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INCREASED PLASMA POST-HEPARIN DIAMINE OXIDASE ACTIVITY AND PLANT STEROL LEVELS IN STREPTOZOTOCIN DIABETIC RAT

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Plasma post-heparin diamine oxidase (DAO) activity and plasma levels of plant sterols were examined in streptozotocin diabetic rats fed with chow containing plant sterols, to investigate the enzyme activity in relation to the morphological changes of small intestine as well as sterol absorption in the diabetic rats. Diabetic rats showed increased small intestinal mass and surface area compared with control rats. Plasma post-heparin DAO activity and plant sterol level were also increased more than 2.5-fold in the diabetic rats. Insulin treatment improved these abnormalities. Plasma DAO activity correlated to both the small intestinal hyperplastic change and plasma plant sterol levels. These results indicate that plasma post-heparin DAO activity may be used as a marker of intestinal hypertrophy as well as ability to absorb dietary sterols.

Although hyperlipidemia in diabetes mellitus is known to be an important risk factor for the development of macrovascular complications (1), the pathophysiology for the development of hyperlipidemia is not well understood. Metabolic abnormalities associated with deficiency of insulin and increase of insulin antagonistic hormones are reported to induce the abnormal lipoprotein metabolism (2). Since diet is thought to be another factor and the absorption of cholesterol in the small intestine is reported to be increased in diabetes mellitus (3, 4), it is important to establish the method to evaluate the contribution of the small intestine for diabetic hyperlipidemia.

Recently, Miettinen et al (5) have reported that serum plant sterol level is a significant marker of cholesterol absorption in human, although few report is published concerning plant sterol levels in diabetes mellitus. Post-heparin plasma level of diamine oxidase (DAO) (EC 1.4.3.6, also called histaminase), a degradating enzyme of histamine and putrescine, is known to be a reliable marker of small intestinal epithelial cell growth (6), and its activity is used as an index of small bowel mucosal mass (7).

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In this report, to test the contribution of small intestinal changes for the sterol absorption in diabetes mellitus, we examined the alteration of post-heparin DAO activity and plasma plant sterol levels in streptozotocin diabetic rats fed with a plant sterol-rich chow, as a marker of intestinal hypertrophy and potential ability to absorb dietary sterols.

MATERIALS AND METHODS

Animals

Seventeen male Sprague-Dawley rats (260-300 g) were obtained from SLC, INC., Japan. Nine animals were made diabetic by the intravenous injection of streptozotocin (60 mg/kg body weight, provided by Upjohn, Co. (Kalamazoo, MI). Four diabetic rats were treated with 6 U of subcutaneous insulin (Human monocompornent insulin, NOVO Nordisk JAPAN Co., Japan) every evening from 2 days after streptozotocin treatment (insulin-treated group), and 5 rats were not treated with insulin (diabetic group). Eight control rats (control group) and streptozotocin-treated rats were fed ad libitum with a standard rat chow (CLEA JAPAN, INC., Japan) containing 1 % of plant sterol and 1 % of cholic acid (wt/wt) for 14 days. Plant sterol (containing 50 % of β-sitosterol and 40 % of campesterol) was purchased from Aldrich, INC., USA. The sterol content in plant sterol containing chow was determined by HPLC method as described below (cholesterol:campesterol:β-sitosterol=1:4.6:6.2). The amounts of chow consumed by these animal were 20.7±1.8 g/day/ animal in control, 30.5±2.6 g/day/animal in insulin-treated, and 39.1±5.8 g/day/animal in diabetic group during the experimental periods, respectively, which were significantly different among the three groups. Determination of DAO activity and the examination of morphological changes of small intestinal tract were done after an overnight fast.

Operation and morphological measurements

Rats were anesthetized with 50 mg/kg BW intraperitoneal pentobarbiturate injection. After cannulization of carotid artery, 5000 U/kg BW heparin (Upjohn Co.) in saline (2000 U/ml), was injected. And then blood samples were taken at 0, 10, 30 and 60 minutes after the heparin injection. Plasma was stored at −70 °C until the enzyme activity was measured. The enzyme activity was kept intact for 2 weeks without a decrease in the activity. After the blood sampling, rats were killed by the cervical dislocation. The entire small intestine was excised, stripped of its mesentery. The lumen of the small intestine was gently washed with 150 ml of Krebs solution. After 50 ml of air flashing to remove luminal Krebs solution, whole small intestine was laid flat on a filter paper to remove outer surface Krebs solution, and then transferred to the tracing paper, and its wet weight was examined. Whole intestine was cut into 4 segments of equal length, and each segment was then opened along its mesenteric insertion and laid flat with mucosal side up. Outline of each segment was traced, and its surface area and whole intestinal longitudinal length were determined using an image analysis system (Type IBAS I, Kontron, INC., Germany). Whole small intestine was dried for 24 hours at 50 °C and weighed.

