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Hyperlipemia-Improving Effects of Ginsenoside-Rb_2 in Cholesterol-Fed Rats

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The effect of ginsenoside-Rb_2 purified from ginseng on lipid metabolism was examined in rats fed on a high cholesterol diet. A single intraperitoneal administration of ginsenoside-Rb_2 produced a significant decrease of total cholesterol, free cholesterol, low density lipoprotein (LDL)-cholesterol, triglyceride, 3-hydroxybutyrate, and acetoacetate levels in the serum. However, a significant increase of high density lipoprotein (HDL)-cholesterol level in the serum was observed after the treatment. Repeated administration of ginsenoside-Rb_2 to rats with hyperlipemia induced by a high-cholesterol diet resulted in a striking decrease in the levels of total cholesterol, free cholesterol, LDL-cholesterol, and triglyceride, whereas the level of HDL-cholesterol was again increased. The changes were larger than those produced by a single intraperitoneal administration. Furthermore, the lipolytic activity of lipoprotein lipase was stimulated with a concomitant decrease in the levels of triglyceride and very low density lipoprotein (VLDL)-triglyceride in the serum. Accumulation of lipid in the adipose tissue was observed. In addition, a slight decrease in hepatic total cholesterol was observed after the treatment, but there was no statistically significant difference between the control and ginsenoside-Rb_2-treated groups. The mechanism of hypolipemic action of ginsenoside-Rb_2 is discussed on the basis of these results.

Keywords——ginsenoside-Rb_2; high-cholesterol-fed rat; hypolipemic action; lipoprotein lipase; adipose tissue; hepatic cholesterol

The extract from the roots of *Panax ginseng* C. A. MEYER has been used as a therapeutic agent for various diseases including hyperlipemia, atherosclerosis, hypertension, and so forth. From a biochemical standpoint, the extract has a variety of effects on lipid metabolism, as shown by Yamamoto *et al.*,[1-4] Sakakibara *et al.*,[5] Gommori *et al.*,[6] and Ikehara *et al.*[7] Studies on the hypolipemic action of ginseng have been pursued by Yamamoto and his colleagues, who showed that oral administration of red ginseng powder reduced plasma total cholesterol, triglyceride, and non-esterified fatty acid in high cholesterol diet-fed rats, while plasma high density lipoprotein (HDL)-cholesterol was elevated.[1-3] Plasma lipid-improving actions were also observed in patients with hyperlipemia.[3,4] Sakakibara *et al.*[5] reported the effects of five purified saponins (ginsenoside-Rb_1, -Rc, -Rd, -Re, and -Rg_1) on the concentrations of serum and liver cholesterol. The results indicated enhancement of cholesterol metabolism by administered saponins, particularly by ginsenoside-Rb_1. On the other hand, we showed previously that semi-purified saponin (fraction 4 and 5) stimulates various reactions of lipid and sugar metabolism in normal rats.[8-12] Additional experimental results indicated that most of the biochemical effects of semi-purified saponin might be due to ginsenoside-Rb_2.[13,14] In connection with these experiments, we have obtained evidence that ginsenoside-Rb_2 treatment of streptozotocin-induced diabetic rats produced an improvement of diabetic hyperlipemia and ketoacidosis.[15] The present paper deals with the effect of ginsenoside-Rb_2

in rats fed on a high cholesterol diet.

Materials and Methods

Animals and Treatments——Male rats of the JCL: Wistar strain (Hokuriku Labour, Ltd., Toyama, Japan), initially weighing 100—110 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 chow diet (powdered CE-2 of CLEA Japan Inc., Tokyo) containing 1% cholesterol and 0.5% cholic acid was given *ad libitum*. Ginsenoside-Rb_2 in saline was administered intraperitoneally to test rats, while control rats were treated with an equal volume of saline. At the indicated time after intraperitoneal administration of ginsenoside, rats were sacrificed by means of a blow on the head and exsanguinated. Blood was collected and allowed to stand for several hours in a cold room at 4 °C. Serum was separated by centrifugation ($1000 \times g$, 10 min, 4 °C). The liver and epididymal adipose tissue were removed quickly, cooled on ice, and weighed rapidly. Portions of the liver and adipose tissue were homogenized in a Potter–Elvehjem type glass homogenizer with a Teflon pestle.

