Long-Term Administration of Highly Purified Eicosapentaenoic Acid Ethyl Ester Improves the Dysfunction of Vascular Endothelial and Smooth Muscle Cells in Male WBN/Kob Rats

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We investigated the effect of long-term administration of highly purified eicosapentaenoic acid ethyl ester (EPA-E), an n-3 polyunsaturated fatty acid, on the dysfunction of the endothelium and smooth muscle cells in male WBN/Kob rats, a model of spontaneous diabetes mellitus. After oral 8-month treatment with EPA-E, the agent significantly and dose-dependently increased the migration activity of vascular endothelial cells and also decreased 5-bromodeoxyuridine (BrdU) uptake by vascular smooth muscle cells at a dose of 0.1 g/kg or higher. In addition, there were significant correlations between the endothelial cell migration or smooth muscle cell proliferation and the 4-hour fasting glucose level. These findings suggest that EPA-E has a suppressive effect on thrombosis and atherosclerosis.

IN A PREVIOUS STUDY, we investigated the preventive effect of highly purified eicosapentaenoic acid ethyl ester (EPA-E), an n-3 polyunsaturated fatty acid, on the development of diabetes, insulin resistance, and blood coagulation abnormalities in male WBN/Kob rats, an animal model of spontaneous diabetes.[1] Also, we reported previously that the data actually demonstrate an amelioration of hyperglycemia and insulin resistance and a beneficial alteration of certain factors known to promote thrombosis and atherosclerosis in the animals with oral 8-month administration of EPA-E.

Thus, using vascular endothelial and smooth muscle cells obtained from the above-mentioned study, we examined the preventive effect of EPA-E on the development of their dysfunction in the present study.

MATERIALS AND METHODS

Animals

Male WBN/Kob rats and male Wistar rats were obtained at 7 months of age from Japan SLC (Shizuoka, Japan) and acclimatized for 1 month for use in this study. The animals were housed individually in aluminum cages (170 × 260 × 180 mm) in a room with controlled temperature (23° ± 2°C) and relative humidity (55% ± 15%) and a 12-hour light/dark cycle, fed a fish meal-free solid diet (MB-3; Funabashi Farm, Chiba, Japan), and provided water ad libitum during the experimental period.

Test Substance

Highly purified EPA-E (93.5% pure; Mochida Pharmaceutical, Tokyo, Japan) was used in this study.

Experimental Design

At 8 months of age, male WBN/Kob rats were randomly assigned to 4 groups of 12 rats each. Twelve male Wistar rats of the same age were used as normal controls. EPA-E was administered daily to the animals at a dosage of 0.1, 0.3, and 1.0 g/kg/d for 8 months by gavage using microsyringes.[1] Distilled water (1.0 mL/kg/d)–treated WBN/Kob rats and Wistar rats served as the vehicle control and normal control, respectively.

Plasma Glucose and Insulin

At the end of 8 months' treatment, heparinized blood samples were obtained from a cervical vein under pentobarbital sodium anesthesia after a 4-hour fast (9 AM to 1 PM). Plasma 4-hour fasting levels of glucose were measured with an autoanalyzer, and those of immunoreactive insulin with a commercial enzyme-linked immunosorbent assay (ELISA) kit.[1]

Oral Glucose Tolerance Test

At the end of the 8-month treatment, an oral glucose tolerance test (OGTT) was performed on half of the rats in each group. After an overnight fast, glucose test (2 g/kg) was administered orally to the animals, and heparinized blood samples were taken from a tail vein without anesthesia at 0, 30, 60, and 120 minutes for measurement of plasma glucose by the above-mentioned method. Samples were also taken at 0, 30, 60, and 120 minutes for measurement of the plasma immunoreactive insulin level with the ELISA kit.

