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Stimulation of Lipid and Sugar Metabolism in Ginsenoside-Rb_2 Treated Rats

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In order to clarify the mechanism of action of ginsenoside-Rb_2 in rats, time course experiments were carried out. The maximum decrease in the hepatic glycogen content was found 8 h after the treatment, but the content had nearly recovered to the control level 24 h after the treatment. In contrast, the level of hepatic glucose-6-phosphate increased, reaching the maximum at 8 h. There was also a dramatic increase in the phosphofructokinase activity, peaking at 12 h. In addition, accumulation of lipid in adipose tissue was observed 10—12 h after the administration of ginsenoside-Rb_2, whereas no significant changes in the total lipid, triglyceride, total cholesterol, phospholipid, glucose, pyruvate, and lactate levels of the liver were observed throughout the experimental period. An increase of hepatic glucose-6-phosphate dehydrogenase activity was observed 2—4 h after the treatment, but this response was transitory.

Keywords——ginsenoside-Rb_2; liver; adipose tissue; glycogen; glucose-6-phosphate; total lipid; phosphofructokinase; glucose-6-phosphate dehydrogenase

As reported previously, treatment of rats with a semi-purified saponin (fraction 4) from the roots of *Panax ginseng* C.A. MEYER had various effects on lipid and sugar metabolism.[1-3] In particular, a single intraperitoneal administration of fraction 4 increased the incorporation of labeled precursor into total lipid of liver and adipose tissue, and the incorporation depended directly on the administered amount. A reduction of carbohydrate in the liver was observed after the administration of fraction 4. However, the extent of the effect depended on the nutritional status of rats. Intraperitoneal administration of fraction 4 also caused an accumulation of lipid in adipose tissue. An attempt was made to identify the active component of fraction 4. Thus, systematic isolation and purification of fraction 4 were carried out, and the effects of six purified saponin (ginsenoside-Rb_1, -Rb_2, -Rc, -Rd, -Re, and -Rg_1) were assessed in terms of the amount of glycogen in the liver at 8 h after the treatment. As a result, it was found that the active component stimulating a decrease of hepatic glycogen is ginsenoside-Rb_2.[4] The present paper describes a detailed study of the effect of ginsenoside-Rb_2 on sugar metabolites and lipid content, and on the activities of gluconeogenic and glycolytic enzymes.

Materials and Methods

Animals and Diet——Male rats of the JCL: Wistar strain (Hokuriku Labour, Ltd., Toyama, Japan), initially weighing 90—100 g, were used in this experiment. The rats were kept in a wire-bottomed cage under a conventional lighting regimen with a dark night. The room temperature (about 25 °C) and humidity (about 60%) were controlled automatically. Animals were fed on commercial feeds (CLEA Japan Inc., Tokyo; protein 24.0%, lipid 3.5%, carbohydrate 60.5%) *ad libitum*. On the 6th day of the feeding period, ginsenoside-Rb_2 (10 mg) was administered intraperitoneally. Control animals were treated with an equal volume of saline. At the indicated time after

intraperitoneal administration of ginsenoside, rats were stunned by means of a sharp blow on the head then decapitated and allowed to bleed freely before excision of tissue. The liver was removed quickly, transferred into liquid nitrogen and weighed rapidly. Fresh liver was 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 coworkers.[5] This preparation was found to be pure by various chemical and physicochemical analyses.

Chemicals——Glucose-6-phosphate dehydrogenase, lactic dehydrogenase, and NADP were obtained from Oriental Yeast Co., Tokyo, Japan. Glucose-6-phosphate and phosphoenolpyruvate were obtained from Sigma Chemical Co., U.S.A. Pyruvate kinase, fructose-6-phosphate, fructose-1,6-diphosphate, NADH, and ATP were purchased from P-L Biochemicals, Inc., U.S.A. 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 Glycogen in the Liver——A portion of the liver was digested with 3 ml of 1 N NaOH in a boiling water bath for 60 min, and glycogen was precipitated by the addition of 2 ml of EtOH and purified according to the method of Roe and Dailey.[6] Liver glycogen was determined by the anthrone–H_2SO_4 method, with glucose as the standard.[7]

Determination of Glucose in the Liver——Liver was homogenized with 9 volumes of cold 0.9% NaCl. A portion of the homogenate was deproteinized with equimolar amounts of $ZnSO_4$ and $Ba(OH)_2$,[8] and precipitates were removed by centrifugation. Glucose in the supernatant was determined using a commercial reagent (Glucose B-Test Wako obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan) based on the glucose–oxidase method.[9]

