Long-Term Administration of Highly Purified Eicosapentaenoic Acid Ethyl Ester Improves Blood Coagulation Abnormalities and Dysfunction of Vascular Endothelial Cells in Otsuka Long-Evans Tokushima Fatty Rats

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Abstract. We investigated the effect of highly purified eicosapentaenoic acid ethyl ester (EPA-E) on blood coagulation abnormalities and dysfunction of vascular endothelial cells in spontaneously diabetic Otsuka Long-Evans Tokushima Fatty rats. The animals were treated with either EPA-E or lard at a daily dose of 0.3 g/kg/day for 52 weeks by gavage, and their coagulation/fibrinolytic parameters, platelet aggregation, and functions of the vascular endothelial cells were examined. EPA-E significantly improved coagulation-related parameters including prothrombin time, activated partial thromboplastin time, fibrinogen level, and activities of factor II, V, VII, VIII, IX, X, and XII, and antithrombin III, and fibrinolysis-related parameters including plasminogen, tissue-type plasminogen activator, α2-plasmin inhibitor, and plasminogen activator inhibitor. It also suppressed ADP- or collagen-induced platelet aggregation and the cholesterol/phospholipid molar ratio in platelet membranes at a dose of 0.3 g/kg. In addition, it significantly increased the migration activity of vascular endothelial cells, and decreased the binding of vascular endothelial cells to vascular endothelial growth factor. In contrast, lard had no effect on hypercoagulation, hypofibrinolysis, and platelet hyperaggregation but significantly aggravated the dysfunction of vascular endothelial cells. These data demonstrate that EPA-E beneficially altered certain factors known to promote thrombosis and atherosclerosis in this animal model.

Key words: Blood coagulation, Vascular endothelial/smooth muscle cells, Eicosapentaenoic acid ethyl ester, Otsuka Long-Evans Tokushima Fatty rats

We reported previously that the long-term administration of highly purified eicosapentaenoic acid ethyl ester (EPA-E), an n-3 polyunsaturated fatty acid, which has an inhibitory effect on platelet aggregation and lipid-lowering effect, causes an improvement of insulin resistance in Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model of spontaneous non-insulin-dependent diabetes mellitus with obesity [1] and that of high sucrose-induced insulin resistance in Dahl salt-sensitive (Dahl-S) rats [2]. It is well recognized that hyperglycemia enhances many risk factors for arteriosclerosis and works as a thrombus-forming factor via causing blood coagulation abnormalities and dysfunction of vascular endothelial cells and thrombocytes [3, 4].

Thus, in the present study, we investigated the effect of EPA-E on blood coagulation abnormalities and
dysfunction of vascular endothelial cells in OLETF rats, using lard as a negative control.

**Materials and Methods**

**Animals**

OLETF rats were obtained at 4 weeks of age from the Tokushima Research Institute, Otsuka Pharmaceutical Co. (Tokushima, Japan). The animals were individually housed in stainless steel cages (320 × 270 × 175 mm) in an animal room with a controlled temperature (23 ± 2°C) and relative humidity (55 ± 15%), and a 12-h light/12-h dark cycle (lights on at 0700 h). They were supplied with a fish meal-free rat chow (MB-3; Funabashi Farm, Funabashi, Japan) and tap water ad libitum during the experimental period. The guidelines for Laboratory Animal Facilities of Jikei University School of Medicine were followed for the care and use of the animals in this study.

**Test substances**

Highly purified EPA-E (93.5% pure; Mochida Pharmaceutical, Tokyo, Japan) and lard (saturated fatty acid, 40.1%; Funabashi Farm, Funabashi, Japan) were used as the test substances.

**Experimental design**

At 8 months of age, male OLETF rats were randomly assigned to three groups of 25 rats each. Animals were treated with EPA-E or lard at a daily dose of 0.3 g/kg/day or with distilled water (0.3 mL/kg/day) for 52 weeks by gavage with microsyringe (Gastight®; Hamilton, Reno, NV, USA). The EPA-E dose was previously found to be effective for suppressing the development of insulin resistance in OLETF rats [1] and Dahl-S rats [2]. The lard dose also was used in the previous studies. Rats treated with distilled water served as the control. Body weight and food consumption were recorded once a week throughout the experimental period.

**Coagulation parameters**

At the end of the 52-week treatment, citrated blood samples were taken under anesthesia with pentobarbital sodium (50 mg/kg body weight, i.p.) by means of a disposable polypropylene syringe from the abdominal aorta and centrifuged at 2,200 × g for 15 min at 4°C to isolate the plasma. Prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen level (Thromboscreen series for PT, APTT, and fibrinogen; Pacific Hemostasis, Huntersville, NC, USA), and plasma activities of factors II, V, VII, VIII, IX, X, XI, and XII (Factor deficiency plasma; International Reagents, Kobe, Japan) were measured by the viscosity method with an automatic coagulometer (KC-40; Heinrich Amelung, Lemgo, Germany). Antithrombin III (AT III; Testzym S AT III: Daiichi Pure Chemicals, Tokyo, Japan) activity was measured by a synthetic substrate method using an autoanalyzer (Roche Cobas Farab; Basel, Switzerland).