Saponin——Ginsenoside-Rb_2 was isolated and purified from the extract of roots of *Panax ginseng* C. A. MEYER according to the procedure of Shibata and co-workers.[16] This preparation was found to be pure by various chemical and physicochemical analyses.

Chemicals——Nicotinamide adenine dinucleotide (NAD) and reduced nicotinamide adenine dinucleotide (NADH) were purchased from P-L Biochemicals, Inc., U.S.A. β-Hydroxybutyrate dehydrogenase was obtained from Sigma Chemical Co., U.S.A. and Intralipid was 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 Total Cholesterol, Free Cholesterol, Low Density Lipoprotein (LDL)-Cholesterol, HDL-Cholesterol, Triglyceride, Non-Esterified Fatty Acid, 3-Hydroxybutyrate, and Acetoacetate in the Serum——Total cholesterol, free cholesterol, triglyceride, and non-esterified fatty acid were determined by using commercial reagents ("Cholesterol B-Test Wako" and "Cholesterol-Test Wako Assistant Reagent" obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan; "TG-Five Kainos" and "NEFA Kainos" obtained from Kainos Laboratories, Inc., Tokyo, Japan). LDL- and HDL-cholesterol were determined by the method of Noma *et al.*[17,18] 3-Hydroxybutyrate was determined spectrophotometrically by measuring the increase of optical density at 340 nm, which was based on the oxidation of 3-hydroxybutyrate by 3-hydroxybutyrate dehydrogenase and NAD.[19] The determination of acetoacetate was based on the decrease in extinction at 340 nm due to the oxidation of NADH.[20]

Separation of Serum Very Low Density Lipoprotein (VLDL) by Density-Gradient Ultracentrifugation——Separation of serum VLDL was conducted according to the method of Havel *et al.* with a slight modification.[21] 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 at 15 °C. Ultracentrifugation was carried out in an SW 41 rotor on a Beckman L5-65 type ultracentrifuge (Beckman Inc., Palo Alto, California). After centrifugation, a syringe was attached to the polyallomer tube and fractions of 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" from Kainos Laboratories, Inc.; "Cholesterol B-Test Wako," "Phospholipids-Test Wako," and "A/G B-Test Wako" obtained from Wako Pure Chemical Industries, Ltd.).

Determination of Total Lipid in the Adipose Tissue——Epididymal adipose tissue was placed in 20 ml of $CHCl_3$–MeOH mixture (2 : 1, v/v). Total lipid was extracted from the tissue by shaking. The residual tissue was then removed, and the $CHCl_3$–MeOH solution was partitioned and washed by the method of Folch *et al.*[22] The organic solution was evaporated and the residue was dried over P_2O_5 overnight. The concentration of total lipid was determined by gravimetry.

Determination of Cholesterol in the Liver——Liver cholesterol was determined by a modification of the method reported by Ichida.[23] A portion of the liver was homogenized with 15 volumes of EtOH. The homogenate was treated at 50—60 °C in a water bath for 30 min and filtered through defatted filter paper (No. 7, Toyo Roshi). The residue was further extracted with about 10 ml of EtOH–ether (3 : 1, v/v) at 50—60 °C for 30 min. The volume of the combined filtrates was adjusted to 10 ml. The concentrations of total and free cholesterol in this extract were determined by using a commercial reagent as described above.

Determination of Lipoprotein Lipase Activity in the Adipose Tissue[24,25]——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 the enzyme solution. The reaction mixture, containing 50 μl of active substrate [10% Intralipid (a commercial emulsion of soybean oil and lecithin) incubated with an equal volume of rat serum 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 as described above.

Results

Single Administration of Ginsenoside-Rb_2

Table I shows the effect of ginsenoside-Rb_2 on the serum constituents after a single intraperitoneal administration. The concentrations of total cholesterol, free cholesterol, and LDL-cholesterol were decreased by 27% (at 20 h), 23—43% (at 16, 20 h), and 39—41% (20, 24 h), respectively. The concentrations of triglyceride, 3-hydroxybutyrate, and acetoacetate were also decreased by 20—26% (8, 16, 24 h), 35—39% (12, 20, 24 h), and 35—67% (4, 12, 16, 20, 24 h), respectively. In contrast, a significant increase of HDL-cholesterol level was observed 20 h after the intraperitoneal administration of ginsenoside-Rb_2 as compared with the control value, but had almost recovered to the control level 24 h after the treatment. However, the non-esterified fatty acid level showed no appreciable change after ginsenoside-Rb_2 administration. Thus, hyperlipemia induced with a high cholesterol diet was improved by ginsenoside-Rb_2 treatment.