Effects of EPA-E on the Function of Vascular Endothelial and Smooth Muscle Cells

Isolation of arterial endothelial cells. The day after administration of the last dose of EPA-E, the animals were killed by exsanguination under anesthesia with pentobarbital sodium (50 mg/kg body weight intraperitoneally), and the abdominal aorta was removed from the normal controls, EPA-E–treated animals, and vehicle controls (n = 6 per group). Arterial endothelial cells were separated from the aorta by the method of Kanayasu et al.[2] Briefly, after removal of adipose tissue around the aorta, the latter was washed with sterile phosphate-buffered saline ([PBS] pH 6.8) and incised with surgical scissors. The incised aorta was then incubated with collagenase (Nitta Gelatin, Osaka, Japan) solution (6,000 U/mL) at 37°C for 5 minutes, after which its endothelial tissue was separated with a scalpel. Finally, the tissue was incubated with 0.02% trypsin (DIFCO Laboratories, Detroit, MT) at 37°C for 5 minutes. Endothelial cells were washed with minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Nissui Pharmaceutical, Tokyo, Japan) pretreated with charcoal and suspended in the medium at a concentration of 4×10^4/mL. The cells were identified as arterial endothelial cells because they reacted positively with anti–factor VIII antibody (Chemicon, Temecula, CA) used for fluorescence immunostaining. The purity of the arterial endothelial cells was greater than 90%.

Determination of migration activity of arterial endothelial cells. According to the method of Hayashi et al,[3] a microchemotaxis chamber (Chemotaxel; Kurabou, Osaka, Japan) was set into each well of a

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24-well culture plate, each well of which had been filled with 0.8 mL of the above-mentioned MEM; then, 0.4 mL of the cell suspension was added to the chamber, and incubation was performed at 37°C for 24 hours under an atmosphere of 5% CO_2/95% air. After incubation, the endothelial cells were fixed with 90% ethanol in water for 5 minutes, stained with hematoxylin solution for 15 minutes, and then washed with distilled water. The chamber with a filter was observed with an inverse microscope at 600× magnification to determine the number of cells that migrated onto the lower surface of the filter. Cell migration was expressed as the total number of cells that migrated per 2,000 pores of the filter. This migration assay was performed in the absence or presence of leukotriene C_4 ([LTC_4] 0.1 pmol/L) as a migration stimulant.

Isolation of vascular smooth muscle cells. According to the method of Chamley-Campbell et al,[4,5] the aorta from which the above-mentioned endothelial cells were separated was used for isolation of smooth muscle cells. Briefly, minced aorta lacking endothelium was incubated with PBS containing 0.1% trypsin (DIFCO) at 37°C for 1 hour with shaking, and the suspension was then centrifuged at 300 ×*g* for 5 minutes at room temperature to obtain smooth muscle cells. This procedure was conducted repeatedly until a sufficient number of cells were obtained for the uptake test described later. The cells were washed with MEM supplemented with 10% fetal bovine serum (Nissui Pharmaceutical) pretreated with charcoal and suspended in the medium at a concentration of 4×10^4/mL. The cells were identified as vascular smooth muscle cells because they were positively stained by anti–α-actin antibody (Chemicon) by fluorescence immunostaining. The purity of the vascular smooth muscle cells was greater than 90%.

Determination of 5-bromodeoxyuridine uptake. As a measure of proliferation activity, we examined 5-bromodeoxyuridine (BrdU) uptake of the smooth muscle cells by the method of Hardonk and Harms.[6] For this procedure, 1 mL of the cell suspension was added to each well of a 24-well microplate (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ), and the microplate was then incubated at 37°C for 22 hours under an atmosphere of 5% CO_2/95% air. Next, 10 µL 10-µmol/L BrdU (Amersham, Buckinghamshire, UK) solution was added to each well, and the incubation was continued for 2 hours under the same conditions described before. After removal of the medium, the cells were treated with 100% methanol at room temperature for 30 minutes to avoid any nonspecific response, washed with PBS containing 0.1% Tween 20, and then treated with a blocking agent solution (Block Ace; Dainippon Pharmaceutical, Osaka, Japan). After the cells were washed with PBS, they were incubated with an anti-BrdU antibody (Amersham) and deoxyribonuclease (Amersham) solution at room temperature for 1 hour. Then, after the cells were washed again with PBS, europium-labeled anti-mouse immunoglobulin G antibody (Pharmacia Biotech, Uppsala, Sweden) was applied to each well and incubation was performed at room temperature for 1 hour. After a final washing of the wells with PBS, the cells were incubated with DELFIA enhancement agent (Pharmacia Biotech) at room temperature for 5 minutes, and europium fluorescence was measured with a DELFIA fluorometer. The BrdU content was calculated from the standard curve prepared earlier.

Statistical Analysis

All data are expressed as the mean ± SD. Dunnett's test[7] was used to analyze the significance of differences between the normal control group (Wistar rats) and the vehicle control (for WBN/Kob rats) or EPA-E–treated groups and between the vehicle control and EPA-E–treated groups. Williams' test[8,9] was used to analyze the dose-dependency of the effects of EPA-E on arterial endothelial cell migration and vascular smooth muscle cell proliferation. A difference from the vehicle control was regarded as statistically significant at a *P* value less than .05.