Determination of Glucose-6-phosphate in the Liver[10]——Frozen liver was powdered in a porcelain mortar continuously chilled with liquid N_2. Aliquots of the powder were transferred to a chilled glass homogenizer. Five volumes of 0.6 N PCA were added and the mixture was homogenized. Precipitated proteins were removed by centrifugation at $3000 \times g$ for 10 min. Aliquots of the supernatant were neutralized to pH 3.5 with K_2CO_3. The solution was allowed to stand in an ice bath for about 15 min and then the supernatant was pipetted off from the precipitate. A portion of the supernatant was used for the assay. The supernatant fluid (1 ml), triethanolamine buffer (0.4 M; pH 7.6), NADP (20 mM), $MgCl_2$ (0.5 M), and glucose-6-phosphate dehydrogenase (0.25 mg protein/ml) were placed in a cuvette, and the increase of optical density at 340 nm was determined with a Hitachi 200-20 spectrophotometer.

Determination of Pyruvate in the Liver[11]——Liver was homogenized with 4 volumes of 5% TCA and then centrifuged at $3000 \times g$ for 10 min. The supernatant fluid obtained was used for the estimation of pyruvate by the 2,4-dinitrophenylhydrazone method.

Determination of Lactate in the Liver[12]——A portion of the liver was homogenized with 5 volumes of 1 N PCA and precipitates were removed by centrifugation at $3000 \times g$ for 10 min. Aliquots of the supernatant were neutralized to pH 3.5 with K_2CO_3. The solution was allowed to stand in an ice bath for about 10 min and then precipitated $KClO_4$ was filtered off. Lactate in the supernatant was determined by a spectrophotometric method, based on measurement of the increase of optical density at 340 nm.

Determination of Enzyme Activities in the Liver——a) Glucose-6-phosphatase: Liver was homogenized with 9 volumes of 0.25 M sucrose. The crude homogenate was centrifuged at $11000 \times g$ for 30 min and the precipitate was discarded. The supernatant fluid was further centrifuged at $105000 \times g$ for 60 min and the supernatant fluid was discarded. The solid precipitate (microsomes) was suspended in ice-cold sucrose/EDTA solution and stored at −20 °C until required. The activity of glucose-6-phosphatase was assayed according to the method of Baginski *et al.* with a slight modification.[13] The assay mixture contained 0.25 M sucrose/1 mM EDTA (pH 7.0), 0.1 M glucose-6-phosphate, and 0.1 M cacodylate buffer (pH 6.5) in a total volume of 0.3 ml. The reaction was started by the addition of a suitably diluted enzyme solution to the assay mixture. After incubation at 37 °C for 5 min, the reaction was terminated by the addition of 2 ml of 2% ascorbic acid/10% TCA solution. The precipitate formed was removed by centrifugation after the mixture had been left to stand in an ice-bath. A 0.5 ml aliquot was pipetted off and the liberated inorganic phosphate in the supernatant was determined by using a commercial reagent (Phosphor B-Test Wako from Wako Pure Chemical Industries, Ltd., Osaka, Japan) based on the molybdenum blue method.[14]

b) Glucokinase: Homogenate of each liver was prepared in 2 volumes of homogenizing medium (0.15 M KCl containing 0.004 M $MgSO_4$, 0.004 M EDTA, and 0.004 M *N*-acetyl cysteine, pH 7.0). This crude homogenate was centrifuged at $105000 \times g$ for 60 min. The supernatant fraction was used for the enzyme assay of glucokinase by the method of Walker and Parry.[15]

c) Glucose-6-phosphate Dehydrogenase: A portion of the liver was homogenized with EDTA–physiological saline solution (0.04 ml/mg wet weight) and then centrifuged at 15000 rpm for 20 min. The enzyme activity of the clear supernatant fluid was determined according to the method employed by Lohr and Waller.[16]

d) Phosphofructokinase: Liver was homogenized in 2 volumes of 50 mM Tris-HCl, pH 8.3, containing 0.25 M sucrose, followed by centrifugation at $16000 \times g$ for 60 min. The crude supernatant fluid was used for the enzyme assay. Phosphofructokinase activity was determined spectrophotometrically by measuring the extinction change due

