Antidiabetic Effect of Glycyrrhizin in Genetically Diabetic KK-A^y Mice

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We, previously demonstrated that one shot administration of glycyrrhizin (Grz) reduced the postprandial blood glucose rise, using Std ddY mice. Subsequently, we evaluated the effects of long-term Grz treatment (2.7, 4.1 g/kg diet) on diabetic symptoms using genetically non-insulin dependent diabetic model mice (KK-A^y). Male KK-A^y mice were divided into 3 groups: the control group, 0.27% Grz diet (2.7 g of Grz/kg diet) group and 0.41% Grz diet (4.1 g of Grz/kg diet) group. The elevation of blood glucose concentration was almost entirely suppressed in mice fed the 0.41% Grz diet 7 weeks after the beginning of test feeding, although it was not suppressed in mice fed the control diet or the 0.27% Grz diet. Water intake in the control and 0.27% Grz diet groups increased gradually, whereas, this was not true in the 0.41% Grz diet group. Grz treatment significantly lowered blood insulin level. Throughout the experiment, Grz did not affect the food intake or body weight among the three groups. The mice fed the 0.41% Grz diet also improved their tolerance to oral glucose loading 9 weeks after the beginning of test feeding. This study shows that Grz has an antidiabetic effect in noninsulin-dependent diabetes model mice.

Key words glycyrrhizin; antidiabetic effect; KK-A^y mice

Noninsulin-dependent diabetes mellitus (NIDDM) is one of the most common chronic diseases in advanced nations including Japan. In some population groups, such as the Pima Indians and Naurians, the disease is often present in epidemic proportions.¹⁾ It is reported that 8 to 10% of adults of European descent will develop NIDDM.¹⁾ Urbanization, obesity and dietary changes are all incriminated in reducing insulin sensitivity, but the specific lifestyle factors involved are not clear.²⁾ The primary metabolic defect is thought to be determined genetically.³⁾ The importance of controlling plasma glucose levels in diabetic subjects has prompted active research to find ways of stabilizing postprandial glucose levels. Prevention of hyperglycemia and hyperinsulinemia by retardation of glucose uptake from the small intestine is one successful approach to improving insulin resistance in diabetes mellitus. There are several approaches to retarding glucose uptake in the small intestine: a) delaying the gastric emptying rate of the gastrointestinal contents, b) inhibiting the digestive enzyme, c) inhibiting active glucose transport, and so on.

The water soluble dietary fibers, guar gum and pectin,^{4,5)} and water soluble polysaccharide, contained in plants such as *Auricularia aricula-judae* QUEL.⁶⁾ and *Lagerstroemia speciosa* L.,⁷⁾ have been reported to increase viscosity of the gastrointestinal contents, and consequently to decrease the gastric emptying rate, and to suppress and/or delay the intestinal digestion and absorption of carbohydrate.

Materials that are able to manipulate glucose uptake in the small intestine are widely found in plants. Some phenolic glycosides, which exist in a wide variety of materials in plants and related artificial materials and are synthesized by glycosidases, were reported to have an affinity for the intestinal glucose transport system and have been shown to slow the rate of glucose absorption from the gut.⁸⁾ Glycyrrhizin (Grz) is a main substance of licorice, which is one of the most important substances utilized as a Kampo medicine for almost 2000 years, in addition to its well-known use as a flavoring agent. Moreover, Grz was reported to have anti-aller-

gic, antiviral, anti-inflammatory activities.^{9–12)} In a previous study, we prepared convenient methods for screening glycosides that decreased the time course of sugar uptake in mice and demonstrated that Grz had the effect of lowering postprandial blood glucose rise.¹³⁾ To stabilize postprandial blood glucose levels, we investigated the effect of Grz ingestion on lowering blood glucose levels in the long term. The purpose of this study was to investigate the antidiabetic effect of Grz on NIDDM model animals, KK-A^y mice.

