GSH and GSSG assays were carried out by employing the method of Hissin and Hilf. Hepatic tissue was homogenized on ice with 1 mM EDTA–50 mM sodium phosphate buffer (pH 7.4). Then, 25 mM metaphosphoric acid was added for protein precipitation. The homogenate was centrifuged at 4 °C at 10000 × g for 30 min to obtain the supernatant for the assays of GSH and GSSG. To assay GSH, 1 mM EDTA–50 mM sodium phosphate buffer (pH 7.4) was added to the supernatant, followed by o-phthalaldehyde. After 20 min at room temperature, fluorescence was estimated at an excitation wavelength of 360 nm and emission wavelength of 460 nm. GSSG was assayed after preincubation with NEM for 20 min, and 0.1 M NaOH was substituted for phosphate buffer. After incubation for 20 min at room temperature, the fluorescence value was estimated at an excitation wavelength of 360 nm and emission wavelength of 460 nm. Protein assay was carried out according to the method of Itzhaki and Gill using bovine serum albumin as a standard.

**Preparation of Nuclear and Post-nuclear Fractions** To prepare nuclear fractions, hepatic tissue was homogenized with ice-cold lysis buffer containing 5 mM Tris–HCl (pH 7.5), 2 mM MgCl₂, 15 mM CaCl₂, and 1.5 mM sucrose, and then 0.1 M dithiothreitol (DTT) and protease inhibitor cocktail were added. After centrifugation (10500 × g for 20 min at 4 °C), the pellet was suspended with extraction buffer containing 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (pH 7.9), 1.5 mM MgCl₂, 0.42 mM NaCl, 0.2 mM EDTA, and 25% (v/v) glycerol, and then 0.1 M DTT and protease inhibitor cocktail were added. The mixture was placed on ice for 30 min. The nuclear fraction was prepared by centrifugation at 20500 × g for 5 min at 4 °C. The post-nuclear fraction was extracted from the liver of each mouse as described below. In brief, hepatic tissue was homogenized with ice-cold lysis buffer (pH 7.4) containing 137 mM NaCl, 20 mM Tris–HCl, 1% Tween 20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture DMSO solution. The homogenate was then centrifuged at 2000 × g for 10 min at 4 °C. The protein concentration of each fraction was determined using a commercial kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

**Western Blot Analyses** For the determination of NF.
xB, PPARα, SREBP-1, and SREBP-2, 30 μg protein of each nuclear fraction was electrophoresed through 8% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). Separated proteins were electrophoretically transferred to a nitrocellulose membrane, blocked with 5% (w/v) skim milk solution for 1 h, and then incubated with primary antibodies to NF-xBp65, PPARα, SREBP-1, SREBP-2, and β-actin, respectively, overnight at 4 °C. After the blots were washed, they were incubated with goat anti-rabbit and/or goat anti-mouse IgG HRP-conjugated secondary antibody for 1.5 h at room temperature. Also, 30 μg protein of each post-nuclear fraction for COX-2 and iNOS was electrophoresed through 10% SDS-PAGE. Each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (Fujifilm, Tokyo, Japan). Band densities were determined using ATTO Densitograph Software (ATTO Corporation, Tokyo, Japan) and quantified as the ratio to β-actin. These protein levels of groups were expressed relative to those of m/m mice (represented as 1).

**Statistical Analysis** Data are expressed as means±S.E. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Dunnett’s test. Values of p<0.05 were considered significant.

**RESULTS**

**Body Weight, Food Intake, and Water Intake** Table 1 shows the changes in the body weight and food and water intakes during the experimental period. Compared to untreated db/db mice, the levels of body weight and food consumption were not changed by morroniside treatment throughout the experimental period. However, the level of water consumption of morroniside (100 mg/kg)-treated mice was significantly lower than that of untreated db/db mice after 8 weeks of treatment.

