Hyperlipemia-Improving Effects of Ginsenoside-Rb2 in Streptozotocin-Diabetic Rats

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The effect of ginsenoside-Rb2 purified from ginseng was investigated in streptozotocin-induced diabetic rats. The rats treated with ginsenoside-Rb2 showed significant decreases in triglyceride, non-esterified fatty acid, and total cholesterol in the serum. A lipid-improving action in the serum was observed in rats with hyperlipemia. Furthermore, the lipolytic activity of lipoprotein lipase was stimulated with a concomitant decrease in the levels of triglyceride and very low density lipoprotein in the serum, while a repressive effect on hormone-sensitive lipase activity was observed. The accumulation of lipid in the adipose tissue was also observed. In addition to the hypolipemic effect, ginsenoside-Rb2 exhibited a lowering action on the serum levels of 3-hydroxybutyrate and acetoacetate in streptozotocin-induced diabetic rats, indicating an improvement of diabetic ketoacidosis.

Keywords——ginsenoside-Rb2; streptozotocin-induced diabetic rat; serum lipid-lowering action; lipoprotein lipase; hormone-sensitive lipase; adipose tissue

In the previous paper, we reported the mechanism of hypoglycemic action produced by ginsenoside-Rb2 in streptozotocin-induced diabetic rats.[1,2] Intraperitoneal administration of ginsenoside-Rb2 to rats was found to stimulate the activities of glucose-6-phosphatase (EC 3.1.3.9) and glucokinase (EC 2.7.1.2) in the liver. These enzymes play an important role in the maintenance of blood glucose level. When glucose utilization is impaired in the liver of streptozotocin-induced diabetic rats, ginsenoside-Rb2 may have a hypoglycemic activity by means of changing the levels of gluconeogenic and glycolytic enzymes and shifting the direction of the overall metabolic flow toward glucose degradation. Additional experimental results indicated that a single intraperitoneal administration of ginsenoside-Rb2 to diabetic rats lowered the serum lipid level.[2] Thus, ginsenoside-Rb2 may be an excellent therapeutic agent for the hyperlipemia due to diabetes. To investigate this possibility, we studied the effect of this compound on the lipid constituents in the serum and adipose tissue, and also on the activity of lipolytic enzymes in the adipose tissue of streptozotocin-induced diabetic rats.

Materials and Methods

Animals——Male rats of the JCL: Wistar strain (Hokuriku Labour, Ltd., Toyama, Japan), initially weighing 120—130 g, were maintained in an air-conditioned room with lighting from 6 a.m. to 6 p.m. The room temperature (about 25 °C) and humidity (about 60%) were controlled automatically. A laboratory pellet chow (obtained from CLEA Japan Inc., Tokyo; protein 24.0%, lipid 3.5%, carbohydrate 60.5%) and water were given freely.

Streptozotocin-Induced Diabetic Rats[3]——Streptozotocin (65 mg/kg body weight) dissolved in 10 mM citrate buffer (pH 4.5) was injected intraperitoneally. Several days after the injection, the blood glucose level was determined and rats with a glucose level of 200 mg/dl or more were used as diabetic rats.

Saponin——Ginsenoside-Rb₂ was isolated and purified from the extract of roots of *Panax ginseng* C. A. MEYER according to the procedure of Shibata and co-workers.[4] This preparation was found to be pure by various chemical and physicochemical analyses.

Treatment with Ginsenoside-Rb₂——Ginsenoside-Rb₂ (10 mg/rat/d) dissolved in saline was administered intraperitoneally to rats for 6 d, while control rats were treated with an equal volume of saline. At 12 h after the last treatment, rats were sacrificed by means of a blow on the head, and exsanguinated. Rats were killed between 2 and 3 p.m. to avoid the effect of circadian variation. The blood was collected in a conical centrifuge tube to determine triglyceride, non-esterified fatty acid, total cholesterol, 3-hydroxybutyrate, acetoacetate, lactate, and lipoprotein. Epididymal adipose tissue was removed quickly, cooled on ice, and weighed rapidly. A portion of the adipose tissue was homogenized in a Potter-Elvehjem type glass homogenizer with a Teflon pestle.