Measurement of plasma DAO activity

DAO activity was measured using Tryding's modification (8) of the method of Okuyama and Kobayashi (9). In brief, under the influence of DAO, [^{14}C]putrescine is converted to γ-aminobutyraldehyde which then forms Δ^{-1}-pyrroline, provided its conversion to γ-aminobutyric acid (GABA) is blocked by the addition of acetaldehyde. One international unit of DAO equals 1 μmol Δ^{-1}-pyrroline per minute at 37 °C. For the plasma DAO assay, duplicate measurements were done using 100 μl aliquot of plasma.

Measurement of plasma cholesterol and plant sterols

Plasma sterol concentration and sterol content of the animal chow were determined by the method described by Kasama, Byun, and Seyama (10). Briefly, for the determination of plasma sterol concentration, 0.1 ml of plasma with 10 μg of desmosterol (5,24-cholestadien-

3β-ol) as an internal standard was treated with 1 M ethanolic KOH and extracted twice with n-hexane. The sterols in the extracts were converted into their benzoate derivatives with benzoyl chloride reagent which was freshly prepared for each assay. The benzoate derivatives of the sterols were re-extracted with 1,2-dichloroethane, and dissolved again in 250 μl of acetonitrile-dichloroethane 2:1 after evaporation under a stream of nitrogen. Fifty μl of the solution was injected into the HPLC system. The separation of the sterols was performed on a reverse-phase column (SBC-ODS 150 × 2.5 mm, Shimadzu, Kyoto, Japan) maintained in an incubator at 50 °C and monitored at 228 nm. The instrument was an LC-6A system (Shimadzu), equipped with a column oven and chromatogram data processor (Chromatopac C-R5A). The solvent used for the elution was acetonitrile-water-acetic acid 97:3:0.2 at a flow rate of 0.5 ml/min. A calibration was done after the derivatization of authentic sterols obtained from Sigma (St. Louis, MO) or Nakalai Tesque (Kyoto, Japan).

Statistical analyses

Analysis of variance was used to test for differences among the tree groups of animals by multivariate analysis of variance. Values are expressed as means±SD. A probability value of $p<0.05$ was used to indicate statistical significance.

RESULTS

The untreated diabetic rats weighed significantly less than the other two groups of rats at the end of the experiment, however the insulin-treated group had similar body weights compared with the control group (Table 1). Plasma glucose levels were increased in both diabetic and insulin-treated group compared with those in control rats, since insulin was not injected the day before experiments to avoid hypoglycemia. The untreated diabetic group showed a significant increase in small intestinal size, evaluated by the measurements of wet weight (control 8.1±0.6 g, diabetic 12.7±1.4 g), dry weight (control 1.58±0.20 g, diabetic 2.13±0.25 g), and surface area (control 100±6.7 cm^2, diabetic 157±9.6 cm^2). Insulin treatment reduced the increase in intestinal weight and surface area in diabetic rats. Diabetic group showed a significant increase in full length of small intestine compared with that of control and insulin-treated groups, respectively. The intestinal length of the insulin-treated group was not significantly different from the control group (control 103±7.2 cm, insulin-treated 107±4.2 cm, diabetic 131±9.7 cm).

TABLE 1
Characteristics of animal groups

	Number	Body weight (g)		Fasting plasma glucose (mg/dl)
		at diabetes induction	at experiment	
Control	8	281 ± 10	297 ± 15	111 ± 19
Insulin	4	282 ± 13	283 ± 26	378 ± 49 [a]
Diabetic	5	280 ± 15	200 ± 35 [a b]	412 ± 113 [a]

Values are expressed as mean ± SD. a: $p<0.001$ vs Control, b: $p<0.001$ vs Insulin.

Marked stimulation of DAO activity was observed in post-heparin plasma, and the maximal activities were obtained between 30 and 60 minutes after the heparin injection. Diabetic group showed a significant increase in DAO activity at 30 and 60 minutes, compared with control group. However, insulin-treated group had significant increase in plasma DAO activity before heparin injection compared with other two groups (control 0.29±0.29 mU/L, insulin-treated 2.13±0.76 mU/L, diabetic 0.56±0.26 mU/L). The DAO activity at 60 minutes stimulated by heparin in insulin-treated group was between those in control and diabetic group (control 46.4±18.2 mU/L, insulin-treated 68.9±25.3 mU/L, diabetic 121.5±31.4 mU/L).

Insulin-treated and diabetic groups showed significant increase of plasma levels of cholesterol compared with control group (control 45.2±12.7 mg/dl, insulin-treated 92.2±38.8 mg/dl, diabetic 88.8±30.4 mg/dl). Plasma concentrations of plant sterols (campesterol and sitosterol) were significantly increased in diabetic group compared with two other groups (campesterol; control 7.2±1.7 mg/dl, insulin-treated 12.8±5.8 mg/dl, diabetic 21.7±6.4 mg/dl, sitosterol; control 3.4±0.9 mg/dl, insulin-treated 5.7±3.0 mg/dl, diabetic 10.1±3.3 mg/dl). Plasma DAO activity at 60 minutes after heparin injection significantly correlates with small intestinal wet weight, small intestinal surface area, as well as plasma levels of plant sterols (campesterol and sitosterol), respectively (Fig. 1). Plasma DAO activity also correlates to