**Serum Glucose, Triglyceride, Total Cholesterol, and Alanine Aminotransferase Levels** The serum glucose, triglyceride, total cholesterol, and alanine aminotransferase levels of db/db mice were markedly higher than those of m/m mice, but no significant changes in the glucose and total cholesterol levels were shown by morroniside administration (Table 2). On the other hand, the elevated serum triglyceride level was significantly decreased in morroniside-treated db/db mice in a dose-dependent manner. The serum alanine aminotransferase level, a liver function parameter, was significantly lower in morroniside-treated compared with vehicle-treated db/db mice.

**Hepatic Glucose, Triglyceride, and Total Cholesterol Contents** The hepatic glucose level of db/db mice was markedly higher than those of m/m normal control mice, but it was lowered nearly to the level of m/m mice by the treatments with morroniside (Fig. 2A). The hepatic triglyceride and total cholesterol levels of the db/db vehicle group were 1.6 and 0.8 times higher than those of m/m mice, respectively, but these elevated lipid levels were significantly reduced by the 20 or 100 mg/kg morroniside administration (Figs. 2B, C).

**Hepatic Biomarkers Associated with Oxidative Stress** In the db/db vehicle-treated mice, hepatic TBA-reactive substance level was increased by 2.1 times compared with m/m mice (m/m vs. db/db vehicle-treated: 0.87 vs. 1.87 nmol/mg tissue, respectively, p<0.05, Fig. 3A). However, the elevated hepatic TBA-reactive substance level was significantly reduced in morroniside-treated db/db mice, and lowered nearly to the level of m/m mice by morroniside treatment (Fig. 3A). Also, the elevated ROS generation in the db/db vehicle-treated group was decreased markedly by morroniside treatment (Fig. 3B). In addition, the db/db vehicle-treated group showed significantly decreased GSH/GSSG ratios compared with the m/m group, which resulted from the decreased GSH and increased GSSG in the liver (Figs. 3C, D). However, this reduction of the hepatic GSH/GSSG ratio in the db/db vehicle-treated group recovered nearly to the level of m/m mice on 100 mg/kg morroniside treatment (Fig. 3E).

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**Table 1. Body Weight, Food Intake, and Water Intake**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg body weight)</th>
<th>Body weight</th>
<th>Food intake (g/d)</th>
<th>Water intake (ml/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Initial (g)</td>
<td>Final (g)</td>
<td>Gain (g/8 weeks)</td>
</tr>
<tr>
<td>m/m</td>
<td>—</td>
<td>18.6±1.8*</td>
<td>25.4±0.9*</td>
<td>6.4±0.1*</td>
</tr>
<tr>
<td>db/db</td>
<td>—</td>
<td>41.4±0.3</td>
<td>55.2±2.4</td>
<td>13.8±1.2</td>
</tr>
<tr>
<td>Vehicle</td>
<td>—</td>
<td>42.5±0.7</td>
<td>57.9±1.3</td>
<td>15.4±0.7</td>
</tr>
<tr>
<td>Morroniside</td>
<td>20</td>
<td>41.6±0.4</td>
<td>57.5±1.3</td>
<td>15.9±0.5</td>
</tr>
<tr>
<td>Morroniside</td>
<td>100</td>
<td>41.6±0.4</td>
<td>57.5±1.3</td>
<td>15.9±0.5</td>
</tr>
</tbody>
</table>

* p<0.05 vs. db/db vehicle-treated values.