Chemicals——Streptozotocin and β-hydroxybutyrate dehydrogenase were purchased from Sigma Chemical Co., U.S.A. Nicotinamide adenine dinucleotide (NAD) and reduced nicotinamide adenine dinucleotide (NADH) were obtained from P-L Biochemicals, Inc., U.S.A., and lactic dehydrogenase was from Oriental Yeast Co., Tokyo, Japan. Intralipid was purchased from the Green Cross Corp., Osaka, Japan. All other reagents were of the highest grade commercially available.

Statistics——The significance of differences between the control and ginsenoside-treated groups was tested by means of Student's t-test.

Determination of Triglyceride, Non-esterified Fatty Acid, Total Cholesterol, 3-Hydroxybutyrate, Acetoacetate, and Lactate in the Serum——Triglyceride, non-esterified fatty acid, and total cholesterol were determined by using commercial reagents ("TG-Five Kainos" obtained from Kainos Laboratories, Inc., Tokyo, Japan; "NEFA Kainos" obtained from Kainos Laboratories, Inc., Tokyo, Japan; "Cholesterol B-Test Wako" obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan). 3-Hydroxybutyrate was determined spectrophotometrically by measuring the increase of optical density at 340 nm resulting from the oxidation of 3-hydroxybutyrate by 3-hydroxybutyrate dehydrogenase and NAD.[5] The determination of acetoacetate was based on the decrease in extinction at 340 nm due to the oxidation of NADH.[6] Lactate was determined by a spectrophotometric method, based on the measurement of the increase of optical density at 340 nm.[7]

Determination of Very Low Density Lipoprotein (VLDL) in the Serum——Serum lipoprotein was separated according to the method of Havel *et al.* with a slight modification.[8] The serum was adjusted to $d = 1.21$ with KBr, and 3.2 ml aliquots were pipetted into polyallomer tubes. A discontinuous gradient was formed by carefully layering 2.4 ml of salt solution $d = 1.063$ above the serum, followed by 2.4 ml of salt solution $d = 1.019$. Finally, the tube was filled with 2.0 ml of $d = 1.006$ salt solution. The samples were centrifuged at $105000 \times g$ for 20 h. Ultracentrifugation was carried out in the SW 41 rotor of a Beckman type L5-65 ultracentrifuge (Beckman Inc., Palo Alto, California). After centrifugation, a syringe was attached to the polyallomer tube and a few drops (about 250 μl) of sample were delivered continuously into test tubes. Triglyceride, total cholesterol, phospholipid, and protein in the fractions were determined by using commercial reagents ("TG-Five Kainos" obtained from Kainos Laboratories, Inc.; "Cholesterol B-Test Wako," "Phospholipids-Test Wako," and "A/G B-Test Wako" obtained from Wako Pure Chemical Industries, Ltd.). VLDL fraction obtained by the present method is identical with that obtained by the cellulose acetate electrophoresis technique.

Determination of Triglyceride, Phospholipid, and Non-esterified Fatty Acid in the Adipose Tissue——Epididymal adipose tissue was placed in 20 ml of $CHCl_3$–MeOH mixture (2 : 1, v/v). The $CHCl_3$–MeOH solution was used for the estimation of triglyceride, phospholipid, and non-esterified fatty acid. Determinations were performed by using commercial reagents as described above.

Determination of Enzyme Activities in the Adipose Tissue——a) Lipoprotein Lipase:[9,10] Epididymal adipose tissue was passed through a grinder and defatted with acetone at room temperature. The resultant fibrous mat was cut into small pieces and extracted for 60 min at 0 °C with 10 mM NH_4OH. The insoluble residue was then removed by centrifugation and the supernatant fluid was used as an enzyme solution. The reaction mixture, containing 50 μl of active substrate [10% Intralipid (a commercial emulsion of soybean oil and lecithin) and an equal volume of rat serum incubated at 37 °C for 30 min], 350 μl of enzyme solution, 50 μl of 0.3 M Tris–HCl buffer (pH 8.5), and 50 μl of 20% bovine serum albumin, was incubated at 37 °C for 60 min. Fatty acid released was estimated by using a commercial reagent ("NEFA-Test Wako" obtained from Wako Pure Chemical Industries, Ltd.).

