Antidiabetic Activity of Lyophyllum decastes in Genetically Type 2 Diabetic Mice

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The antidiabetic activity of Lyophyllum decastes (Tricholomataceae) was investigated in KK-Ay mice, an animal model of genetically type 2 diabetes with hyperinsulinemia. The water extract of Lyophyllum decastes (LD) (500 mg/kg body weight) reduced the blood glucose of KK-Ay mice 7 h after a single oral administration (p<0.05) when compared with control. LD reduced the blood glucose of KK-Ay mice 3 weeks after repeated administration (p<0.05), and also significantly lowered the serum insulin of KK-Ay mice under similar conditions (p<0.01). However, LD did not affect the blood glucose in normal mice. LD tended to decrease of the blood glucose in an insulin tolerance test. In addition, the muscle content of facilitative glucose transporter isofrom 4 (GLUT4) protein content in the plasma membrane fraction from muscle significantly increased in the orally LD-treated KK-Ay mice when compared to that of the controls (p<0.01). These results suggest that the antidiabetic activity of LD is derived, at least in part, from a decrease in insulin resistance, due to the increase of GLUT4 protein content in the plasma membrane of the muscle.

Key words antidiabetic activity; KK-Ay; Lyophyllum decastes; GLUT4; insulin resistance

Despite considerable progress in the management of diabetes mellitus by synthetic drugs, the search for indigenous natural antidiabetic agents is ongoing. The plant kingdom offers a wide field to look for effective oral hypoglycemics. More than 400 species have been reported to display hypoglycemic effects, but only a few of them have been investigated.1—3

Glucose transport across the plasma membrane is mediated by carrier proteins termed glucose transporters.4,5) Recent cDNA cloning has demonstrated that the facilitative glucose transporters comprise a family of structurally related proteins with differing tissue distribution.6) The gene expression and protein content of glucose transporters have been found to be altered under pathological conditions such as diabetes mellitus.6—8)

The fruits of Lyophyllum decastes (FRIES) SINGER. (Tricholomataceae), have been used in traditional medicine.9) However, adequate characterization of their effect is yet to be done and no study has been performed on type 2 diabetes models. In the present study, we examined the effect of Lyophyllum decastes on blood glucose in type 2 diabetic mice, and also the effect of insulin resistance in order to clarify the antidiabetic mechanism.

MATERIALS AND METHODS

Fruits of Lyophyllum decastes were supplied from Ohji Paper Co., Ltd. (Mie, Japan) and used in the present experiment. One hundred grams of fruits was extracted with 0.7 l of water (100 °C, 1 h, 1 times). The water extracts were lyophilized (LD) and stored at room temperature until use.

Animals Adult male ddY mice (SLC, Shizuoka, Japan) weighing 22—25 g and KK-Ay mice (Clea, Tokyo, Japan), 12 weeks old, were used. KK-Ay mice with blood glucose level above 300 mg/100ml were considered to be diabetic and used in this study. The mice were housed in an air-conditioned room at 22±2°C with a 12 h light—12 h dark cycle (light: 9:00 a.m. to 9:00). The animals were kept in the experimental animal room for 7 d with free access to food (CE-2, Clea, Tokyo) and water (tap water). Blood samples were withdrawn from the cavernous sinus with a capillary to determine blood glucose levels under non-anesthesia and non-fasting. LD was dissolved in distilled water. The studies were started at 10:00—11:00 a.m., and blood samples after repeated administration of LD were taken at 10:00—11:00 a.m. LD was administered orally on a compulsory basis (repeated administration, once a day for 3 weeks).

Insulin Tolerance Test Insulin tolerance tests were performed at the end of the repeated administration. After overnight (18 h) fasting, the insulin (0.5 U/kg body weight) solution was administered subcutaneously (s.c). Blood samples were collected before administration of the insulin and 30, 60 and 120 min after its administration of the insulin.

Determination of Blood Glucose and Insulin Blood glucose levels in mice were determined by the glucose oxidase method,10) and serum insulin was measured by the double antibody method.11) All the data were expressed as mean±S.E.M. and Student’s t test and analysis of variance (ANOVA) was used for the statistical analysis. The values were considered to be different when the p value was less than 0.05.

Isolation of Hindlimb Muscle The effect of LD on GLUT4 protein content was studied at the end of the repeated administration. After overnight (18 h) fasting, the mice were given insulin (0.5 U/kg) subcutaneously and 0.5 h later the hindlimb muscle was resected for the experiment.

Plasma Membrane (PM) Fraction of Skeletal Muscle The muscle tissue was placed in a buffer (5 mm sodium azide, 0.25 m sucrose, 0.1 m phenylmethylsulfonyl fluoride (PMSF), 10 mm NaHCO3 (pH 7.0)) at 4 °C. Subfractionation of muscle membrane was as described by Baron et al.,12) whose procedure was modified from that of Klip et al.13,14) The muscle was homogenized and was centrifuged at 1000 g for 10 min, and the supernatant was saved. The resulting pel-
let was resuspended in the buffer and rehomogenized with a
glass homogenization tube. The supernatant was combined
with the first supernatant, and centrifuged at 9000 g for 10
min. The resulting supernatant was then centrifuged at
190000 g for 60 min. These membranes were applied to a
discontinuous sucrose gradient containing 25%, 30%, and
35% sucrose (wt/vol) solutions and centrifuged at 190000
g for 16 h. Plasma membranes were collected in 25% sucrose
gradients, resuspended in the buffer, pelleted by centrifuga-
tion at 190000 g for 60 min, and resuspended in the buffer.