MATERIALS AND METHODS

Chemicals Glycyrrhizin monoammonium salt (Grz, Fig. 1) was purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Casein, cornstarch, mineral mixture, vitamin mixture, and cellulose powder were purchased from Oriental Yeast Co. (Tokyo, Japan). Soybean oil and DL-methionine were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animals and Diets Male 4 week-old KK-A^y/Ta Jcl (type

Fig. 1. The Structure of Glycyrrhizin

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Table 1. Composition of the Experimental Diets

	Dietary group		
	Control	0.27% Grz	0.41% Grz
	g/kg diet		
Casein	200	200	200
Cornstarch	541	541	541
Soybean oil	100	100	100
Mineral mixture ^{a)}	40	40	40
Vitamin mixture ^{b)}	22	22	22
Cellulose powder	95	92.3	90.9
L-Methionine	2	2	2
Glycyrrhizin	0	2.7	4.1
Total energy, MJ	16.2	16.2	16.2

a) Composition of the mineral mixture was as follows (g/kg): CaPO₄·2H₂O, 145.6; KH₂PO₄, 257.2; NaH₂PO₄, 93.5; NaCl, 46.6; calcium lactate, 350.9; ferric citrate, 31.8; MgSO₄, 71.7; ZnCO₃, 1.1; MnSO₄·5H₂O, 1.2; CuSO₄·5H₂O, 0.3; KI, 0.1.
b) Composition of the vitamin mixture was as follows (per kg): retinyl acetate, 170 mg; cholecalciferol, 2.5 mg; all-rac- α -tocopheryl acetate, 5 g; menadione, 5.2 g; thiamine-HCl 1.2 g; riboflavin, 4 g; pyridoxine-HCl 800 mg; cyanocobalamin, 0.5 mg; L-ascorbic acid, 30 g; D-biotin, 20 mg; folic acid, 200 mg; calcium pantothenate, 5 g; p-aminobenzoic acid, 5 g; nicotinic acid, 6 g; inositol, 6 g; choline chloride, 200 g; cellulose powder, 731.407 g

2 diabetes) mice were obtained from Clea Japan (Tokyo). The mice were individually housed in stainless steel wire cages. All the mice were kept in an air-conditioned room at 22±2 °C with a lighting schedule of 12 h-light (0600–1800) and 12 h-darkness. The composition of the basal diet (control diet) is indicated in Table 1. Grz was added to the basal diet in place of cellulose (Grz diet). Animals were fed a control diet with free access for 2 weeks of a preliminary test period. Diets for the mice were prepared weekly and stored in a freezer at –20 °C. At 6 weeks of age, the mice were divided into 3 groups of 8 each matched for initial body weight and blood glucose concentration. All had free access to the individual diet and water. Care and treatment of experimental animals conformed to Kyoto University guidelines for the ethical treatment of laboratory animals.

Animal Experiment Body weight, food intake and water intake was recorded daily. Fasting blood glucose concentration of the mice was measured weekly following 5 h (0900–1400) of food deprivation. Blood samples were collected from the tail vein without anesthesia. The serum was immediately separated by centrifugation (9000×g for 5 min) to determine the fasting blood glucose concentration. On the final day of the experiment, the mice were sacrificed by decapitation without anesthesia following 5 h of food deprivation. Blood samples were collected from the neck vein. The serum was immediately separated by centrifugation (9000×g for 5 min) to determine the 5 h-fast physiological characteristics in the blood after 9 weeks of Grz feeding. The heart, liver, spleen, kidneys, perinephrial fat, epididymal fat and gastrocnemius muscle were quickly removed and weighed.

Blood Glucose Response to the Oral Glucose Tolerance Test After 9 weeks of feeding the experimental diets, an oral glucose tolerance test was performed. Each mouse was orally administered 200 μ l of a 20% glucose solution (1 g of glucose/kg of body weight) following 18 h (2000–1400) of food deprivation. Blood samples were collected from the tail vein before the glucose load and at 30, 60 and 120 min thereafter. The serum was immediately separated by centrifugation for measurement of the blood glucose concentration.

Analytical Procedures The concentration of blood glucose was determined by the glucose oxidase method with a commercial kit (Glucose C-II-Test, Wako Pure Chemical Ind., Osaka, Japan). The level of blood insulin was measured with a kit (Glazyme Insulin-EIA-Test, Wako Pure Chemical Ind.). Blood free fatty acid, total cholesterol and triacylglycerol levels were also measured using commercial kits (NEFA C-Test, Cholesterol C-Test and Triglyceride G-Test, Wako Pure Chemical Ind.).

Statistical Analysis Results are expressed as means±S.E.M. One-way analysis of variance (ANOVA) with Fisher's PLSD post hoc test was used to access the statistical significance of difference, with a *p* value of <0.05 used as a measure of significance.

RESULTS

Body Weight, Food Intake and Water Intake Body weight and food intake did not change significantly among the 3 groups throughout the test period (Figs. 2A, B), and both total food intake and final body weight were not statistically different among 3 them (Table 2). As presented in Fig. 2C, the water intake in the 0.41% Grz diet group of mice was significantly lower than those in the control diet group from 7 weeks after the onset of the experiment to the end. The overall mean of the water intake was about 25% lower in the 0.41% Grz mice than in the control mice (Table 2).

