Effect of Alpha-Linolenic Acid on Blood Glucose, Insulin and GLUT4 Protein Content of Type 2 Diabetic Mice

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Alpha-linolenic acid (LL) was tested for antidiabetic activity in KK-Ay mice, an animal model of type 2 diabetes. Repeated administration of LL (300 mg/kg) to KK-Ay mice significantly suppressed the increment of blood glucose at 21 d (p<0.05). However, no affect on the blood glucose level in normal mice was seen, indicating that LL is useful in treating type 2 diabetes. In addition, LL improved hyperinsulinemia in KK-Ay mice (p<0.01). LL also significantly decreased the blood glucose at 120 min in the insulin tolerance test (p<0.05). The muscle content of facilitative glucose transporter isoform 4 (GLUT4) protein content in the total membrane fraction from KK-Ay mouse muscle significantly increased in the LL-treated mice when compared to that in the controls (p<0.01). From these findings, it seems likely that the hypoglycemic effect of LL 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.

Key words —— alpha-linolenic acid, KK-Ay mice, antidiabetic activity, insulin resistance, GLUT4

INTRODUCTION

In the early 1970s, epidemiologic studies conducted in Greenland Eskimos by Bang and colleagues led to the hypothesis that fish oil rich in omega-3 poly unsaturated fatty acid (omega-3PUFA) is associated with a low incidence of type 2 diabetes and coronary heart disease (CHD).1–3) The effect of fish oil intake has, in turn, been attributed to omega-3PUFA, especially eicosapentaenoic acid (EPA; C20:5 omega-3) and docosahexaenoic acid (DHA; C22:6 omega-3).4) DHA contains 22 carbon atoms with 6 double bonds, the first one at position 3 from the methyl terminal. Alpha-linolenic acid (LL) belongs to the omega-3 family, and is metabolized to EPA and DHA by desaturase and elongase. Type 2 diabetes has been reported to reduce EPA and DHA in both liver and muscle phospholipid.5)

Glucose transport across the plasma membrane is mediated by carrier proteins termed glucose transporters.6,7) Recent cDNA cloning has demonstrated that the glucose transporters comprise a family of structurally related proteins with differing tissue distribution.8) The protein content of glucose transporters has been found to be altered under pathological conditions such as diabetes mellitus.9,10) In the present study, we examined the effect of alpha-linolenic acid on blood glucose and insulin of KK-Ay mice, an animal model of type 2 diabetes. Investigating the protein content of the skeletal muscle glucose transporters (GLUT4) to identify the mechanism by which LL improves the hyperglycemic condition, we found that the substance decreases the blood glucose and insulin, and increases GLUT4 protein content in the total membrane fraction from mouse skeletal muscle.

MATERIALS AND METHODS

Alpha-Linolenic Acid —— Alpha-linolenic acid was purchased from Wako Pure Chemical Industries Co., Ltd. (Osaka, Japan) and stored at −20°C until use. The antioxidant used was dl-α-tocopherol (40 mg/kg body weight) with alpha-linolenic acid in the repeated administration (control: α-tocopherol only).

Antidiabetic Activity —— Normal male mice (ddY, 6 weeks old) and KK-Ay mice (6 weeks old) were used. The mice were given free access to drinking
water and food. LL was dispersed in distilled water and administered to the mice orally. As a control experiment, the distilled water solution was also administered to the mice.

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

**Determination of Blood Glucose and Insulin** —— The blood was drawn from the eye with a capillary. The glucose level of the drawn blood was determined by the glucose oxidase method and serum insulin was measured by the double-antibody method.

**Isolation of Hindlimb Skeletal Muscle** —— After overnight fasting, the mice were given insulin (0.5 U/kg) subcutaneously and, 2 h later, the hindlimb skeletal 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 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM NaHCO₃ (pH 7.0)] at 4°C. Subfractionation of muscle membrane was performed as described by Baron and colleagues whose procedure was modified from that of Klip and colleagues. The muscle was homogenized and was centrifuged at 1000 g for 10 min, and the supernatant was saved. The resulting pellet 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 was centrifuged at 190000 g for 16 h. Plasma membrane were collected 25% sucrose gradients, resuspended in the buffer, pelleted by centrifugation at 190000 g for 60 min, and resuspended in the buffer.