b) Hormone-Sensitive Lipase:[10,11] Homogenization and centrifugation of each adipose tissue were carried out in a cold room at 4 °C. Extracts of fat pads were prepared by homogenizing the tissue in 3 ml of 0.25 M sucrose per g of tissue in a glass chamber with a Teflon pestle. The homogenate was centrifuged at $12000 \times g$ for 10 min. The fat cake accumulated at the top of the tube was discarded. The remaining supernatant was saved for assay of hormone-sensitive lipase activity. Lipolytic activity was measured by placing 0.1 ml of an extract in a glass-stoppered tube with 0.1 ml of 2% Intralipid, 0.02 ml of 20% extracted albumin (pH 7.4), 0.2 ml of 0.03 M phosphate buffer (pH 7.4), and sufficient water to make a final volume of 2.5 ml. After 60 min of incubation at 37 °C, fatty acid released was estimated by using a commercial reagent ("NEFA-Test Wako" obtained from Wako Pure Chemical Industries, Ltd.).

Determination of Protein——Protein was determined by the method of Itzhaki and Gill, with bovine serum albumin as a standard.[12]

Results

Effect of Ginsenoside-Rb$_2$ on the Lipid Constituents in the Serum

Table I shows the serum constituent levels of rats of the ginsenoside-Rb$_2$-treated and control groups. The rats treated with ginsenoside-Rb$_2$ showed a significant decrease in triglyceride level; as shown in Table I, the triglyceride level was about 48% less at the 6th day in the ginsenoside-Rb$_2$-treated group as compared with the control group. Similarly, administration of ginsenoside-Rb$_2$ to rats resulted in a significant decrease in the non-esterified fatty acid from 976.5 μeq/l to 726.6 μeq/l. The level of total cholesterol was also about 21% lower after 6 administrations in the ginsenoside-Rb$_2$-treated group as compared with the control group. Furthermore, a conspicuous decrease was observed in the levels of serum 3-hydroxybutyrate, acetoacetate, and lactate. The 3-hydroxybutyrate level, which was 0.42 μmol/ml, decreased to 0.30 μmol/ml in rats treated with ginsenoside-Rb$_2$. The level of acetoacetate decreased to 0.10 μmol/ml on average. The level of lactate was also about 17% lower after 6 administrations.

TABLE I. Effect of Ginsenoside-Rb$_2$ on Serum Constituents

	Triglyceride (mg/dl)	Non-esterified fatty acid (μeq/l)	Total cholesterol (mg/dl)	3-Hydroxy-butyrate (μmol/ml)	Acetoacetate (μmol/ml)	Lactate (μmol/ml)
Normal rat	65.1±6.9	662.4±22.6	100.4±3.1	0.14±0.04	N.D.	0.34±0.02
Diabetic rat						
Control	343.6±71.8 (100)	976.5±77.4 (100)	152.7±11.9 (100)	0.42±0.02 (100)	0.28±0.04 (100)	0.94±0.05 (100)
Rb$_2$	178.5±24.0[a] (52)	726.6±57.8[a] (74)	120.5±6.1[a] (79)	0.30±0.03[b] (71)	0.10±0.03[b] (36)	0.78±0.03[a] (83)