Fasting Blood Glucose Concentration Changes in the 5 h-fasting blood glucose concentration are shown in Fig. 2D. Five hour-fasting blood glucose levels in all groups gradually increased with age from the beginning of the test to the 6 th week. The fasting blood glucose levels in the control group jumped up significantly in the 7 th week, although the levels of the mice fed the Grz diet did not. In the 8 th week, the blood glucose levels in 0.27% Grz increased to equal the control diet group. In and after the 8 th week, the blood glucose level in the 0.41% Grz diet group was significantly (*p*<0.05) lower than the other groups.

Blood Glucose Response to the Oral Glucose Tolerance Test The blood glucose concentrations at various times after the oral glucose administration are shown in Fig. 3. The postprandial blood glucose levels in the 0.41% Grz group were significantly (*p*<0.05) lower than those in the other two groups 60 min after the glucose administration. No difference in blood glucose response was observed at any point after the glucose administration to the 0.27% Grz group when compared with the control group.

Physiological Characteristics in the Blood on the Last Day of Experiment As shown in Table 3, the 5 h-fasting blood glucose and insulin level in the 0.41% Grz group was significantly (*p*<0.05) lower than the control and 0.27% Grz group. The concentrations of free fatty acid, total cholesterol and triacylglycerol in the blood were not significantly different among the 3 groups (Table 3). No significant difference was apparent in the absolute and relative weight of the heart, liver, kidney, peripheral fat, epididymal fat or gastrocnemius muscle (data not shown).

Fig. 2. Weekly Changes of Body Weight (A), Food Intake (B), Water Intake (C) and the 5 h Fasting Blood Glucose Levels (D) in KK-Ay Mice

Mice were fed on the control diet (□), 0.27% Grz diet (○), or 0.41% Grz diet (●) in Table 1 for 9 weeks. The symbols and bars represent means $\pm$ S.E.M. of 6–8 mice. The symbols in the same time with no common superscript letters are statistically significant at $p < 0.05$.

Table 2. Final Body Weight, Total Food Intake, and Total Water Intake

	Dietary group		
	Control	0.27% Grz	0.41% Grz
Body weight	41.3 $\pm$ 0.7	42.0 $\pm$ 0.8	40.1 $\pm$ 1.1
Food intake (g/64 d)	416.0 $\pm$ 14.7	413.7 $\pm$ 15.1	394.5 $\pm$ 16.1
Water intake (g/64 d)	1079.1 $\pm$ 95.0	947.0 $\pm$ 67.6	769.4 $\pm$ 87.1*

Final body weight, total food intake, and total water intake 9 weeks after feeding test diet represent means $\pm$ S.E.M. of 6–8 mice. Values in the same row with asterisk (*) are significantly different at $p < 0.05$.

DISCUSSION

Grz is a main substance of licorice, which is one of the most important substances utilized as a Kampo medicine for almost 2000 years, a flavoring agent and a sweetener. In a previous study, we demonstrated that Grz had a significant inhibitory effect on the postprandial blood glucose rise in normal Std ddY mice.¹³⁾ In addition, we confirmed that Grz significantly restricted the postprandial blood glucose rise in KK-A^y mice (data not shown). Therefore, we investigated the antidiabetic effect of Grz in an NIDDM model animal, KK-A^y mice through the long-term feeding of Grz. KK-A^y mice have genetically determined obesity and such diabetic syndromes as hyperglycemia, hyperinsulinemia, glucosuria, and severe insulin resistance, all of which increase with age at

Fig. 3. Effect of Grz on the Blood Glucose Response to the Oral Glucose Tolerance Test in KK-A^y Mice

Mice were fed on the control diet (□), 0.27% Grz diet (○), or 0.41% Grz diet (●) in Table 1 for 9 weeks. Thereafter they were orally given a 20% glucose solution (1 g of glucose/kg body wt) following 18 h of food deprivation. The symbols and bars represent means $\pm$ S.E.M. of 6–8 mice. At each time, symbol with asterisk (*) is significantly different at $p < 0.05$.

least up to 16 weeks.