**Table 2. Hematological Analyses**

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose (mg/kg body weight)</th>
<th>Glucose (mg/dl)</th>
<th>Triglyceride (mg/dl)</th>
<th>Total cholesterol (mg/dl)</th>
<th>Alanine aminotransferase (IU/l)</th>
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<tbody>
<tr>
<td>m/m</td>
<td>—</td>
<td>219.7±12.0*</td>
<td>89.6±5.7*</td>
<td>74.0±1.3*</td>
<td>8.4±0.8*</td>
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<tr>
<td>db/db</td>
<td>—</td>
<td>765.2±44.8</td>
<td>298.7±24.6</td>
<td>168.5±8.3</td>
<td>35.3±4.3</td>
</tr>
<tr>
<td>Vehicle</td>
<td>—</td>
<td>753.8±34.2</td>
<td>229.8±24.8*</td>
<td>160.4±11.2</td>
<td>24.0±2.5*</td>
</tr>
<tr>
<td>Morroniside</td>
<td>20</td>
<td>713.6±32.3</td>
<td>175.4±19.1*</td>
<td>173.8±4.2</td>
<td>22.8±2.1*</td>
</tr>
</tbody>
</table>

* p<0.05 vs. db/db vehicle-treated values.
Expressions of NF-κBp65, COX-2, and iNOS in Hepatic Tissues

The NF-κBp65, COX-2, and iNOS protein expressions in the db/db vehicle-treated group were significantly up-regulated compared to those of the m/m group. These increased protein expressions related to inflammation and ROS generation in the liver were markedly reduced by the administration of morroniside, which indicates the deactivation of NF-κB and down-regulation of NF-κB-related...
COX-2 and iNOS (Fig. 4).

Expressions of PPARα, SREBP-1, and SREBP-2 in Hepatic Tissues

The hepatic PPARα protein expression of db/db mice was slightly lower than those of the m/m group, but significantly elevated than the 100 mg/kg morroniside treatment for 8 weeks (Fig. 5A). The protein expressions of hepatic SREBP-1 and SREBP-2 in db/db mice were significantly higher than those of m/m mice, respectively. However, these elevated SREBP protein expressions in db/db mice were significantly down-regulated in the morroniside 100 mg/kg-treated db/db mice (Figs. 5B, C).

DISCUSSION

Morroniside is carbocyclic iridoid glycoside, and it has been reported to exhibit powerful antioxidative and α-glucosidase-inhibitory activities.16,23) We reported the beneficial effect of morroniside on streptozotocin-induced type 1 diabetic renal damage in our previous study.17) Morroniside was screened out in an on-going project to identify the main active compound of Corni Fructus, one of the eight component medicinal herbs contained in Hachimi-Jio-gan which have been used as traditional medicine for the treatment of diabetes. We have successively identified the main renoprotective active components of Corni Fructus through in vitro and in vivo studies.14,17,26) Subsequently, the effect of morroniside on the type 2 diabetic liver, especially in the presence of dyslipidemia, which is not well known, was investigated in the present study.

The liver is an important organ for maintaining glucose and lipid homeostasis, and plays a critical role in regulating endogenous glucose production from de novo synthesis (gluconeogenesis) or the catabolism of glycogen (glycogenolysis). Increased rates of hepatic glucose production are largely responsible for the development of overt hyperglycemia.23) C57BLKS/J db/db mice were used in the present study to identify the effect of morroniside on hepatic dyslipidemia. The db/db mice develop diabetes mellitus due to a failure to respond to leptin, resulting from a mutation in their receptor gene expressed in the hypothalamus, although gene expression and leptin secretion are markedly augmented in these mice, resulting in leptin resistance.23) The db/db mice were also characterized by obesity, sustained hyperglycemia, hyperlipidemia, and hyperinsulinemia as a result of destroyed leptin receptors.20) Subsequently, the genotypes of db/db mice led to a lack of signaling by leptin, which regulates food intake and systemic fuel metabolism.

