Chinese Prescription Kangen-karyu Ameliorates the Development of Diabetic Hepatic Damages via Regulating Oxidative Stress and Inflammation in the Liver of db/db Mice

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The prevention and treatment of diabetic complications are considered to be the most important for the general care of diabetic patients. We have been conducting pre-clinical animal experiments related to diabetes using Kangen-karyu, a Chinese prescription, to examine its therapeutic potential. In the present study, we further studied the anti-diabetic mechanism of Kangen-karyu, especially on the regulation of hyperglycemia-induced hepatic oxidative stress and inflammation in db/db mice. Kangen-karyu (100 or 200 mg/kg body weight/day, per os (p.o.)) was administered every day for 18 weeks to db/db mice, and its effect was compared with vehicle-treated db/db and m/m mice. The administration of Kangen-karyu decreased the elevated serum and hepatic glucose concentration in db/db mice. The elevated expressions of p22phox and Nox-4 proteins (reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits) were significantly decreased after Kangen-karyu treatments. The oxidative stress-related markers in hepatic tissue (reactive oxygen species, reduced glutathione/oxidized glutathione ratio, and thiobarbituric acid-reactive substance) were also significantly ameliorated by the Kangen-karyu treatments. The db/db mice exhibited the up-regulation of nuclear factor-κBp65, cyclooxygenase-2, and inducible nitric oxide synthase enzymes in the liver; however, Kangen-karyu treatment significantly reduced those expressions. Taking these into consideration, our findings support the therapeutic evidence for Kangen-karyu ameliorating the development of diabetic hepatic damages via regulating oxidative stress and inflammation.

Key words Kangen-karyu; db/db mouse; hepatic damage; oxidative stress; inflammation

The incidence of diabetes mellitus is increasing worldwide at an alarming rate due to population growth, obesity, and aging; thus, its complications are also on the increase. The prevention and treatment of diabetic complications are considered to be the most important for the general care of diabetic patients. The basic causes of complications include tissue metabolism disorders that result from chronic hyperglycemia and hyperlipidemia, which results in damage to many organs, such as the vascular tissue, retina, nerves, kidney, and liver.1,2

Major characteristics of metabolic disorders are glucotoxicity and lipotoxicity, which are commonly correlated with oxidative stress and proinflammatory signaling.3 For instance, chronic exposure to elevated glucose and fatty acid concentrations can cause damage to different types of cell through a variety of mechanisms (glucotoxicity and lipotoxicity), but oxidative stress may be a common link in cell dysfunction.3,4 Traditional Chinese prescriptions have received much attention as potential sources of novel therapeutic agents due to their multiple beneficial effects and absence of toxic and/or side effects.5

Kangen-karyu (Guan-Yuan-Ke-Li in Chinese, and has developed in Japan by the modification of herbal constituents of Kan-shin No. 2 (Guan-xin No. 2 in Chinese)) is a crude drug consisted of six herbs (Paeoniae Radix, Cnidii Rhi- zoma, Carthami Flos, Cyperi Rhizoma, Saussureae Radix, and Salviae Miltiorrhizae Radix), and has been clinically used as a treatment for cardiovascular diseases such as angina pectoris and cerebrovascular diseases.6 We have been conducting pre-clinical animal experiments related to diabetes using Kangen-karyu to examine its therapeutic potential. Previously, we reported that Kangen-karyu showed beneficial effects on type 1 diabetes and its related complications through the suppression of protein expressions related to advanced glycation endproducts and oxidative stress.7 Kangen-karyu also exerts its renoprotective potential mainly through its antioxidant properties during the development of diabetic nephropathy in type 2 diabetes.8 In our most recent research, Kangen-karyu improved hyperlipidemia and lipid deposition via the regulation of hepatic sterol regulatory element binding protein (SREBP)-1 expression in a type 2 diabetic db/db mouse model.9 These results suggest that Kangen-karyu acts as a regulator in inflammatory reactions and abnormal lipid metabolism in type 2 diabetic mice.

In the present study, we further studied the anti-diabetic mechanism of Kangen-karyu, especially on the regulation of hyperglycemia-induced hepatic oxidative stress and inflammation in db/db mice, which has not yet been clarified.