RESULTS

Incidence of Diabetes in Male WBN/Kob Rats

The 4-hour fasting glucose levels for the various groups are shown in Table 1. At the end of the 8-month treatment period, the incidence of diabetes in the vehicle control group was 100%. On the other hand, the rate in EPA-E groups that received 0.1, 0.3, and 1.0 g/kg was 92%, 50%, and 17%, respectively. All Wistar rats, used as the normal controls, were nondiabetic.

Plasma Insulin

The EPA-E groups that received 0.3 and 1.0 g/kg showed a significant and dose-dependent increase in insulin compared with the vehicle group (Table 1).

OGTT

The EPA-E groups showed a significant and dose-dependent decrease in plasma glucose before and at 30, 60 (except 0.1-g/kg group), and 120 (except 0.1-g/kg group) minutes after glucose loading compared with the vehicle control group. In contrast, rats that received 0.3 or 1.0 g/kg showed a significant and dose-dependent increase in plasma immunoreactive insulin at each determination point compared with the vehicle control group (Table 2).

Migration Activity of Arterial Endothelial Cells

The vehicle control group displayed a significant decrease in the migration activity of arterial endothelial cells in the absence or presence of LTC_4 compared with the Wistar rats. The EPA-E groups showed a significant and dose-dependent ($P = .0001$) increase in the migration activity of the cells without or with LTC_4 compared with the vehicle control group. In addition, at a dose of 0.1 g/kg, there was still a significant decrease in the migration ability of WBN/Kob endothelial cells without LTC_4 compared with endothelial cells from Wistar rats, whereas at a dose of 0.3 and 1.0 g/kg, no significant difference was found. Furthermore, there was no significant difference in migration in the presence of LTC_4 between Wistar cells and EPA-E–treated WBN/Kob cells at any dose (Fig 1).

BrdU Uptake by Vascular Smooth Muscle Cells

The vehicle control group showed a significant increase in BrdU uptake by vascular smooth muscle cells compared with the Wistar rats. The EPA-E groups showed a significant and dose-dependent ($P = .0001$) decrease in BrdU uptake by the

Table 1. Four-Hour Fasting Plasma Glucose and Insulin Levels in Male WBN/Kob Rats Treated Orally With EPA-E for 8 Months

Treatment	4-Hour Fasting Glucose (mg/dL)	Immunoreactive Insulin (ng/mL)
Vehicle control	361 ± 72	0.6 ± 0.2
EPA-E 0.1 g/kg/d	297 ± 72	0.8 ± 0.2
EPA-E 0.3 g/kg/d	201 ± 98*	1.1 ± 0.3*
EPA-E 1.0 g/kg/d	136 ± 53*	1.6 ± 0.4*

NOTE. The data (n = 12) were analyzed by Dunnett's test. Animals that showed >200 mg/dL as a 4-hour (9 AM-1 PM) fasting plasma glucose level were evaluated to be diabetic. The vehicle control group was treated orally with distilled water (1.0 mL/kg/d).

*$P < .01$ *v* vehicle.

Table 2. Plasma Glucose and Insulin Levels After an Oral Glucose Load in Male WBN/Kob Rats Treated Orally With EPA-E for 8 Months

Parameter	Time (min) 0	30	60	120‡
Plasma glucose (mg/dL)				
Vehicle control	299 ± 62	695 ± 122	717 ± 154	518 ± 135
EPA-E 0.1 g/kg/d	220 ± 66*	541 ± 122*	622 ± 103	491 ± 157
EPA-E 0.3 g/kg/d	89 ± 11†	287 ± 43†	366 ± 60†	261 ± 60†
EPA-E 1.0 g/kg/d	86 ± 6†	269 ± 25†	351 ± 29†	229 ± 43†
Plasma immunoreactive insulin (ng/mL)				
Vehicle control	0.4 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.5 ± 0.2
EPA-E 0.1 g/kg/d	0.7 ± 0.2	1.4 ± 0.2	1.1 ± 0.3	0.9 ± 0.3
EPA-E 0.3 g/kg/d	1.1 ± 0.2†	2.2 ± 0.6	1.8 ± 0.4†	1.3 ± 0.4†
EPA-E 1.0 g/kg/d	1.6 ± 0.3†	3.9 ± 2.0†	3.0 ± 1.0†	1.7 ± 0.4†

NOTE. The data (n = 6) were analyzed by Dunnett's test. The vehicle control group was treated orally with distilled water (1.0 mL/kg/d).
*$P < .05$, †$P < .01$ *v* vehicle.
‡Time after glucose (2 g/kg) load.

cells compared with the vehicle control group. However, none of the doses of EPA-E reduced the proliferation to the level found for Wistar rats (Fig 2).