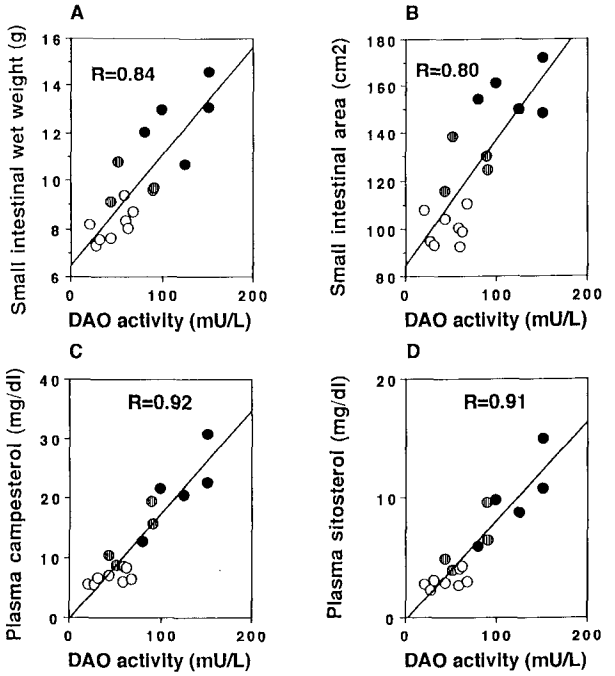

Fig 1. Correlations of concentrations of plasma DAO activity at 60 minutes with small intestinal wet weights (A), small intestinal surface area (B), concentrations of plasma campesterol (C), and sitosterol (D). ○ =non-diabetic, ◍ =insulin-treated diabetic, ● =untreated diabetic rats.

plasma glucose (r=0.75) and cholesterol (r=0.64) level, but the relationship is rather weak than that with plant sterols (data not shown).

DISCUSSION

This study demonstrates that plasma DAO activity correlates not only to the size of small intestine but also to plasma plant sterol concentrations after 14 day-feeding with the laboratory chow containing 1 % of plant sterol in rats. Untreated diabetic rats had intestinal hyperplasia and increased plasma plant sterol levels. The mechanisms of small intestinal hypertrophy in experimentally induced diabetic animals were not clearly understood. It has been suggested that the hyperphagia in these animals is responsible for the small intestinal hyperplasia, however, diabetic rats fed with diets isocaloric to control rats have been reported to show also small intestinal hyperplasia (11-13), indicating that uncontrolled diabetic state itself may induce the intestinal growth in the absence of hyperphagia. Although polyamines (14) and some growth factors (15, 16) were thought to have key roles in the development of intestinal hyperplasia, the exact mechanisms can not be speculated from this study.

The enzyme diamine oxidase has a unique anatomical distribution (17). In many mammalian species, DAO in post-heparin plasma is thought to be released almost exclusively from the small intestinal epithelial cells (6, 18). The physiological role of the small intestinal DAO is yet undefined. Though it is believed to play a key role in the small intestinal replicative processes, this enzyme may play another role: such as the regulation of exogenous and endogenous amines concentrations in the plasma (19). Fogel et al (20) reported the decrease in the tissue levels of DAO activity in the small intestines of diabetic rats at 4 and 9 weeks after streptozotocin injection. However, they did not assess the small intestinal changes, and the plasma levels of post-heparin DAO activity. Since we determined that plasma levels of post-heparin DAO activity was maximally stimulated (6) at 2 weeks after the streptozotocin treatment, the difference of the experimental conditions may explain the discrepancy of the results.

This study first demonstrated the striking increase of plasma levels of plant sterols in diabetic rats fed with chow containing plant sterols. Cholesterol levels were also increased in diabetic animals. Matsubara et al (21) reported that streptozotocin-induced diabetic rats fed with chow containing 1% of cholesterol showed marked hypercholesterolemia, and that this hypercholesterolemia was improved by N-(α-methylbenzyl)linoleamide (an acyl-CoA: cholesterol acyltransferase inhibitor) treatment. His co-workers (22) reported that, insulin treatment on the streptozotocin-induced diabetic rats reduced small intestinal acyl-CoA: cholesterol acyltransferase activity to the levels found in control animals, and postulated that

the enhancement of CoA-dependent cholesterol esterification in the intestine might be one of the major factors responsible for hypercholesterolemia in diabetes. Although little is known concerning with the specificity for the absorption of sterols by small intestine, plasma levels of plant sterols are reported to be in parallel with cholesterol absorption (23). Therefore, the increased plasma levels of plant sterols in diabetic animals may indicate the increased potential ability for the absorption of cholesterol, which may be accounted for by the hyperplastic changes in small intestine, since plasma levels of plant sterols were closely correlated to DAO activity in post-heparin plasma. The plasma concentrations of plant sterols were within a range of sitosterolemia; a rare inherited lipid storage disease characterized with xanthomatosis and premature atherosclerosis. Thus, this model animal may give us valuable information in relation to pathological roles of the increased plasma levels of the abnormal sterols in this rare disorder (24).

These findings indicate that the measurement of plasma post-heparin diamine oxidase activity can be used to assess the morphological changes of small intestinal tract, and also suggest that the activity may be a marker for the potential ability to absorb dietary sterols in diabetes mellitus.

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