MATERIALS AND METHODS

**Materials** Protease inhibitor cocktail, 4,6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid, TBA), ethylene-diaminetetraacetic acid (EDTA), reduced glutathione (GSH), oxidized glutathione (GSSG), and 10% normal-buffered formalin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR, U.S.A.). The Bio-Rad protein assay kit and pure nitrocellulose membrane were purchased from Bio-Rad Laboratories (Tokyo, Japan). β-Actin and phenylmethylsulfonyl fluo-
ride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Rabbit polyclonal antibodies against nuclear factor-κB (NF-κB) p65, p22^phox, intracellular adhesion molecule (ICAM)-1, and monocyte chemotactic protein (MCP)-1, and mouse monoclonal antibody against cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and histone were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). Anti-reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox)-4 (Santa Cruz, CA, U.S.A.). Anti-reduced nicotinamide adenine dinucleotide (NADH) oxidase (COX)-2, inducible nitric oxide synthase (iNOS), and monocyte chemotactic protein (MCP)-1, and mouse monoclonal antibody against cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and histone were purchased from Santa Cruz Biotechnology, Inc. ECL Western Blotting Detection Reagents were purchased from GE Healthcare (Piscataway, NJ, U.S.A.). Goat anti-rabbit and goat anti-mouse immunoglobulin G (IgG) horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antibodies against nucleotidase, including ATP, ADP, and AMP, were purchased from Santa Cruz Biotechnology, Inc. (LifeSpan BioSciences, Seattle, WA, U.S.A.) was also used. Rabbit polyclonal antibodies against nucleotidase, including ATP, ADP, and AMP, were purchased from Santa Cruz Biotechnology, Inc. Rabbit polyclonal antibodies against nucleotidase, including ATP, ADP, and AMP, were purchased from Santa Cruz Biotechnology, Inc. (IgG) horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. ECL Western Blotting Detection Reagents were purchased from GE Healthcare (Piscataway, NJ, U.S.A.).

Preparation of Kangen-karyu Extract  Kangen-karyu in the form of a dried powder extract was from Iskra Co., Ltd. (Tokyo, Japan). The composition of Kangen-karyu used in this study was: 2.25 g of Paeoniae Radix (Paeonia lactiflora PALLAS root), 2.25 g of Cnidii Rhizoma (Cnidium officinales MAKINO rhizome), 2.25 g of Carthami Flos (Carthamus tinctorius L. petal), 1.125 g of Cyperi Rhizoma (Cyperus rotundus L. rhizome), 1.125 g of Aucklandiae Radix (Aucklandia lappa DCNE. root), and 4.5 g of Salviae Miltiorrhizae Radix (Salvia miltiorrhiza BUNGE root). This prescription was extracted with 25 volumes of water at 100 °C for 1 h. After filtration, the solution was evaporated under a reduced pressure to give an extract at a yield of 44%, by weight, of the starting materials. A typical high-performance liquid chromatography of Kangen-karyu is given in Fig. 1. Each sample was dissolved in 50% aqueous ethanol with sonication, and filtered through a Cosmonice filter (PVDF, 0.45 mm; Nacalai Tesque, Inc., Kyoto, Japan). Reverse-phase high-performance liquid chromatography was performed using a Cosmosil 5C18-AR II column (250×4.6 mm i.d.; Nacalai Tesque, Inc.) with elution gradients of 4—30% (39 min) and 30—75% (15 min) CH3CN in 50 mM H3PO4 at a flow rate of 0.8 ml/min. The UV absorbance from 200 to 400 nm was monitored, and the fluorescence intensity was determined at an excitation wavelength of 486 nm and emission wavelength of 530 nm. 25 mM DCFH-DA was added to homogenates. After incubation for 30 min to obtain the supernatant for the assays.

Measurement of Glucose Levels in Hepatic Tissue The hepatic glucose level was determined using the method of Momose et al. with minor modifications. In brief, hepatic tissue was homogenized with ice-cold physiological saline, and the homogenate was deproteinized with 0.15 M NaOH and 5% ZnSO4. The supernatant was obtained by centrifugation at 16000×g for 15 min, and the glucose level was determined using a Wako kit (Glucose CII-Test). Assessment of Hepatic Reactive Oxygen Species (ROS) Generation and TBA-Reactive Substance (TBARS) Levels ROS generation was measured using the method of Ali et al. Hepatic tissues were homogenized on ice with 1 mM EDTA—50 mM sodium phosphate buffer (pH 7.4), and then 25 mM DCFH-DA was added to homogenates. After incubation for 30 min, changes in fluorescence values were determined at an excitation wavelength of 486 nm and emission wavelength of 530 nm. The hepatic TBARS content, an oxidative stress biomarker, was determined employing the method of Mihara and Uchiyama.