Consistent with another report,20) the body weight, food intake, and water intake of db/db mice in our current study were markedly higher than those of m/m mice due to augmented food consumption in the former. The administration of morroniside for 8 weeks led to no difference in body weight and food intake; however, water intake was significantly reduced in morroniside 100 mg/kg-treated mice (Table 1). These results suggest that the oral administration of morroniside may improve the typical diabetic symptom, an excessive intake of water. We observed a significant elevation of serum glucose, triglyceride, total cholesterol, and alanine aminotransferase levels in db/db compared with m/m mice. The levels of serum glucose and total cholesterol were slightly decreased by the administration of morroniside, but its administration led to a marked reduction in the levels of serum triglyceride and alanine aminotransferase of db/db mice (Table 2). In the case of hepatic tissue samples, the elevated glucose, triglyceride, and total cholesterol levels in the livers of db/db mice were significantly reduced by morroniside treatment (Fig. 2). The effects of morroniside on hepatic tissue were more significant than on serum parameters, which may reflect the fact that the liver is the major organ affected by morroniside in db/db mice compared to other organs, such as the pancreas or kidney.

Subsequently, the effects of morroniside on factors related to ROS and inflammation in hepatic tissues were investigated. Hyperglycemia and hepatic glucose over-production led to oxidative stress and tissue damage due to mechanisms involving repeat acute changes in cellular metabolism.31,32) A large amount of data emphasize four key metabolic pathways as being major contributors to hyperglycemia-induced cell damage33,34); (1) increased polyol pathway flux; (2) increased AGE formation; (3) activation of PKC isoforms; and (4) increased hexosamine pathway flux. Hyperglycemia results in the increased enzymatic conversion of glucose to the polyalcohol sorbitol, with concomitant decreases in NADPH and glutathione.32) The resulting loss of antioxidative equivalents leads to enhanced sensitivity to oxidative stress associated with intracellular ROS production. The increased ROS can affect gene expression, which leads to attempts to protect cells from the oxidant-induced damage of proteins, DNA,
and lipids. Hyperglycemia also causes oxidative stress due to the increased mitochondrial production of superoxide, including the depletion of NADPH and consequent disturbance of glutathione and nitric oxide metabolism. These oxidative stresses are responsible for regulation of the transcriptional pathways of NF-κB, which is a transcription factor thought to play an important role in the onset of inflammation. The activation of NF-κB by oxidative stress is induced by a cascade of events leading to the activation of inhibitor κB (IκB) kinase, which phosphorylates IκB, leading to its degradation and the translocation of NF-κB to the nucleus. NF-κB activation can lead to the enhanced expression of proinflammatory cytokines, chemokines, adhesion molecules, inflammatory receptors, and inflammatory enzymes such as iNOS and COX-2. Therefore, the modulation of NF-κB activation may provide a direct way of inhibiting inflammatory mediators.

In our study, the hepatic TBA-reactive substance and ROS levels in db/db mice were 2.1 and 1.7 times higher than those of m/m mice, respectively (Figs. 3A, B), suggesting increased oxidative stress in the liver of db/db mice. In addition, db/db vehicle-treated mice showed a significant reduction of the GSH/GSSG ratio, which is a useful barometer of the oxidative stress imposed on a cellular system (Fig. 3E). However, the elevated hepatic TBA-reactive substance and ROS levels in db/db mice were significantly reduced, and the reduced ratio of GSH/GSSG was increased by morroniside treatment almost to the level of normal control mice (Figs. 3A, B, E). From the analysis of hepatic protein expression, the administration of morroniside could reduce the elevated hepatic NF-κBp65, COX-2, and iNOS levels (Figs. 4A, B, C). These results suggest that the administration of morroniside can alleviate hepatic damage induced by ROS through the deactivation of NF-κB and subsequent restoration of the antioxidative state.

In conclusion, morroniside showed antioxidant and anti-inflammatory activities and an ameliorating effect on dyslipidemia via the inhibition of NF-κB activation, up-regulation of PPARα, and down-regulation of SREBP expressions in the liver of db/db mice. These results suggest that the administration of morroniside can improve liver dysfunction caused by dyslipidemia and oxidative stress in type 2 diabetic mice. Consequently, we suggest that morroniside, an iridoid glycoside from Corni Fructus, may reduce the risk of type 2 diabetes by the amelioration of metabolic disorders including dyslipidemia and hyperglycemia, as well as inflammatory responses.

REFERENCES