to the conversion of fructose-6-phosphate into lactate.[17] The enzyme solution 0.05 ml, Tris-HCl buffer (0.1 M; pH 8.5) 2.00 ml, $MgSO_4$ (10 mg/ml; KCl 10 mg/ml) 0.10 ml, phosphoenolpyruvate (10 mg/ml) 0.10 ml, fructose-1,6-diphosphate (10 mg/ml) 0.10 ml, fructose-6-phosphate (10 mg/ml) 0.20 ml, ATP (10 mg/ml) 0.20 ml, NADH (10 mg/ml) 0.10 ml, pyruvate kinase (2 mg/ml) 0.03 ml, and lactic dehydrogenase (5 mg/ml) 0.01 ml were placed in a cuvette and the decrease of optical density at 340 nm was determined with a Hitachi 200-20 spectrophotometer.

Determination of Protein——Protein was determined by the method of Itzhaki and Gill, with bovine serum albumin as a standard.[18]

Extraction of Total Lipid in the Liver and Adipose Tissue——Liver and epididymal adipose tissue were quickly removed after decapitation of the rat, cooled on ice and weighed. The adipose tissue was placed immediately in 20 ml of $CHCl_3$–MeOH mixture (2 : 1, v/v). The cooled liver was homogenized with 3 volumes of ice-cold 0.9% NaCl solution in a Potter–Elvehjem type glass homogenizer with a Teflon pestle. The homogenate was filtered through 4 layers of gauze and 1 ml of the filtrate was mixed with 20 ml of $CHCl_3$–MeOH (2 : 1, v/v). Total lipid was extracted from both tissues by shaking. The residual tissues were then removed, and the $CHCl_3$–MeOH solution was partitioned and washed by the method of Folch *et al.*[19] 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 Triglyceride, Total Cholesterol, and Phospholipid in the Liver——Liver was homogenized with 3 volumes of ice-cold 0.9% NaCl solution. The homogenate was filtered through 4 layers of gauze and 1 ml of the filtrate was mixed with 20 ml of $CHCl_3$–MeOH (2 : 1, v/v). The $CHCl_3$–MeOH solution was used for the estimation of triglyceride, total cholesterol, and phospholipid. Determination was performed by using a commercial reagent (TG-Five Kainos from Kainos Laboratories, Ltd., Tokyo, Japan; Cholesterol B-Test Wako from Wako Pure Chemical Industries, Ltd., Osaka, Japan; Phospholipids–Test Wako from Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Results

Time Course of the Effect of Ginsenoside-Rb_2 on Metabolite Concentrations in the Liver

Table I shows the metabolite concentrations in the liver of rats treated with ginsenoside-Rb_2. A slight decrease (23% compared to the control) of the glycogen content was seen 2 h after the treatment. The maximum decrease in the hepatic glycogen content was observed 8 h after the ginsenoside-Rb_2 treatment. The content had nearly recovered to the control level

Table I. Effect of Ginsenoside-Rb_2 on Metabolite Concentrations in the Liver

Time after treatment (h)	Glycogen (mg/tissue)	Glucose (mg/tissue)	Glucose-6-phosphate (mg/tissue)	Pyruvate (mg/tissue)	Lactate (mg/tissue)
Control	82.7±12.4 (100)	12.8±0.9 (100)	0.27±0.03 (100)	0.16±0.01 (100)	14.8±1.0 (100)
2	63.3±14.5 (77)	—	0.36±0.04 (133)	0.19±0.01 (119)	—
4	72.1±12.2 (87)	14.1±0.8 (110)	0.36±0.03 (133)	0.17±0.01 (106)	16.3±0.6 (110)
6	57.4±16.5 (69)	—	0.40±0.05 (148)	0.17±0.01 (106)	—
8	13.8±4.4[b] (17)	14.4±0.8 (113)	0.49±0.04[b] (181)	0.17±0.01 (106)	16.2±1.0 (109)
10	—	15.1±0.8 (118)	—	—	15.1±1.7 (102)
12	79.1±21.9 (96)	14.5±0.6 (113)	0.40±0.03[a] (148)	0.13±0.01 (81)	12.9±1.6 (87)
16	—	14.1±1.2 (110)	0.35±0.04 (130)	0.13±0.01 (81)	13.0±1.1 (88)
24	90.5±12.8 (109)	12.5±0.7 (98)	0.32±0.05 (119)	0.15±0.01 (94)	11.0±0.9 (74)

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*) $p<0.01$.