**Western Blot Analysis**

The antibody used in the Western blotting (East Acres, U.S.A.) was raised against a synthetic peptide corresponding to the COOH-terminal domain of mouse GLUT4 (12 amino acid peptide), as reported by James et al.15) (No reaction against brain, or liver. Does not cross-react with GLUT1 or GLUT2 tested). To prepare the total membrane particulate fractions, the mice muscles were excised and 1—2 g of muscle slices was homogenized in 25
ml of 10 mM Tris–HCl, 1 mM phenylmethyl sulphonyl fluo-
ride and 1000 units/ml of aprotinin.16) The homogenates were
then centrifuged at 700 g for 10 min at 4 °C to sediment the
fraction containing the nuclei and mitochondria. The result-
ning supernatant was centrifuged at 13000 g for 20 min at 4 °C
to yield a pellet designated as the membrane fraction of the
muscle in this study. The membrane fractions (30 μg) pre-
pared were suspended in 1% sodium dodecyl sulfate (SDS)
and 50 mM dithiourretiol and subjected to SDS-polyacryl-
amide (9%) gel electrophoresis. Electrophoretic transfer to
nitrocellulose paper and detection of the immunocomplex
with enhanced chemiluminescence (Amersham, Bucking-
hamshire, U.K.) were carried out as has been previously de-
scribed.17) The sheet was exposed on RX X-ray film and in-
tensifying screen (Fuji, Tokyo). The prestained molecular
weight standard (Bio-Rad, Richmond, VA, U.S.A.) was used
for estimation of the molecular weight. The experiments
were performed at least twice for each tissue with similar re-
sults.

**RESULTS**

**Effect of LD on Blood Glucose in KK-Ay** The effect of
LD injected p.o to KK-Ay mice is shown in Fig. 1 (single admin-
istration). LD (500 mg/kg body weight) decreased blood
glucose 7 h after the administration (p<0.05). Tolbutamide
(a known sulfonylurea hypoglycemic agent) (50 mg/kg)-
treated mice showed lower blood glucose 7 h after the admin-
istration (p<0.01). Effect of the repeated administration of
LD on blood glucose is shown in Fig. 2. LD-treated animals
(500 mg/kg) showed lower blood glucose levels from 3 weeks
after the administration (p<0.05). The serum insulin level in
LD-treated KK-Ay mice was decreased 3 weeks after the ad-
ministration (p<0.01) (Fig. 4). However, LD did not affect
the blood glucose or serum insulin in normal mice (Figs. 3,
4).

**Insulin Tolerance Test** LD (500 mg/kg body weight,
p.o) tended to decrease in blood glucose after 30 min com-
pared with controls (Fig. 5).

**Muscle GLUT4 Protein** Effects of LD on muscle PM
fraction of GLUT4 protein levels in both control and LD-
treated KK-Ay mice are demonstrated in Fig. 6. The quanti-
tation of GLUT4 protein in membrane in muscle was as-
assessed by Western blotting in the mice. Quantitation of the GLUT4 glucose transporter band isolated from nitrocellulose paper demonstrated that the relative amount of GLUT4 protein in the muscle from LD treated mice was 156% of that observed in the control mice (p<0.01, **p<0.001, ns, not significant).

DISCUSSION

This study clearly showed that the water extract of the fruits of LD produces a consistent hypoglycemic effect. We examined the therapeutic effects of LD on hyperglycemia in KK-Ay mice, an animal model of type 2 diabetes mellitus. These mice, which are known for genetically induced diabetes and which include ob/ob mice and KK mice, were hyperinsulinemia as a result of insulin resistance. Their treatment of mice with LD resulted in hypoglycemia with reduced serum insulin. These results indicate that LD improves hyperinsulinemia.

The toxicity of LD seems to be very low (LD_{50}>=2000 mg/kg body weight) (data not shown). Moreover, LD-treated (2000 mg/kg) mice did not show any obvious stimulus action, further suggesting that LD is a medicine with lower toxicity.

LD had also tended to lower blood glucose in the insulin tolerance test and hyperinsulinemia was improved. Insulin (0.5 U/kg)-treated KK-Ay mice did not have lower blood glucose because of insulin resistance in the peripheral tissues, indicating that LD lessens this resistance. Insulin resistance in peripheral tissues is known as one of the pathogenic factors of type 2 diabetes, together with the impairment of glucose-induced insulin secretion from pancreatic beta cells. Therefore, it is important that LD improves insulin resistance.

We also examined the effect of LD on GLUT4 glucose transporter in mouse muscle, since it has been reported that GLUT4 plays a crucial role in the muscle process of glucose uptake. LD increased GLUT4 protein content of muscle in KK-Ay mice. It is known that GLUT4 and GLUT1 are present in skeletal muscle, however, LD did not affect GLUT1 protein content in this muscle (data not shown). From these findings, it is likely that the hypoglycemic effect of LD is derived, at least in part, from the decrease in insulin resistance, due presumably to the increase of GLUT4 protein content in total membrane of the muscle.

Further study would show how LD could become a useful drug in the treatment of diabetes through this unique therapeutic mechanism. The above experimental results suggest that the antidiabetic activity of LD supports the traditional medical use of type 2 diabetes.

REFERENCES