**Fibrinolytic parameters**

Tissue-type plasminogen activator (t-PA, Chromozym PL; Roche Diagnostics, Mannheim, Germany) level and activities of plasminogen (PLG, Testzym S-2251; Daiichi Pure Chemicals), α₂-plasmin inhibitor (α₂-PI, Testzym S APL; Daiichi Pure Chemicals), and plasminogen activator inhibitor (PAI, Stachrom PAI; Diagnostica Stago, Asnieres, France) of the plasma samples used for the coagulation analysis were measured by a synthetic substrate method with the autoanalyzer.

**Platelet aggregation**

A portion of the above-mentioned citrated blood was centrifuged at 120 × g for 10 min at 25°C to prepare platelet-rich plasma (PRP), and additional centrifugation at 1,600 × g for 10 min at 25°C was done to obtain platelet-poor plasma (PPP). The platelet count in PRP was measured with a fully automated hemocytometer (E-2000; Toa Medical Electronics, Kobe, Japan), and the PRP was diluted with PPP to adjust the number of platelets to 2.5 × 10⁵/μL. Aggregation in response to ADP (1 and 5 μM, Meiji Yakuhin, Tokyo, Japan) or collagen (0.5 and 1 μg/mL, Nycomed Arzneimittel, Munich, Germany) was recorded with a platelet aggregometer (NBS Hema Trace 801; MC Medical, Tokyo, Japan) by a nephelometric method using PRP. The maximum rate of aggregation induced by ADP or collagen was then calculated.
Platelet lipid (cholesterol and phospholipids) analysis

Two mL of a citrated blood sample as described above was added to a test tube containing 160 μL of 77 mmol/L EDTA-2K. The samples were then centrifuged at 400 × g for 10 min at 4°C to prepare PRP, followed by additional centrifugation at 2,200 × g for 10 min at 4°C to obtain platelets. These platelets were suspended in an ice-cold buffer consisting of 0.154 mol/L NaCl, 0.154 mol/L Tris-HCl (pH 7.4), and 77 mmol/L EDTA-2K (pH 7.4) (45 : 4 : 1, v/v/v), and the suspension was then centrifuged at 2,200 × g for 10 min at 4°C. The pellet was suspended in physiological saline, and the platelet count in the suspension was determined with a hemocytometer (F-800; Toa Medical Electronics). The platelet count in the suspension was adjusted to 5 × 10^5/μL by dilution with physiological saline. Washed platelets were stored at −80°C until extraction of lipids could be performed. The washed platelets were disrupted with an ultrasonicator (Labsonic U; B. Braun Biotech, Melsungen, Germany), after which 1 mL of sonicated platelet suspension was incubated with 12 mL of chloroform-methanol (2 : 1, v/v) at room temperature for 60 min. The treated suspension was then incubated with 2.5 mL of 0.05% sulfuric acid at room temperature for 10 min to extract lipids. The solvent fraction containing lipids was isolated by centrifugation at 1,100 × g for 15 min at 25°C and then dried by evaporation under vacuum. Using the extracted lipids, we determined the contents of total cholesterol (enzymic cholesterol esterase-cholesterol oxidase-peroxidase method, Iatrolipo TC; Iatron Laboratories) and phospholipids (permanganate salt method, Phospholipids Test Wako; Wako Pure Chemical) and calculated the cholesterol/phospholipid (C/P) molar ratio.

Isolation of arterial endothelial cells

On the day after administration of the last dose, the animals were killed by exsanguination under anesthesia with pentobarbital sodium (50 mg/kg body weight, i.p.), and the abdominal aorta was removed from vehicle controls (n = 18), lard-treated animals (n = 16), and EPA-E-treated animals (n = 17). Arterial endothelial cells were separated from the aorta by the method of Kanayasu et al. [5]. Briefly, after cutting off adipose tissue around the aorta, the latter was washed with sterile PBS (pH 6.8) and incised with surgical scissors. The incised aorta was then incubated with collagenase (Nitta Gelatin, Osaka, Japan) solution (6,000 units/mL) at 37°C for 5 min, after which its endothelial tissue was separated with a scalpel. Finally, the tissue was incubated with 0.02% trypsin (Difco Laboratories, Detroit, MI, USA) at 37°C for 5 min. Endothelial cells were washed with minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Nissui Pharmaceutical, Tokyo, Japan) that had been pretreated with charcoal and were then suspended in the medium at a concentration of 4 × 10^4/mL. The cells were identified to be arterial endothelial cells because they reacted positively with anti-factor VIII antibody (Chemicon International, Temecula, CA, USA) used for fluorescence immunostaining. The purity of the arterial endothelial cell preparation was more than 90%.