TABLE II. Effect of Ginsenoside-Rb_2 on Enzyme Activities in the Liver

Time after treatment (h)	Glucose-6-phosphatase (μmol/min/mg protein)	Glucokinase (ΔOD/h/mg protein)	Glucose-6-phosphate dehydrogenase (ΔOD/h/mg protein)	Phosphofructokinase (ΔOD/h/mg protein)
Control	17.0±1.3 (100)	306.0±18.0 (100)	6388±469 (100)	30.0±6.6 (100)
2	13.5±0.7[a] (79)	291.6±33.0 (95)	9071±1516 (142)	—
4	13.5±0.8[a] (79)	243.6±9.0[a] (80)	8624±383[b] (135)	47.4±5.4 (158)
6	11.7±0.7[b] (69)	237.6±21.6[a] (78)	6708±447 (105)	—
8	8.8±0.8[c] (52)	205.8±13.2[c] (67)	5877±511 (92)	51.6±9.6 (172)
10	—	—	—	81.6±19.8[a] (272)
12	15.5±1.2 (91)	333.5±21.1 (109)	—	97.8±17.4[b] (326)
16	—	—	—	61.2±15.6 (204)
24	17.0±1.9 (100)	272.3±99.3 (89)	5430±127 (85)	40.2±16.2 (134)

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*) $p<0.001$.

24 h after the treatment. There was a drastic increase in the glucose-6-phosphate level at 8 h. The level was still high 12 h after the treatment but the increase was no longer significant after 24 h. In contrast, glucose, pyruvate, and lactate content in the liver showed no appreciable changes after ginsenoside-Rb_2 administration.

Time Course of the Effect of Ginsenoside-Rb_2 on the Enzyme Activities in the Liver

The activity of hepatic glucose-6-phosphatase, a rate-limiting enzyme of gluconeogenesis, was decreased by ginsenoside-Rb_2 reaching the minimum 8 h after the treatment, but had almost recovered to the control level 24 h after the treatment (Table II). A significant decrease was also observed in the level of glucokinase when ginsenoside-Rb_2 was administered. Thus, the ratio of glucose-6-phosphatase to glucokinase activity was not changed by the treatment with ginsenoside-Rb_2. Furthermore, the activity of hepatic phosphofructokinase, a rate-limiting enzyme of glycolysis, was increased from 4 to 8 h after the intraperitoneal administration of ginsenoside-Rb_2. There was also a sharp increase in the phosphofructokinase activity, which reached the maximum 12 h after the treatment. The increase was no longer significant after 24 h. There was a transient increase in the level of glucose-6-phosphate dehydrogenase in the liver from 2 to 4 h after the intraperitoneal administration of ginsenoside-Rb_2 (Table II).

Time Course of the Effect of Ginsenoside-Rb_2 on the Lipid Content in the Liver and Adipose Tissue

As shown in Table III, the maximal level of total lipid content in the adipose tissue was observed 10 h after the ginsenoside-Rb_2 treatment. At 8 and 12 h after the treatment, there were increase of 38 and 56%, respectively, in the total lipid content, and 16 h after the administration of ginsenoside-Rb_2, there was still a 40% increase over the control animals. However, administration of ginsenoside-Rb_2 to rats caused no appreciable changes of the

TABLE III. Effect of Ginsenoside-Rb_2 on the Lipid Content in the Liver and Adipose Tissue

Time after treatment (h)	Liver				Adipose tissue
	Total lipid (mg/tissue)	Triglyceride (mg/tissue)	T. cholesterol (mg/tissue)	Phospholipid (mg/tissue)	Total lipid (mg/tissue)
Control	218.9±7.9 (100)	22.7±1.1 (100)	6.0±0.4 (100)	100.9±4.5 (100)	208.3±16.1 (100)
4	206.4±8.0 (94)	23.9±1.4 (105)	5.9±0.4 (98)	112.4±8.2 (111)	270.8±24.0 (130)
8	211.1±11.2 (96)	24.7±2.0 (109)	6.1±0.3 (102)	120.2±7.2 (119)	287.5±43.8 (138)
10	201.7±10.9 (92)	23.7±1.0 (104)	5.8±0.4 (97)	118.9±6.6 (118)	356.7±9.0[b] (171)
12	196.4±7.8 (90)	25.5±1.0 (112)	6.1±0.3 (102)	117.8±4.4 (117)	324.0±20.5[a] (156)
16	187.2±6.0 (86)	22.9±1.8 (101)	6.3±0.3 (105)	112.4±5.6 (111)	291.1±35.8 (140)
24	218.1±9.8 (100)	21.3±1.4 (94)	6.2±0.3 (103)	108.8±6.2 (108)	228.8±24.8 (110)

Values are means±S.E. of 6 rats.
Figures in parentheses are percentages of the control value.
a) Significantly different from the control value, $p<0.01$, *b*) $p<0.001$.

total lipid, triglyceride, total cholesterol, and phospholipid levels of the liver.