DISCUSSION

We have clearly demonstrated an amelioration of hyperglycemia and insulin resistance by oral 8-month administration of EPA-E to male WBN/Kob rats in a previous study.[1] This result was supported by evidence that EPA-E was useful for preventing the development of insulin resistance in Otsuka Long-Evans Tokushima fatty rats, a model of spontaneous non–insulin-dependent diabetes mellitus with obesity, and of high-sucrose–induced insulin resistance in Dahl salt-sensitive rats.[10,11] In this study using vascular endothelial and smooth muscle cells obtained from EPA-E–treated WBN/Kob rats of our previous study,[1] we clarified that EPA-E significantly increased the migration activity of endothelial cells in the absence or presence of LTC_4 and decreased BrdU uptake by smooth muscle cells. From the fact that endothelial cell migration is an important process in the regeneration of the endothelial lining of injured blood vessels, the former finding suggests that EPA-E repairs the vascular endothelial cell injury. This result is supported further by the observation by Kanayasu et al[2] that eicosapentaenoic acid (EPA) stimulated porcine smooth muscle cell migration in vitro. The latter finding suggests that the agent suppressed the proliferation of smooth muscle cells. This result is in conformity with the experiment by Terano et al[12] showing that EPA inhibited the proliferation of rat cultured vascular smooth muscle cells by modulating various steps of the signal transduction elicited by platelet-derived growth factor. These investigators suggested that the observed effect may be due to a change in membrane fluidity through the increase in EPA-E. In addition,

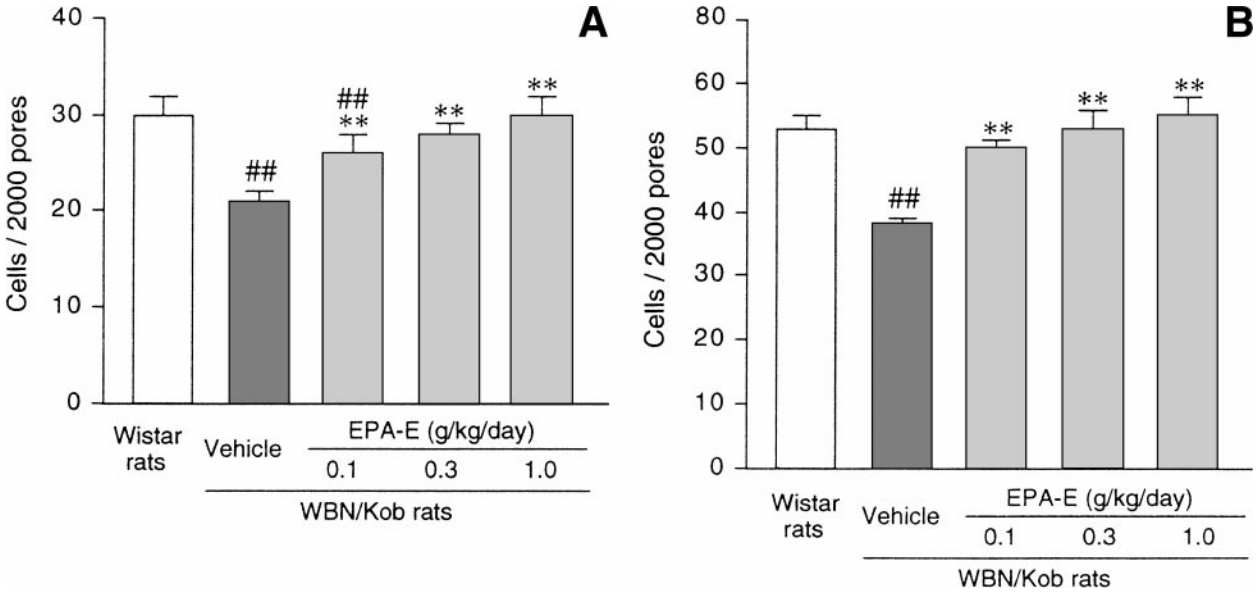

Fig 1. Migration activity of arterial endothelial cells in male WBN/Kob rats treated orally with EPA-E for 8 months (A, without stimulation; B, with 0.1 pmol/L LTC_4 stimulation). Each column represents the mean ± SD (n = 6). **$P < .01$ *v* vehicle (Dunnett's multiple test). ##$P < .01$ *v* Wistar rats (Dunnett's multiple test). The action of EPA-E on arterial endothelial cell migration was significant in a dose-dependent manner at $P = .0001$ (Williams' test). The vehicle control group was treated orally with distilled water (1.0 mL/kg/d).