A study of the hyperlipemic-lowering action of ginsenoside-Rb_2 was performed by using rats having total cholesterol levels of 85—380 mg/dl. The data in Fig. 1 show that total cholesterol level was correlated with the free cholesterol, LDL-cholesterol, HDL-cholesterol, and triglyceride levels in the rats not treated with ginsenoside-Rb_2. In particular, the decrease in the total cholesterol level was associated significantly with the decrease of LDL-cholesterol level ($r=0.98$). Similar relationships were observed with free cholesterol and triglyceride. On the other hand, the total cholesterol level was decreased with a concomitant increase in the level of HDL-cholesterol ($r=-0.78$). Twenty hours after the intraperitoneal administration of 10 mg of ginsenoside-Rb_2, there was a clear reduction of hyperlipemia. In particular, treatment of rats with ginsenoside-Rb_2 greatly reduced the level of free cholesterol. A marked decrease was also observed in the level of LDL-cholesterol.

Repeated Administration of Ginsenoside-Rb_2

Figure 2 shows the serum constituent levels of rats of the ginsenoside-Rb_2-treated and control groups. The rats of the ginsenoside-Rb_2-treated group showed a significant decrease of total cholesterol level; as shown in Fig. 2, the total cholesterol level was about 40—50% lower after 3 and 6 administrations of ginsenoside-Rb_2. Similarly, administration of ginsenoside-Rb_2 to rats resulted a significant decrease of free cholesterol from 18.0 mg/dl after 3 administrations to 14.9 mg/dl after 6 administrations. The level of LDL-cholesterol was also about 55—61% lower after 3—6 administrations in the ginsenoside-Rb_2-treated group as compared with the control group. In contrast, a striking increase of HDL-cholesterol level was observed after 6 intraperitoneal administrations of ginsenoside-Rb_2; the mean values in the control and ginsenoside-Rb_2-treated groups were 13.2 and 19.5 mg/dl, respectively. The repeated administrations were more effective than a single intraperitoneal administration.

Furthermore, a significant decrease of triglyceride level in the serum of the ginsenoside-Rb_2-treated group was noticed after 6 administrations. As shown in Fig. 2, triglyceride (initially 64.6 mg/dl) decreased to 37.9 mg/dl in rats treated with ginsenoside-Rb_2. A relative decrease of VLDL with respect to triglyceride was observed in the ginsenoside-Rb_2-treated group as compared with the control group (Fig. 3). The lipid content extracted from epididymal adipose tissue was about 45% higher when ginsenoside-Rb_2 was administered (205 ± 25 *vs.* 298 ± 30 mg/tissue). These results suggest that ginsenoside-Rb_2 causes the accumulation of lipid in adipose tissue as a result of its stimulating action on the uptake of circulating chylomicrons and lipoprotein triglyceride.

TABLE I. Time Course of Effect of Ginsenoside-Rb_2 on Serum Constituents after Single Administration

Time after treatment (h)	T. chol. (mg/dl)	Free chol. (mg/dl)	LDL-chol. (mg/dl)	HDL-chol. (mg/dl)	TG (mg/dl)	NEFA (μeq/l)	3-Hydroxybutyrate (μmol/ml)	Acetoacetate (μmol/ml)
Control	329.5±18.1 (100)	55.8±3.7 (100)	250.6±20.7 (100)	18.6±0.6 (100)	63.9±4.2 (100)	879.1±61.5 (100)	0.23±0.02 (100)	0.051±0.005 (100)
4	299.2±12.3 (91)	45.9±3.0 (82)	216.5±11.4 (86)	19.9±0.9 (107)	65.1±9.8 (102)	814.0±58.0 (93)	0.24±0.02 (104)	0.033±0.005[a] (65)
8	296.4±39.8 (90)	35.8±5.5 (64)	211.4±35.5 (84)	19.0±1.1 (102)	47.3±4.2[a] (74)	850.5±116.6 (97)	0.26±0.03 (113)	0.032±0.007 (63)
12	344.2±18.2 (104)	46.6±4.0 (84)	185.4±23.3 (74)	20.2±1.2 (109)	62.8±6.1 (98)	776.8±37.0 (88)	0.14±0.02[b] (61)	0.023±0.007[b] (45)
16	313.9±14.5 (95)	42.8±3.0[a] (77)	192.5±19.3 (77)	19.5±1.2 (105)	49.3±2.0[a] (77)	805.4±72.2 (92)	0.18±0.02 (78)	0.020±0.007[b] (39)
20	241.1±26.4[a] (73)	31.8±4.5[b] (57)	148.4±26.7[a] (59)	22.6±1.7[a] (122)	55.2±3.0 (86)	762.5±107.9 (87)	0.14±0.03[a] (61)	0.017±0.004[c] (33)
24	283.9±25.9 (86)	40.5±6.7 (73)	152.9±25.5[a] (61)	19.8±1.1 (106)	51.2±3.3[a] (80)	737.5±85.0 (84)	0.15±0.02[a] (65)	0.021±0.003[c] (41)