Discussion

To elucidate the mechanism of action of ginsenoside-Rb_2, the time courses of sugar metabolites were investigated. A slight decrease in the glycogen content was observed 2 h after the intraperitoneal administration of ginsenoside-Rb_2, and the decrease was greatest at 8 h after the ginsenoside-Rb_2 treatment (Table I). Thus, the decreasing effect of semi-purified saponin (fraction 4) on the hepatic glycogen content, which was demonstrated in the previous study,[1-3] was confirmed in the present experiment using ginsenoside-Rb_2. In contrast, administration of ginsenoside-Rb_2 to rats increased the level of glucose-6-phosphate, which reached the maximum at 8 h (Table I). These changes are in reciprocal relation to those of hepatic glycogen content. However, glucose level in the liver showed no appreciable change when ginsenoside-Rb_2 was administered (Table I). Accordingly, it may be considered that ginsenoside-Rb_2 switches the metabolic flow toward the anaerobic glycolytic pathway by the degradation of glycogen in the liver. These data further support the view that the enzymes on the glycolytic pathway play an important role in the overall pattern of metabolic changes produced by ginsenoside-Rb_2. Indeed, a significant increase in the activity of phosphofructokinase, a rate-limiting enzyme of glycolysis, was observed 12 h after the treatment(Table II).

We also observed an accumulation of lipid in adipose tissue 10—12 h after the administration of ginsenoside-Rb_2, though no significant change in the total lipid, triglyceride, total cholesterol, and phospholipid levels of the liver was observed throughout the experimental period (Table III). From these observations, in conjunction with the experiments on sugar metabolism, it may be concluded that ginsenoside-Rb_2 induces an accumulation of lipid in adipose tissue as a result of its stimulating action on the glycogenolytic and glycolytic pathways.

It is generally accepted that lipoprotein lipase specifically hydrolyzes triglyceride of

chylomicron and lipoprotein complexes, and this hydrolysis regulates the uptake of triglyceride into adipose tissue.[20-22] Thus, further information on the effect of ginsenoside-Rb_2 on lipoprotein lipase is required. On the other hand, lipogenesis is markedly influenced by the nutritional status of the animals. Fasting of the animals results in a reduction of lipogenesis.[23] Upon refeeding, lipogenesis returns to the normal level.[24] Previous published data from our laboratory also showed that the nutritional status of the animals affected the lipogenic capacity of a semi-purified saponin.[2] Therefore, attention should be paid to the factor of nutritional status in future studies.

Many steps are involved in the synthetic pathway of lipogenesis and it is of interest to know which one is most strikingly influenced by ginsenoside-Rb_2 treatment. The most probable one may be the reaction from acetyl-CoA, which is considered to be rate-limiting for the biosynthesis of fatty acids. Thus, assay of acetyl-CoA carboxylase after ginsenoside-Rb_2 treatment might also be of interest. On the other hand, Srikantaiah *et al.* have pointed out the importance of the $NADPH/NADP^+$ ratio *in vivo* in the control of fatty acid biosynthesis.[25] The finding that treatment with ginsenoside-Rb_2 increased the activity of glucose-6-phosphate dehydrogenase suggests that ginsenoside-Rb_2 might modify NADPH generation.

Another important observation was that the administration of the ginsenoside-Rb_2 to rats decreased the activity of hepatic glucose-6-phosphatase. A decrease of glucokinase activity was also observed (Table II). However, preliminary data from our laboratory indicate that ginsenoside-Rb_2 caused a significant increase of glucokinase activity in the liver of streptozotocin-diabetic rats, although there was no statistically significant difference in the activity of glucose-6-phosphatase. A significant decrease in blood glucose level was observed in streptozotocin-diabetic rats when ginsenoside-Rb_2 was administered. Considering these observations, it may be that hypoglycemic activity is brought about by means of changes in the levels of glycolytic and gluconeogenic enzymes. Further work along this line is planned.

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