Values are means ± S.E. of 6 rats. Figures in parentheses are percentages of the control value. Significantly different from the control value, a) $p < 0.05$, b) $p < 0.01$. N.D., not detectable.
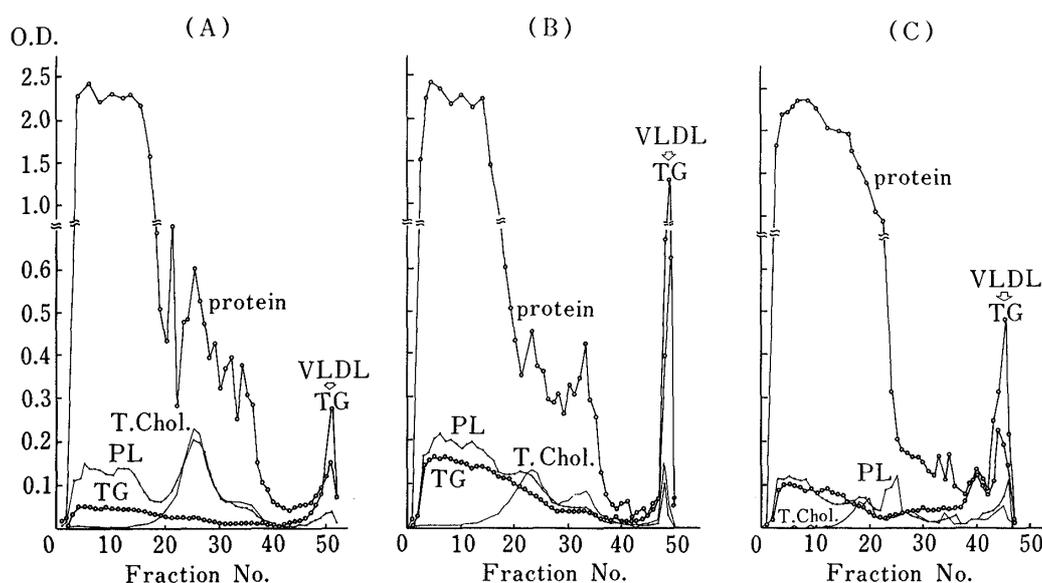

Fig. 1. Effect of Ginsenoside-Rb$_2$ on the Ultracentrifugation Pattern of Lipoprotein in the Serum

Values are means of 6 rats. (A) normal rat, (B) diabetic rat (control group), (C) diabetic rat (ginsenoside-Rb$_2$-treated group).
TG, triglyceride; PL, phospholipid; T.Chol., total cholesterol.

TABLE II.   Effect of Ginsenoside-Rb$_2$ on the VLDL Constituents in the Serum

	Triglyceride (mg/dl)	Total cholesterol (mg/dl)	Phospholipid (mg/dl)	Protein (mg/dl)
Normal rat	64.7	8.2	9.8	14.4
Diabetic rat				
Control	165.8	11.0	10.2	45.9
	(100)	(100)	(100)	(100)
Rb$_2$	84.0	6.8	11.0	24.9
	(51)	(62)	(108)	(54)

Values are means of 6 rats.   Figures in parentheses are percentages of the control value.

TABLE III.   Effect of Ginsenoside-Rb$_2$ on the Lipid Constituents
in the Adipose Tissue

	Triglyceride (mg/tissue)	Phospholipid (mg/tissue)	Non-esterified fatty acid (mg/tissue)
Normal rat	175.9 ± 11.2	17.8 ± 1.6	2.63 ± 0.23
Diabetic rat			
Control	110.4 ± 7.0	13.2 ± 1.8	2.30 ± 0.30
	(100)	(100)	(100)
Rb$_2$	172.2 ± 18.9[b]	18.5 ± 1.3[a]	2.26 ± 0.19
	(156)	(140)	(98)

Values are means ± S.E. of 6 rats.   Figures in parentheses are percentages of the control value.
Significantly different from the control value, a) $p < 0.05$, b) $p < 0.01$.

TABLE IV.   Effect of Ginsenoside-Rb$_2$ on the Lipolytic Activity
in the Adipose Tissue

	Lipoprotein lipase (neq/h/mg protein)	Hormone-sensitive lipase (neq/h/mg protein)
Normal	388 ± 15	12.4 ± 1.3
Diabetic rat		
Control	244 ± 18	14.4 ± 2.3
	(100)	(100)
Rb$_2$	400 ± 40[a]	13.0 ± 2.6
	(164)	(90)

Values are means ± S.E. of 4 rats.   Figures in parentheses are percentages of the control value.
a) Significantly different from the control value, $p < 0.01$.

In connection with this experiment, a relative decrement of VLDL was observed in the ginsenoside-Rb$_2$-treated group (Fig. 1). The concentration of triglyceride in the VLDL decreased by 49% as compared with the control group. A decrease was also observed in the amounts of total cholesterol and protein. However, there was no significant change in the level of phospholipid (Table II).