Abbreviations: T. chol., total cholesterol; Free chol., free cholesterol; LDL-chol., low density lipoprotein cholesterol; HDL-chol., high density lipoprotein cholesterol; TG, triglyceride; NEFA, non-esterified fatty acid. Rats received 10 mg of ginsenoside-Rb_2 intraperitoneally, while control rats were treated with an equal volume of saline. At the indicated time after treatment, rats were killed. Values are means±S.E. of 6 rats. Figures in parentheses are percentages of the control value. *a*) Significantly different from the control value, $p<0.05$. *b*) $p<0.01$. *c*) $p<0.001$.

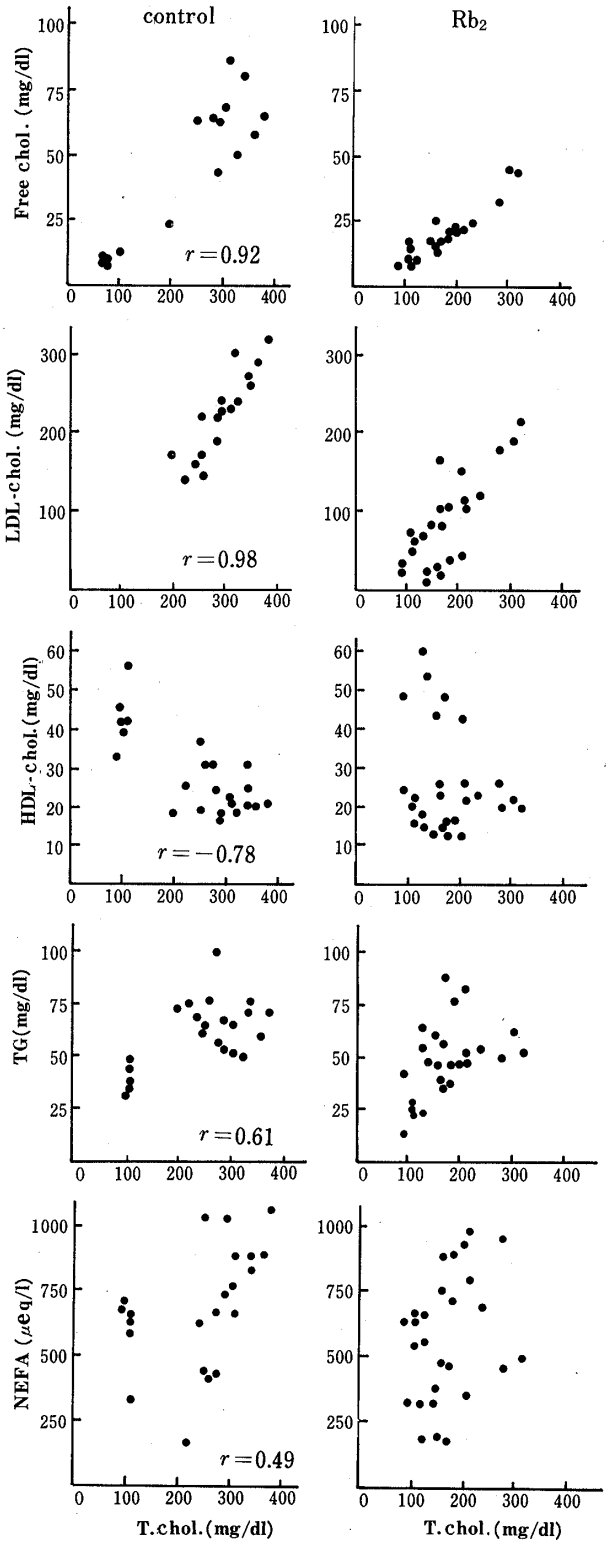

Fig. 1. Relation between Total Cholesterol Level and Free Cholesterol, LDL-Cholesterol, HDL-Cholesterol, Triglyceride, or Non-Esterified Fatty Acid in the Serum