Determination of Hepatic GSH and GSSG Levels GSH and GSSG assays were carried out applying the method of Hissin and Hilf. Hepatic tissues were homogenized on ice with 1 mM EDTA—50 mM sodium phosphate buffer (pH 7.4). Then, 25% 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 the addition of α-phthalaldehyde. 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. GSSG was assayed after preincubation with N-ethylmaleimide for 20 min, and 0.1 mm 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 assays were carried out according to the method of Izhaki and Gill[4] using bovine serum albumin as a standard.

Preparation of Nuclear and Post-Nuclear Fractions
Nuclear protein extraction was performed according to the method of Komatsu.[15] In brief, hepatic tissues were 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 mm dithiothreitol (DTT) and protease inhibitor cocktail. The homogenate was then centrifuged 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 PMSF, and protease inhibitor cocktail. The homogenate was then centrifuged at 20000 × 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.).

Immunoblotting Analyses
For the determination of NF-κBp65, 30 μg of protein of each nuclear fraction was electrophoresed through an 8% sodium dodecylsulfate polyacrylamide gel (SDS-PAGE). Separated proteins were transferred to a nitrocellulose membrane, blocked with 5% (w/v) skim milk and then 0.1 M dithiothreitol (DTT) and protease inhibitor cocktail. The membrane was then washed, incubated with anti-rabbit or anti-mouse IgG HRP-conjugated secondary antibody for 1.5 h at room temperature, and detected by chemiluminescence with LAS-4000 (Fujifilm, Tokyo, Japan). Band densities were determined using ATTO Densitograph Software (ATTO Corp., Tokyo, Japan) and quantified as the ratio to histone and/or 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.M. Significance was assessed employing one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (SPSS 11.5.1 for Windows, 2002, SPSS Inc., U.S.A.). Values of p < 0.05 were considered significant.

RESULTS

General Characteristics
After 18-week treatment, db/db mice showed a significant increase in body weight gain and liver weight compared with m/m mice (Table 1). However, there were no significant weight changes among the vehicle-, Kangen-karyu 100 mg/kg body weight-, and Kangen-karyu 200 mg/kg body weight-treated db/db mice groups. Also, db/db mice exhibited type 2 diabetic characteristics such as hyperglycemia and hyperleptinemia compared with m/m mice, but Kangen-karyu administration significantly reduced serum glucose and leptin concentrations at a dose of 200 mg/kg. The serum ALT and AST, hepatic functional parameters, in db/db vehicle mice were elevated compared to m/m mice, whereas these augmented levels showed a slight decrease (Table 1).

Hepatic Glucose Level
In hepatic tissue, the elevated glucose content of db/db mice (11.55 mm/g tissue for m/m mice vs. 18.32 mm/g tissue for db/db mice) was significantly lowered in both 100 and 200 mg/kg of Kangen-karyu-treated mice (16.12 mm/g tissue (p < 0.05) and 14.92 mm/g tissue (p < 0.01), respectively).

NADPH Oxidase in Hepatic Tissue
As shown in Fig. 2, db/db control mice significantly increased the expression of NADPH oxidase subunits p22phox and Nox-4 proteins. The elevated hepatic p22phox expression was significantly decreased below the m/m value after Kangen-karyu treatments (100, 200 mg/kg body weight) for 18 weeks (Fig. 2A). Kangen-karyu led to a significant decrease in Nox-4 level at a dose of 200 mg/kg body weight (Fig. 2B).

Oxidative Stress-Related Markers in Hepatic Tissue

Table 1. Biochemical Analyses

<table>
<thead>
<tr>
<th>Item</th>
<th>m/m</th>
<th>db/db Veh</th>
<th>KK100</th>
<th>KK200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g/18 weeks)</td>
<td>4.7±0.2***</td>
<td>18.9±1.1</td>
<td>17.9±1.5</td>
<td>18.7±1.4</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.47±0.1***</td>
<td>4.08±0.20</td>
<td>3.90±0.36</td>
<td>3.62±0.59</td>
</tr>
<tr>
<td>(g/100 g body weight)</td>
<td>5.75±0.37**</td>
<td>7.75±0.41</td>
<td>7.41±0.61</td>
<td>7.02±1.24</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>136.6±4.1***</td>
<td>483.3±10.3</td>
<td>442.8±24.6</td>
<td>436.6±13.4*</td>
</tr>
<tr>
<td>Serum leptin (ng/ml)</td>
<td>1.95±0.31***</td>
<td>19.87±0.91</td>
<td>19.27±1.11</td>
<td>15.43±0.63**</td>
</tr>
<tr>
<td>Serum ALT (IU/l)</td>
<td>36.80±2.27*</td>
<td>92.92±15.67</td>
<td>83.81±8.24</td>
<td>80.85±12.95</td>
</tr>
<tr>
<td>Serum AST (IU/l)</td>
<td>11.17±0.46**</td>
<td>56.74±3.97</td>
<td>50.96±6.61</td>
<td>42.19±7.34</td>
</tr>
</tbody>
</table>