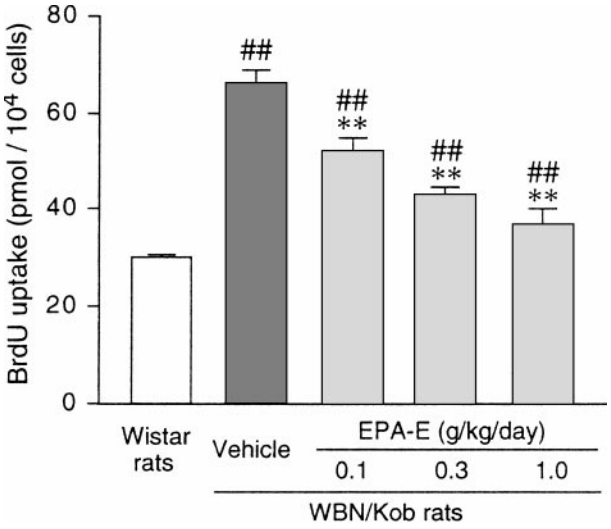

Fig 2. BrdU uptake by vascular smooth muscle cells in male WBN/Kob rats treated orally with EPA-E for 8 months. Each column represents the mean ± SD (n = 6). **$P < .01$ *v* vehicle (Dunnett's multiple test). ##$P < .01$ *v* Wistar rats (Dunnett's multiple test). The action of EPA-E on vascular smooth muscle cell proliferation was significant in a dose-dependent manner at $P = .0001$ (Williams' test). The vehicle control group was treated orally with distilled water (1.0 mL/kg/d).

the migration activity of vascular endothelial cells obtained from male WBN/Kob rats treated orally with EPA-E, lard, olive oil, or safflower oil at a dose of 0.3 g/kg/d for 6 months was 30 ± 2, 20 ± 1,* 19 ± 1,* or 22 ± 5* cells per 2,000 pores in the absence of LTC_4, respectively, and 59 ± 1, 41 ± 1,* 40 ± 1,* or 41 ± 2* cells per 2,000 pores in the presence of LTC_4, respectively. The data demonstrated a significant difference in the migration of vascular endothelial cells between EPA-E–treated rats and rats that received the other fatty acids. Therefore, it is conceivable that the effect on migration is unique to EPA-E.

It is well known that nitric oxide (NO) inhibits the proliferation of vascular smooth muscle cells, inflammation, and platelet aggregation in addition to causing relaxation of vascular smooth muscle cells. Therefore, a decrease in the NO-producing ability of vascular endothelial cells would promote the progression of arteriosclerosis; conversely, an increase in NO production would suppress the disease. We demonstrated previously that EPA-E improved the function of vascular endothelial cells in terms of increasing endothelial NO synthase activity in Dahl-S rats fed a high-sucrose diet containing 1.00% salt.[13] In view of these facts, the above-mentioned actions of EPA-E may be partly attributable to an increase in NO production. Considering the results obtained from the previous study and this study together, there was a significant negative correlation (in the absence or presence of LTC_4, $r = -.852$ or $-.749$, respectively, $P = .0001$) between the migration activity and the plasma 4-hour fasting glucose level. Also, there was a significant negative correlation ($r = -.883$, $P = .0001$) between BrdU uptake by vascular smooth muscle cells and the plasma 4-hour fasting glucose level. However, further studies are needed to clarify the details of the mechanism of EPA-E action against diabetes mellitus. In conclusion, our data indicate that EPA-E could enhance the migration activity of vascular endothelial cells and suppress the proliferation of vascular smooth muscle cells, either via its direct action on these cells or by an indirect action consisting of an amelioration of hyperglycemia and insulin resistance.

*$P < .01$ *v* EPA-E group by Dunnett's test; unpublished data, Mochida Pharmaceutical, 1993.

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