Rats received 10 mg of ginsenoside-Rb_2 intraperitoneally, while control rats were treated with an equal volume of saline. At 20 h after the treatment, rats were killed.

Control; left side; Rb_2, right side.

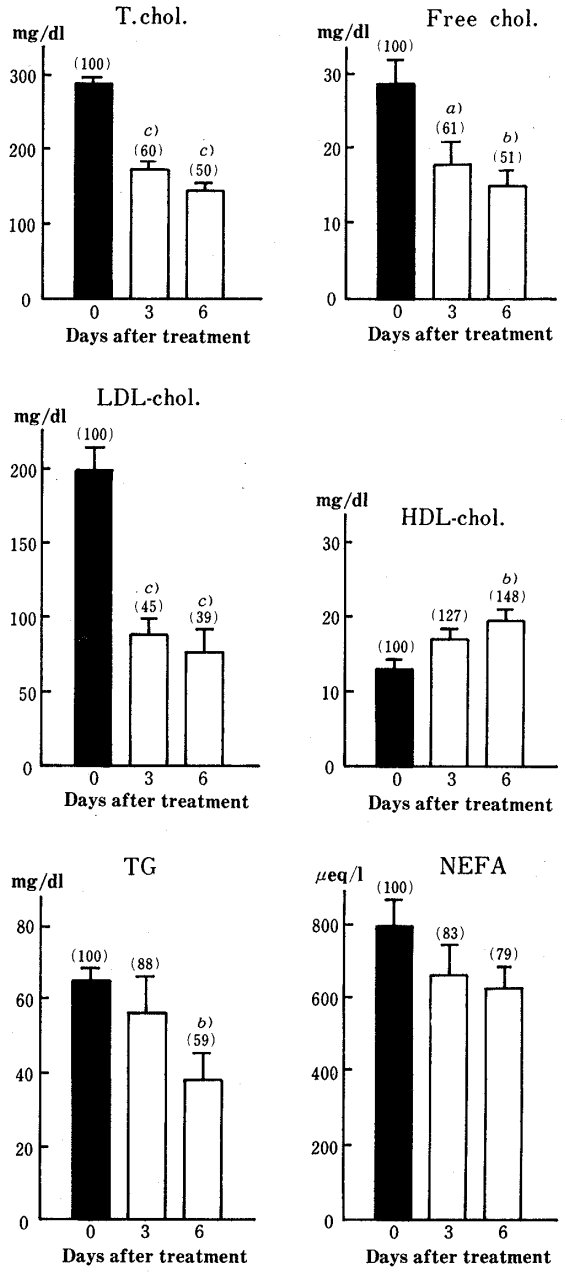

Fig. 2. Effect of Ginsenoside-Rb_2 on Serum Constituents after Repeated Administration

Ginsenoside-Rb_2 (10 mg/rat/d) was administered intraperitoneally to rats for 3 or 6 d, while control rats were treated with an equal volume of saline. At 20 h after the last treatment, rats were killed. Values are means ± S.E. of 6 rats. Figures in parentheses are percentages of the control value.

a) Significantly different from the control value, $p<0.05$, *b*) $p<0.01$, *c*) $p<0.001$.