m/m: Misty mice; db/db: Veh. db/db vehicle-treated mice; db/db: KK100; db/db: Kangen-karyu (100 mg/kg body weight)-treated mice; db/db: KK200; db/db: Kangen-karyu (200 mg/kg body weight)-treated mice. Data are the means ± S.E.M. Significance: *p < 0.05, **p < 0.01, ***p < 0.001 vs. db/db vehicle-treated group.
ROS and TBARS levels in hepatic tissue of db/db mice were 1.3- and 1.4-fold higher than those of m/m mice, as shown in Figs. 3A and C. However, Kangen-karyu markedly suppressed these levels from the dose of 100 mg/kg body weight; especially the reduction of the TBARS level in 200 mg/kg Kangen-karyu-treated mice was more marked than the difference between vehicle-treated db/db mice and m/m mice (Figs. 3A, C). The GSH/GSSG ratio was slightly decreased, without significance, but the Kangen-karyu 200 mg/kg body weight group showed a significant increase from the value of vehicle-treated db/db mice (Fig. 3B).

**Anti-inflammatory Activity in Hepatic Tissue** In order to evaluate the anti-inflammatory activity induced by ROS generation in hepatic tissue, we examined NF-κBp65, COX-2, and iNOS by employing immunoblotting analyses (Fig. 4). The NF-κBp65, COX-2, and iNOS protein levels of diabetic db/db mice were 1.5-, 2.6-, and 3.1-fold higher, respectively, than the m/m mouse values, whereas oral Kangen-karyu administration for 18 weeks significantly decreased these protein expressions. Especially, NF-κBp65 and COX-2 levels in 200 mg/kg body weight Kangen-karyu-treated mice successfully reached normal levels (Figs. 4A, B). In addition, db/db mice showed slight increases in MCP-1 and ICAM-1 proteins from the m/m values, without significance; however, Kangen-karyu administration suppressed these protein levels below the normal values (Figs. 4D, E).

**DISCUSSION**

Type 2 diabetes is a metabolic disease characterized by elevation of the blood glucose concentration, lipid abnormalities, and vascular complications. In our previous report, we investigated the hypolipidemic efficacy of Kangen-karyu in type 2 diabetic db/db mice through the regulation of hepatic SREBP-1 and lipogenic enzyme gene expressions. Here, we report new insights into the efficacy of Kangen-karyu on the glucotoxicity, especially on the regulation of hyperglycemia-induced hepatic oxidative stress and inflammation, in db/db mice.

Biochemical mechanisms of glucotoxicity have been proposed to involve the generation of chronic oxidative stress. In the process of ROS generation, the Nox family of NADPH oxidase has been strongly supported as a major source. Nox-derived ROS play a physiological role in responses to stimulation by various growth factors, cytokines, and hormones including insulin, and have pathophysiological roles in endothelial dysfunction, inflammation, apoptosis, fibrosis, angiogenesis, and important processes underlying diabetes and liver injury. Structurally, NADPH oxidase comprises a membrane-associated cytochrome b55, composed of one p22phox (where phox stands for phagocyte oxidase) and one gp91phox subunit and at least four cytosolic subunits (p47phox, p67phox, p40phox, and the small GTPase Rac1 or Rac2). Mechanistically, in obesity and type 2 diabetes such as db/db model mice, which are associated with nonalcoholic steatohepatitis, NADPH oxidase complexes are involved in steatosis and insulin resistance: (1) gp91phox (known as Nox-2), p22phox, and Nox-4 may increase c-Jun N-terminal kinase pathway activity, serine/threonine phosphorylation of insulin receptor substrate 1, and activation of transcriptional factors such as activator protein-1, leading to steatosis and insulin resistance; (2) increased Nox-4 promotes endoplasmic reticulum stress and hepatocyte apoptosis, and (3) gp91phox and Nox-4 increase transforming growth factor-β, inflammation/fibrosis, and the proliferation of stellate cells.