Effect of Ginsenoside-Rb$_2$ on the Lipid Constituents in the Adipose Tissue

As shown in Table III, a striking increase in triglyceride content in the adipose tissue was observed after 6 administrations of ginsenoside-Rb$_2$. The phospholipid content also increased by 40% when ginsenoside-Rb$_2$ was administered. However, non-esterified fatty acid in the

adipose tissue showed no appreciable change.

Effect of Ginsenoside-Rb₂ on the Lipolytic Enzyme Activities in the Adipose Tissue

The treatment of rats with ginsenoside-Rb_2 altered the lipolytic activity of lipoprotein lipase, which is of interest in view of the importance of uptake of circulating chylomicrons and lipoprotein triglyceride. As shown in Table IV, the lipolytic activity of lipoprotein lipase increased about 64% when ginsenoside-Rb_2 was administered and reached an almost normal fed-rat level. In contrast, administration of ginsenoside-Rb_2 to rats tended to decrease the activity of hormone-sensitive lipase (Table IV).

Discussion

The most common lipid abnormality in diabetes is hypertriglycemia. The extent to which these abnormalities occur and their mechanism in different types of diabetes remain to be defined fully. Diabetics often also have an elevated non-esterified fatty acid level, and high non-esterified fatty acid can provide excess substrate for hepatic triglyceride synthesis.[13-16]

In the present experiment, ginsenoside-Rb_2 decreased the triglyceride level, indicating an improvement of hypertriglycemia (Table I). In addition, the treatment of rats with ginsenoside-Rb_2 caused decrements in non-esterified fatty acid and total cholesterol (Table I). As compared with the significant decrease in the serum triglyceride level, however, the changes in the levels of non-esterified fatty acid and total cholesterol were smaller. It seems clear that ginsenoside-Rb_2 has an improving effect on hyperlipemia induced by streptozotocin. As reported previously, a single administration of ginsenoside-Rb_2 reduced serum triglyceride, non-esterified fatty acid, and total cholesterol.[2] The present study demonstrated that repeated administration of ginsenoside-Rb_2 had a more effective hyperlipemic-lowering action.

Our observations also suggest that a striking change of triglyceride content in the adipose tissue occurs after repeated administration of ginsenoside-Rb_2 (Table III). Rat epididymal adipose tissue is known to contain at least two distinct lipases, hormone-sensitive lipase and lipoprotein lipase. Lipoprotein lipase regulates the rate of uptake of plasma triglyceride by adipose tissue, because it catalyzes the hydrolysis of circulating chylomicrons and lipoprotein triglyceride that occurs during the uptake of fatty acid components. A study on the effect of ginsenoside-Rb_2 on the lipoprotein lipase activity in adipose tissue showed that the lipolytic activity of this enzyme was increased by the repeated administration of ginsenoside-Rb_2, whereas the activity of hormone-sensitive lipase decreased (Table IV). Furthermore, VLDL, which is proposed to play a major role in endogenous triglyceride transport in the circulation, decreased (Fig. 1). These results suggest that ginsenoside-Rb_2 stimulates the uptake of circulating triglyceride into adipose tissue through enhancement of the lipoprotein activity.

In addition to the hypolipemic effect, ginsenoside-Rb_2 exhibited a lowering action on the serum levels of 3-hydroxybutyrate and acetoacetate in streptozotocin-induced diabetic rats, indicating an improvement of diabetic ketoacidosis (Table I). These experiments suggest the usefulness of ginsenoside-Rb_2 in the atherosclerotic disease of diabetes.

Previously, we reported that ginsenoside-Rb_2 stimulates various metabolic reactions involved in lipid and sugar metabolism in normal rats.[17,18] The effects included stimulations of the glycolytic and lipogenic pathways, which could explain the accumulation of triglyceride in adipose tissue. Additional experimental results indicated that by the repeated administration of ginsenoside-Rb_2 in hyperlipemia induced by high-cholesterol diet feeding, an increment in the lipoprotein lipase activity was associated with the increasing lipid content in adipose tissue.[19] These actions of ginsenoside-Rb_2 may be regarded as an antiatherogenic activity. Moreover, these phenomena are consistent with those observed in the streptozotocin-

induced diabetic rats in the present study. Such alterations show that ginsenoside-Rb$_2$ induced a metabolic pattern compatible with the usual behavior of the overall metabolic pathways under various physiological circumstances.

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