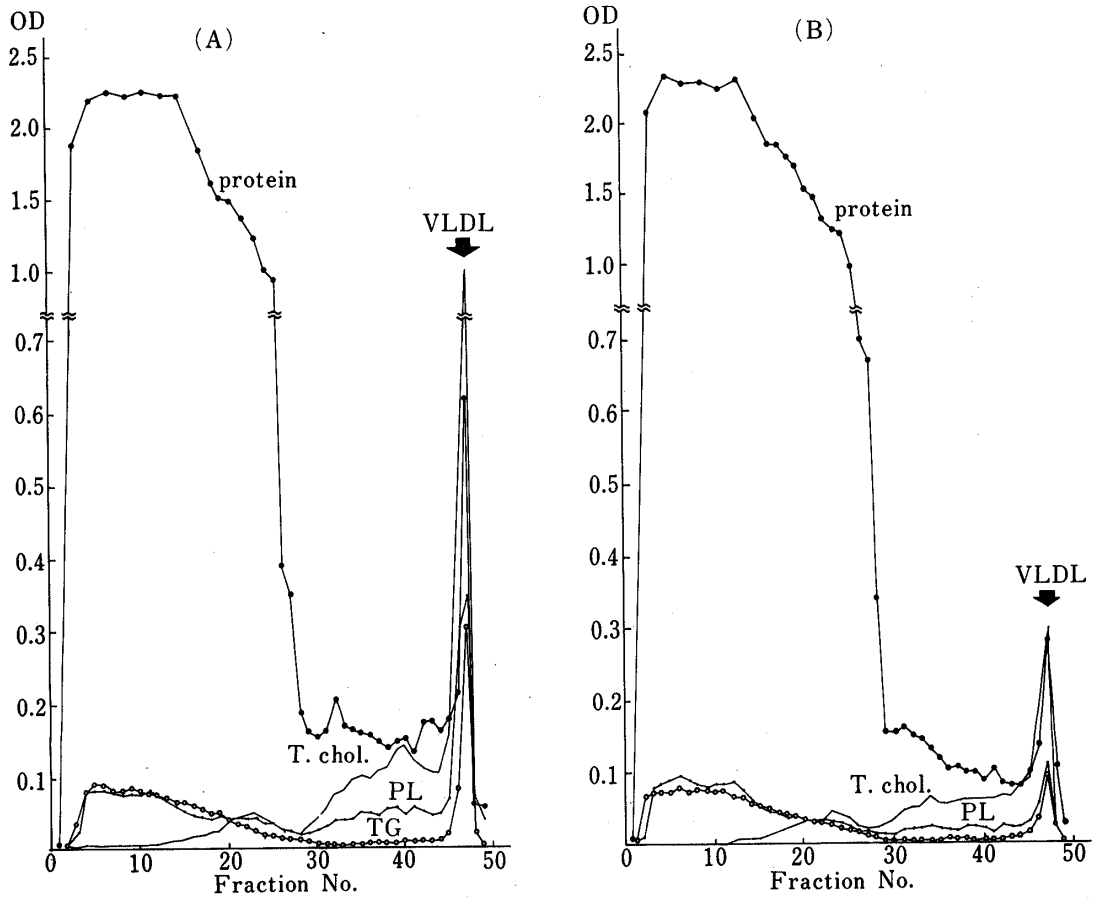

Fig. 3. Effect of Ginsenoside-Rb_2 on the Ultracentrifugation Pattern of Lipoprotein in the Serum

Ginsenoside-Rb_2 (10 mg/rat/d) was administered intraperitoneally to rats for 6 d, while control rats were treated with an equal volume of saline. At 20 h after the last treatment, rats were killed. (A): control group, (B): ginsenoside-Rb_2-treated group.

TABLE II. Effect of Ginsenoside-Rb_2 on Total and Free Cholesterol Concentrations in the Liver

Material	Total cholesterol (mg/tissue)	Free cholesterol (mg/tissue)
Control	42.6±1.9 (100)	22.6±1.4 (100)
Rb_2	37.3±3.4 (88)	22.1±2.4 (98)

Ginsenoside-Rb_2 (10 mg/rat/day) was administered intraperitoneally to rats for 6 d, while control rats were treated with an equal volume of saline. At 20 h after the last treatment, rats were killed. Values are means±S.E. of 6 rats. Figures in parentheses are percentages of the control value.