It has also been reported that the expression of p22phox is increased in parallel with elevation of the levels of lipid peroxidation. Accordingly, we have performed immunoblotting...
analyses of p22phox and Nox-4 in hepatic tissue of db/db mice. In the present study, Kangen-karyu improved hyperglycemic states in serum and hepatic tissue, being more prominent in hepatic tissue, suggesting that the effect of Kangen-karyu involves the control of glucose homeostasis and development of glucotoxicity in hepatic tissue. Additionally, the increased expressions of hepatic p22phox and Nox-4 were significantly decreased by the administration of Kangen-karyu, which also reflected the hepatic results regarding ROS, GSH/GSSG, and TBARS levels. Therefore, the efficacy of Kangen-karyu may be related to the suppression of ROS-generating NADPH oxidase induced by hyperglycemia, which is a potential source of oxidative stress in diabetes.

Chronic hyperglycemia also favors the increased expression of iNOS via the activation of NF-κB, which is involved in ROS generation.26 It is well-known that NF-κB plays a key role in the regulation of transcription and expression of many genes, such as COX-2 and iNOS, which are related to the inflammatory responses.27—29 Previously, we showed that Kangen-karyu administration led to a decline in hepatic NF-κB and COX-2 protein expressions in a high-fructose-induced metabolic syndrome rat model.30 In the present study, Kangen-karyu significantly suppressed hepatic NF-κB and COX-2 protein expressions, as well as iNOS protein, in a type 2 diabetic db/db mouse model.

The hyperglycemic condition also stimulates expressions of the chemokines and adhesion molecules MCP-1 and ICAM-1 in the liver, which are enhanced by the induction of NF-κB.31,32 MCP-1 and ICAM-1, chemokines and adhesion molecules, mediate monocytes/macrophages which are extravasculated from the blood stream and attracted to the target tissues. MCP-1 is produced predominantly by macrophages and endothelial cells and is a potent chemotactic factor for monocytes.33—35 Recently, an increase in MCP-1 expression was shown to contribute to insulin resistance through the induction of an inflammatory response and hepatic steatosis in obesity.36 It has also been reported that increased COX-2 expression via NF-κB results in the activation of a prostaglandin/cyclic adenosine monophosphate (cAMP) pathway, and may regulate MCP-1 expression, contributing to the modulation of hepatic tissue inflammation.37 ICAM-1 is an immunoglobulin-like cell adhesion molecule playing a major role in the regulation of interactions with immune cells, and whose expression is up-regulated at sites of inflammation. Its ligands of lymphocyte function-associated antigen-1 and Mac-1 are expressed on leukocytes, and it has been shown to function as a co-stimulatory molecule. T-cell activation through T-cell receptor engagement requires co-stimulatory molecules and also adhesion molecules such as ICAM-1. ICAM-1 also mediates leukocyte invasion from the blood into tissue during inflammatory processes.38 The present study showed that MCP-1 and ICAM-1 protein expressions in db/db mice were suppressed to the normal levels by the administration of Kangen-karyu, although there were no significant differences between m/m and vehicle-treated db/db mice. Therefore, we suggest that the effect of Kangen-karyu on hepatic inflammation was, at least in part, dependent on the potential antioxidative activity in the liver of db/db mice, and further experiments will be necessary.

In conclusion, we have demonstrated the efficacy of Kangen-karyu against hepatic glucotoxicity such as oxidative stress and its related inflammation in insulin resistance of type 2 diabetic db/db mice, summarized in Fig. 5. Although the detailed mechanisms of Kangen-karyu against insulin resistance were not clarified in the present study, paeoniflorin,
one of the components of Kangen-karyu (Fig. 1), has been reported to suppress the production of proinflammatory mediators such as tumor necrosis factor-α, MCP-1, and free fatty acids, to have the potential to improve chronic inflammatory conditions in obesity, and improve obesity-related insulin resistance. Lithospermic acid B possesses a strong capacity to inhibit aldose reductase, the first enzyme of the polyol pathway implicated in the secondary complications of diabetes. In addition, rosmarinic acid shows a protective potential against oxidative damage in hepatic cells, indicating that it could be beneficial against liver disease, where it is known that oxidative stress plays a crucial role. Taking these into consideration, our findings may support the therapeutic evidence for Kangen-karyu ameliorating the development of diabetic hepatic damages via regulating oxidative stress and inflammation.

REFERENCES