**Western Blot Analysis** —— The antibody used for 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. (No reaction against brain, or liver. No cross-reactivity with GLUT1 or GLUT2 tested). The membrane fractions (40 µg) prepared were suspended in 1% SDS and 50 mM dithiothreitol and subjected to SDS-polyacrylamide (9%) gel electrophoresis. Electrophoretic transfer to nitrocellulose paper and detection of the immunocomplex with enhanced chemiluminescence (Amersham, Buckinghamshire, U.K.) were carried out as has been previously described. The sheet was exposed on RX X-ray film and intensifying screen (Fuji, Tokyo, Japan). 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 results.

**Statistical Analysis** —— All data are expressed as mean ± S.E.M. Analysis of variance (ANOVA) and Student’s t-test was used for statistical analysis. Values were considered to be significantly different when the p-value was less than 0.05.

**RESULTS AND DISCUSSION**

Despite a fat-enriched diet, the incidence of diabetes mellitus among Eskimos is low. The present study clearly showed that LL produces consistent antidiabetic activity in KK-Ay mice. The KK-Ay mice used showed early stage symptoms of type 2 diabetes. Results in the KK-Ay mice are summarized in Fig. 1. LL showed antidiabetic activities at a dosage of 300 mg/kg 21 d after the administration. The body weight did not change between control and LL-treated group at 21 d (Control:}

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*Fig. 1. Effect of LL on Blood Glucose in KK-Ay Mice*  
LL (300 mg/kg) was administered orally to KK-Ay mice once a day. Blood samples were taken for glucose determinations. Each value represents the mean ± S.E. of 3–5 mice. Significantly different from control, *p*<0.05. Significantly different from prevalue, *p*<0.01 (by ANOVA).
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35.7±0.9 g, LL: 34.0±0.3 g) significantly. In normal mice, no change in blood glucose was observed in LL-treated mice (data not shown). Thus it appears that LL could be useful as a dietary cure for type 2 diabetes. The serum insulin concentration in LL-treated KK-Ay mice decreased (p<0.01) (Fig. 2).

Blood glucose concentrations during the insulin tolerance test are shown in Fig. 3. Blood glucose was significantly decreased in LL-treated animals at 120 min after insulin injection compared with control group (p<0.05). KK-Ay mice, including ob/ob mice and KK mice, genetically induced diabetes and are hyperinsulinemic as a result of insulin resistance. Therefore, these findings indicate that LL may improve hyperinsulinemia by decreasing insulin resistance. Effect of LL on skeletal muscle GLUT4 protein levels in KK-Ay mouse is shown in Fig. 4. Densitometric scanning of the bands revealed that the amounts of skeletal muscle GLUT4 protein in LL-treated mice was increased to 250% compared to that in control mice (p<0.01). It is known that GLUT4 and GLUT1 are present in skeletal muscle. However, LL did not affect GLUT1 protein content in skeletal muscle (data not shown). From these findings, it is likely that the hypoglycemic effect of LL 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 may indicate how LL could become useful as a dietary cure of diabetes.

Fig. 2. Effect of LL on Insulin Concentration in KK-Ay Mice (21 d)
LL (300 mg/kg) was administered orally to KK-Ay mice once a day. After 21 d, blood samples were taken for insulin determinations. Each value represents the mean ± S.E. from 3–5 mice. Significantly different from control, **p<0.01.

Fig. 3. Effect of LL on Insulin Tolerance Test in KK-Ay Mice (21 d)
After overnight fasting, the insulin (0.5 U/kg body weight) solution was administered subcutaneously. Blood samples were collected before the administration of the insulin and at 30, 60 and 120 min later. Each value represents the mean ± S.E. of 4–5 mice. Significantly different from control, *p<0.05. Significantly different from prevalue, # p<0.01 (by ANOVA).

Fig. 4. Effect of LL on GLUT4 Content in Mouse Skeletal Muscle
After overnight fasting, the mice were given insulin (0.5 U/kg) subcutaneously and, 2 h later, the hindlimb muscle was resected for the experiment. Western blot analysis of GLUT4 protein content was shown in Material and Method. Each value represents the mean ± S.E. from 3 mice. Significantly different from control, **p<0.01.
REFERENCES