Determination of migration activity of arterial endothelial cells

According to the method of Hayashi et al. [6], a microchemotaxis chamber (Chemotaxicell; Kurabou, Osaka, Japan) was set into each well of a 24-well culture plate, each of which had been filled with 0.8 mL of the above-mentioned MEM; and then 0.4 mL of the cell suspension was added to the chamber, and incubation was carried out at 37°C for 24 h under an atmosphere of 5% CO₂/95% air. After incubation, the endothelial cells were fixed with 90% ethanol in water for 5 min, stained with hematoxylin solution for 15 min, 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 had migrated onto the lower surface of the filter. Cell migration was expressed as the total number of cells that had migrated per 2,000 pores of the filter. This migration assay was conducted in the absence or presence of leukotriene C₄ (LTC₄, 0.1 pmol/L) as a migration stimulant.

Binding of endothelial cells to vascular endothelial growth factor (VEGF)

The vascular endothelial cells were homogenized in PBS with an ultrasonicator (Labsonic U), and the cell membrane fraction was obtained by centrifugation (10,000 × g for 3 min at 4°C). By use of this fraction, we measured the binding of vascular endothelial cells
to VEGF according to the surface plasma resonance method [7] with a Biosensor Analytical System (NL-SPR 670; Nippon Laser and Electronics Laboratories, Nagoya, Japan). Briefly, 1 mL of a 1 μg/mL solution of VEGF in PBS was placed on a gold-coated glass plate, and the plate was placed in the instrument. Next, PBS at 37°C was circulated through the instrument, and the resonance, which is considered to be the value at 0 min, was determined. One mL of cell-membrane fraction was then added to the plate and incubated at 37°C. At 10 min after initiation of the reaction, the resonance value was recorded. The binding of endothelial cells to VEGF was calculated as the resonance units bound (the difference in resonance value between 0 and 10 min). A plate coated with BSA was used as a control reference.

**Statistical analysis**

Data were expressed as the mean ± SD. The significance of differences between the vehicle control and EPA-E-treated group or lard-treated group was analyzed by Dunnett’s test [8]. The analyses were carried out with SAS software (SAS Institute, Cary, NC, USA). A statistically significant difference was defined as a P value less than 0.05.

**Fig. 1-1.** Coagulation time and coagulation factors in male OLETF rats treated orally with EPA-E for 52 weeks. Each column represents the mean ± SD (the control, EPA-E, and lard groups: 18, 17, and 15 animals, respectively). **P<0.01 vs control (Dunnett’s test).**
**Results**

*General description*

The control, EPA-E, and lard groups had 7, 8, and 10 dead animals, respectively, by the end of the experimental period. Occurrence of deaths in all three groups was mainly due to renal failure. The control, EPA-E, and lard groups were comparable in body weight gain and food consumption during the experimental period.

*Coagulation parameters*

The EPA-E group showed significant increases in PT, APTT, and AT III activity and significant decreases in fibrinogen content and activities of factors II, V, VII, VIII, IX, X, XI, and XII compared with the control group. In contrast, these parameters in the lard group were similar to those in the control group (Fig. 1).

*Fibrinolytic parameters*

The EPA-E group indicated significant increases in

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**Fig. 1-2.** Coagulation factors and coagulation inhibitory factor in male OLETF rats treated orally with EPA-E for 52 weeks. Each column represents the mean ± SD (the control, EPA-E, and lard groups: 18, 17, and 15 animals, respectively). **P<0.01 vs control (Dunnett’s test).**
Fig. 2. Fibrinolysis factors and fibrinolysis inhibitory factors in male OLETF rats treated orally with EPA-E for 52 weeks. Each column represents the mean ± SD (the control, EPA-E, and lard groups: 18, 17, and 15 animals, respectively). **P<0.01 vs control (Dunnett’s test).

Fig. 3. ADP- or collagen-induced platelet aggregation in male OLETF rats treated orally with EPA-E for 52 weeks. Each column represents the mean ± SD (the control, EPA-E, and lard groups: 18, 17, and 15 animals, respectively). **P<0.01 vs control (Dunnett’s test).
plasminogen and t-PA, and significant decreases in α2-PI and PAI compared with the control group. These parameters in the lard group were similar to those in the control group (Fig. 2).