Lipoprotein lipase is known to regulate 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.[26,27] Therefore, to confirm the above effect of ginsenoside-Rb_2, its action on the lipolytic activity of lipoprotein lipase was determined. As shown in Fig. 4, the lipolytic activity of lipoprotein lipase was stimulated with a concomitant decrease in the level of serum triglyceride ($r=-0.67$). Increment in the lipoprotein lipase activity was associated with increase in the lipid content in

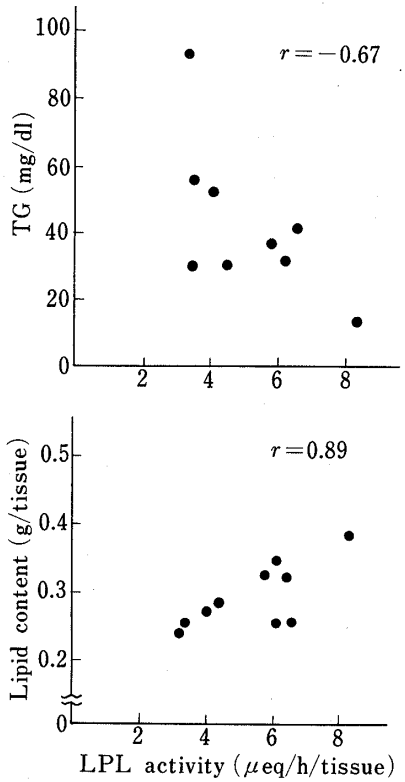

Fig. 4. Relation between Lipoprotein Lipase Activity and Serum Triglyceride or Adipose Tissue Lipid Content

Ginsenoside-Rb_2 (10 mg/rat/d) was administered intraperitoneally to rats for 6 d. At 20 h after the last treatment, rats were killed.

adipose tissue ($r=0.89$).

On the other hand, a slight decrease was observed after the treatment in the level of total cholesterol of the liver, but there was no statistically significant difference between the ginsenoside-Rb_2-treated and control groups (Table II).

Discussion

Nutritional and clinical studies have revealed that high cholesterol levels in the blood may be one of the risk factors of coronary heart disease and atherosclerosis. It has been noted that there is a close relationship between atherosclerosis and increase and/or decrease of serum lipids. In particular, VLDL and LDL may be risk factors, and HDL may be a preventive factor.[28]

Yamamoto *et al.*[1,3] found that in rats fed on a high cholesterol diet, the concentrations of serum cholesterol and triglyceride were decreased and the excretion of sterol was increased by the administration of ginseng principle fraction 4 (saponin content, *ca.* 1/2) and ginseng powder. Moreover, in hyperlipemic patients, prolonged ginseng administration (24 months, *p.o.*) caused a decrement of total cholesterol, an increment of HDL-cholesterol, and an improvement of the atherogenic index (total cholesterol minus HDL-cholesterol/HDL-cholesterol).[4] Sakakibara *et al.*[5] reported the enhancement of cholesterol metabolism by administered saponins, particularly by ginsenoside-Rb_1.

In this work we present evidence that a single administration of ginsenoside-Rb_2 produced an improvement of hyperlipemia induced by a high cholesterol diet in rats. However, the hyperlipemic-lowering action of ginsenoside-Rb_2 appeared to be dependent on the level of blood lipid (Table I, Fig. 1).

Furthermore, we observed that repeated administration of ginsenoside-Rb_2 to hyperlipemic rats decreased the levels of total cholesterol, free cholesterol, and LDL-cholesterol with a concomitant increase in the level of HDL-cholesterol (Fig. 2). Ginsenoside-Rb_2 improved the atherogenic index in rats fed on a high cholesterol diet. Moreover, we found that

there was a significant decrease in the triglyceride level (Fig. 2). Thus, repeated administrations were more effective than a single intraperitoneal administration. These actions of ginsenoside-Rb_2 may be considered as an anti-atherogenic action.

Compared with the significant decrease in the serum cholesterol level, cholesterol in the liver was affected to a lesser extent (Table II). This suggests that ginsenoside-Rb_2 may accelerate serum cholesterol turnover through increased cholesterol degradation and excretion in the feces notwithstanding increased hepatic cholesterogenesis, which was observed in the case of *Panax ginseng* administration to normal rats.[1] Further work to investigate this is planned.

We also observed that repeated administration of ginsenoside-Rb_2 to rats fed on a high cholesterol diet stimulated the lipolytic activity of lipoprotein lipase with a concomitant decrease in the levels of triglyceride and VLDL-triglyceride in the serum (Figs. 2—4). Moreover, we showed that the increment in the lipoprotein lipase activity was associated with increasing lipid content in the adipose tissue (Fig. 4). The adipose tissue may be a target of ginsenoside-Rb_2 action. These phenomena are consistent with those in streptozotocin-induced diabetic rats.[15]

These results suggest that ginsenoside-Rb_2 might be clinically useful in cases of hyperlipemia.

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