In this study, by feeding the control diet to KK-A^y mice, the blood glucose level and water intake kept rising with gradual increase in weight gain and food intake for 9 weeks. Elevation of the 5 h-fasting blood glucose level in diabetic

Table 3. Five hour-Fast Physiological Characteristics in the Blood on the Last Day of Experiment

	Dietary group		
	Cont.	0.27% Grz	0.41% Grz
Glucose (mg/dl)	394.6±20.4	366.4±31.3	256.7±44.0*
Insulin (μ U/ml)	64.8±15.9	62.5±10.8	29.9±7.2*
Free fatty acid (mEq/l)	0.879±0.215	0.513±0.121	0.744±0.138
Total cholesterol (mg/dl)	92.0±14.4	67.1±10.9	71.7±13.7
Triacylglycerol (mg/dl)	141.9±27.4	173.0±65.6	150.0±13.2

Five hour-fast physiological characteristics in the blood on the last day of experiment represent means±S.E.M. of 6–8 mice. Values with asterisk (*) are significantly different at $p<0.05$.

mice was suppressed by the addition of Grz to the control diet (Fig. 2D), accompanied by a decrease in water intake (Fig. 2C). The increase in water intake proportional to the elevation of blood glucose content was also characteristic in the KK-A^y mice as generally observed in diabetes. The increase in water intake necessary to depress the elevation of osmotic pressure due to a high blood glucose level was presumed to be reduced by the supply of Grz to diabetic mice. This suggests that the blood glucose level is reduced and secondarily accompanied by the decrease in water intake since abundant glucose is not at once taken into the blood.

The 5 h-fasting blood insulin level in the 0.41% Grz diet group, measured on the last day of the experiment, was the lowest ($p<0.05$) among the three test groups (Table 3). In addition, Grz improved glucose tolerance after 9 weeks of feeding of Grz in KK-A^y mice (Fig. 3), suggesting that these mice have the features of non-insulin dependent (type 2) diabetes: they are known to have insulin resistance in peripheral tissue and/or to be impaired in the insulin receptor.^{6,7,14,15} The inhibitory effect of this substance on food intake was also examined to determine whether reduced food intake would be a major factor causing the antihyperglycemic action in diabetic animals. Grz at the 0.41% level did not affect food intake or body weight (Table 2).

The mechanism by which Grz exerts the antidiabetic effect on diabetic mice is not clear. Grz is one of the triterpene saponins. Shimizu *et al.* reported that extracts from the leaves of *Gymnema* plants, which consists of triterpene saponins like gymnemic acid, suppressed the postprandial blood glucose rise by inhibiting the Na⁺-glucose cotransport system.¹⁶ Since the chemical structure of Grz and extracts from *Gymnema* are common, we hypothesized that the mechanism lowering postprandial blood glucose rise by Grz was common to that of the extracts from *Gymnema* plants, by due to preventing the glucose active transport system in the small intestine. Grz might prevent rapid and drastic postprandial blood glucose rise, which leading to restraining of the reduction of insulin sensitivity in KK-A^y mice. The metabolic fate of absorbed Grz was also well studied.¹⁸ Grz and/or glycyrrhetic acid have been reported to act as a physiological activator, an antiallergic, antiviral or anti-inflammatory substance. Orally administered Grz is converted to glycyrrhetic acid by glycyrrhizin-hydrolyzing bacteria in the intestine and the resulting glycyrrhetic acid is absorbed.¹⁸ Glycyrrhetic

acid may play an important role because of its pharmacological action and its side effects; electrolyte imbalance (hypernatraemia and hypokalaemia), edema, increased body weight and suppression of the renin-angiotensin-aldosterone system. In our preliminary test, we investigated the relationship of Grz dose and the growth of KK-A^y mice. At the dose of 2.5% Grz, daily food intake and the body weight of the test mice began to decrease one week after feeding this diet. However, below the dose of 1% Grz, food intake and body weight changed in the same way. At the dose of 0.27% and 0.41% Grz, the dose used in this study, these factors became the same level of the mice fed control diet. From these results, we assumed the dose of Grz used in this test did not induce any side effects. Our data suggested that absorbed Grz and/or glycyrrhetic acid may act as an antidiabetic substance without inducing side effects.

In conclusion, Grz treatment for 9 weeks suppressed the rise in fasting blood glucose and insulin levels, and improved the glucose tolerance in KK-A^y mice without affecting food intake or final body weight. The results of this study clearly demonstrate that dietary supplementation of Grz at the 0.41% level could prevent the progress of NIDDM in KK-A^y mice. Unfortunately, we do not yet know how Grz works; however, we are now investigating its mechanism of action. Our data indicate that Grz has therapeutic potential and useful activities for the treatment of hyperglycemia associated with NIDDM.

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