Platelet aggregation

The EPA-E group showed a significantly decreased platelet aggregation induced by ADP or collagen compared with the control group. On the other hand, the lard group resembled the control group in ADP- or collagen-induced platelet aggregation (Fig. 3).

Platelet lipid analysis

The EPA-E group showed a significantly decreased cholesterol content and a significantly increased phospholipid level in platelets compared with the control group. As a result, the C/P ratio was significantly elevated in the EPA-E group. In contrast, these parameters in the lard group were similar to those in the control group (Fig. 4).

Migration activity of arterial endothelial cells

The EPA-E group showed a significant increase in migration activity of the arterial endothelial cells in the absence or presence of LTC₄ compared with the control group. In contrast, the lard group showed a significant decrease in migration activity of the cells with or without LTC₄ compared with the control group (Fig. 5).

**Fig. 4.** Cholesterol (CHO), phospholipid (PL), and CHO/PL (C/P) molar ratio in male OLETF rats treated orally with EPA-E for 52 weeks. Each column represents the mean ± SD (the control, EPA-E, and lard groups: 18, 17, and 15 animals, respectively). **p<0.01 vs control (Dunnett’s test).

**Fig. 5.** Migration activity of arterial endothelial cells (upper panel: without stimulation, lower panel: with 0.1 pmol/l LTC₄ stimulation) in male OLETF rats treated orally with EPA-E for 52 months. Each column represents the mean ± SD (the control, EPA-E, and lard groups: 18, 17, and 15 animals, respectively). **p<0.01 vs control (Dunnett’s test).
Binding of endothelial cell to VEGF

The EPA-E group showed a significant decrease in the binding of vascular endothelial cells to VEGF compared with the control group. In contrast, the lard group indicated a significant increase in that to VEGF compared with the control group (Fig. 6).

Discussion

We reported previously that EPA-E was useful for preventing the onset of insulin resistance in OLETF rats [1] and in Dahl-S rats fed a high-sucrose diet [2], and that the product improved the function of vascular endothelial cells via enhancement of endothelial nitric oxide synthase activity in Dahl-S rats fed a high-sucrose diet containing 1.00% salt [9]. Recently, we demonstrated that the long-term administration of EPA-E improves hyperglycemia, insulin resistance, and blood coagulation abnormalities in WBN/Kob rats, a model of spontaneous diabetes mellitus [10]. In the present study, we investigated whether the long-term treatment of EPA-E would ameliorate blood coagulation abnormalities and dysfunction of vascular endothelial and cells in OLETF rats, a model of spontaneous non-insulin-dependent diabetes mellitus with obesity. In this study we used lard as a negative control.

EPA-E intake led to significant increases in PT, APTT, and AT III activity and to significant decreases in fibrinogen content and factor II, V, VII, VIII, IX, X, XI, and XII activities. These results suggest that EPA-E ameliorated the hypercoagulation due to the diabetic state. In addition, EPA-E treatment also resulted in significant increases in plasminogen and t-PA and, conversely, to decreases in α2-PI and PAI. These findings suggest that EPA-E lessened the decrease in fibrinolytic capacity attributable to diabetes. Furthermore, EPA-E significantly inhibited ADP- or collagen-induced platelet aggregation and induced a significant decrease in the C/P molar ratio in platelets. These results indicate that EPA-E inhibits ADP- or collagen-induced platelet aggregation by causing an increase in platelet membrane fluidity. The above-mentioned results coincided with our previous data obtained in WBN/Kob rats treated with EPA-E [10]. In contrast, it became evident that lard, increasing the insulin resistance in OLETF rats, did not increase or decrease the above-mentioned parameters in this study.

Next, we measured the migration activity of the vascular endothelial cells as an index of their function. EPA-E significantly increased the migration activity of the vascular endothelial cells in the presence or absence of LTC4, a potent chemotactic factor of the cells. Since endothelial cell migration is an important process in wound repair occurring on the luminal surface of blood vessels, this result suggests that EPA-E has a repairing action on the vascular endothelial cell injury. In contrast, lard significantly decreased the migration activity of the cells. This finding suggests that lard may be detrimental to the regeneration of injury in blood vessels.

Finally, to clarify the action mechanism of EPA-E on the vascular endothelial cells, we measured the binding of endothelial cells to VEGF. EPA-E significantly decreased this binding, whereas lard significantly increased it. These results suggest that EPA-E might suppress the proliferation of vascular endothelial cells through signal transduction via the VEGF receptor. However, further studies are needed to clarify the details of the mechanism of EPA-E action on dysfunction of the vascular endothelial cells due to diabetes mellitus.

In conclusion, our data clearly demonstrate multiple and beneficial actions of EPA-E on the detrimental changes in platelets and endothelial cell function, and factors of coagulation/fibrinolysis that occur in OLETF rats with diabetes.
References


