Long-Term Administration of Highly Purified Eicosapentaenoic Acid Ethyl Ester Prevents Diabetes and Abnormalities of Blood Coagulation 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 development of diabetes, insulin resistance, and abnormalities of blood coagulation in male WBN/Kob rats, a model of spontaneous diabetes mellitus. After 8-month oral EPA-E treatment, the incidence of diabetes at a dose of 0.1, 0.3, and 1.0 g/kg was 92%, 50%, and 17%, respectively. Its incidence was suppressed significantly and dose-dependently at a dose of 0.3 g/kg or higher compared with the rate (100%) for the vehicle control. Additionally, EPA-E significantly and dose-dependently decreased the elevation of plasma glucose after an oral glucose load and increased the glucose infusion rate (GIR) during the euglycemic insulin-glucose clamp test at a dose of 0.1 g/kg or higher compared with the vehicle control. Furthermore, EPA-E significantly and dose-dependently ameliorated coagulation-related parameters, including the prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen level, and factor II, V, VII, VIII, IX, X, XI, and XII and antithrombin III (AT III) activities, and fibrinolysis-related parameters, including plasminogen, tissue-type plasminogen activator (t-PA), α_2-plasmin inhibitor (α_2-PI), and plasminogen activator inhibitor (PAI), and also suppressed ADP- or collagen-induced platelet aggregation and the cholesterol to phospholipid (C/P) molar ratio in platelet membranes at a dose of 0.1 g/kg or higher. These data demonstrate multiple actions of the product in these laboratory animals. These include changes in platelet function, coagulation/fibrinolysis factors, plasma immunoreactive insulin secretion, and plasma glucose/insulin resistance.

HIGHLY PURIFIED eicosapentaenoic acid ethyl ester (EPA-E), an n-3 polyunsaturated fatty acid that exhibits an inhibitory effect on platelet aggregation[1] and a lipid-lowering effect,[2-4] has been used for the treatment of arteriosclerosis obliterans and hyperlipidemia in Japan. Recently, we reported that EPA-E was useful for preventing insulin resistance in Otsuka Long-Evans Tokushima Fatty (OLETF) rats,[5] a model of spontaneous non–insulin-dependent diabetes mellitus with obesity, as well as high-sucrose–induced insulin resistance in Dahl salt-sensitive (Dahl-S) rats.[6] Additionally, we demonstrated that the combination of EPA-E and cilnidipine, a Ca^{2+} channel blocker of the 1,4-dihydropyridine class, improved insulin sensitivity in Dahl-S rats fed a high-sucrose (60%) diet containing 1.00% salt compared with the combination of EPA-E and another Ca^{2+} channel blocker, amlodipine or nifedipine.[7] Furthermore, EPA-E improved the function of vascular endothelial cells in terms of increasing endothelial nitric oxide synthetase activity.[7]

More recently, using WBN/Kob rats, an animal model of spontaneous diabetes,[8] we defined the effects of diabetes on coagulation, fibrinolysis, and platelet aggregation.[9] Thus, in the present study, we investigated the preventive effect of EPA-E on the development of diabetes, insulin resistance, and abnormalities of blood coagulation in male WBN/Kob rats.

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MATERIALS AND METHODS

Animals

Male WBN/Kob rats and male Wistar rats were obtained at 7 months of age from Japan SLC (Shizuoka, Japan), acclimatized for 1 month, and then used in this study. The animals were individually housed in aluminum cages (170 × 260 × 180 mm) in a room controlled at a temperature of 23° ± 2°C and 55% ± 15% relative humidity with 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.

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

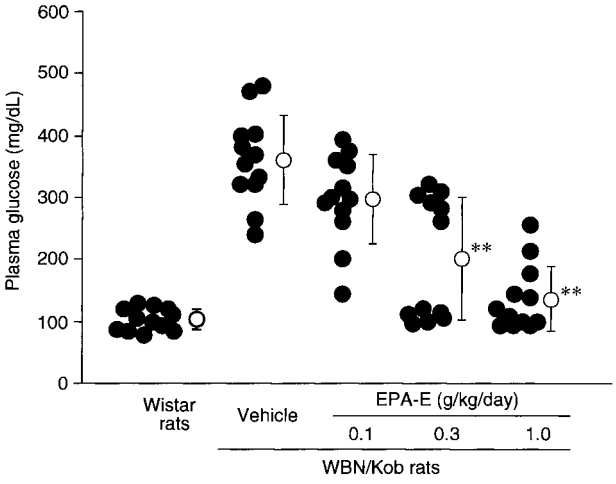

Fig 1. Incidence of diabetes in male WBN/Kob rats treated orally with EPA-E for 8 months. (●) Plasma glucose after 4-hour fasting (9 AM-1 PM) in individual rats; (○) mean ± SD (n = 12) for each group. Data were analyzed by Dunnett's test. **$P < .01$ *v* vehicle.

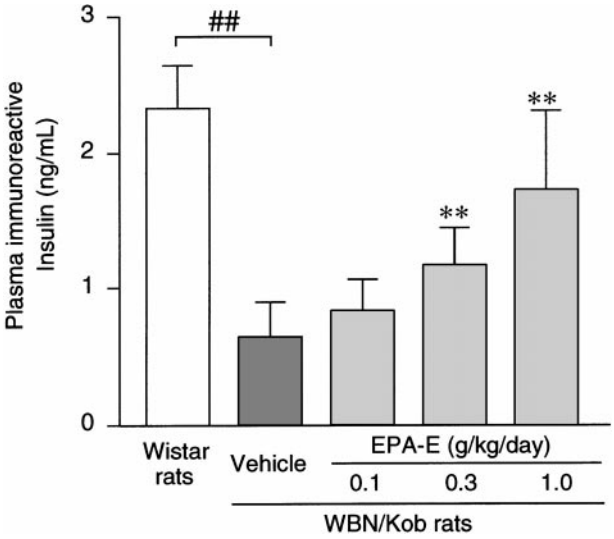

Fig 2. Plasma insulin in male WBN/Kob rats treated orally with EPA-E for 8 months. Each column represents the mean ± SD (n = 12). **$P < .01$ *v* vehicle (Dunnett's multiple test). ##$P < .01$ for the indicated groups (Student's *t* test).

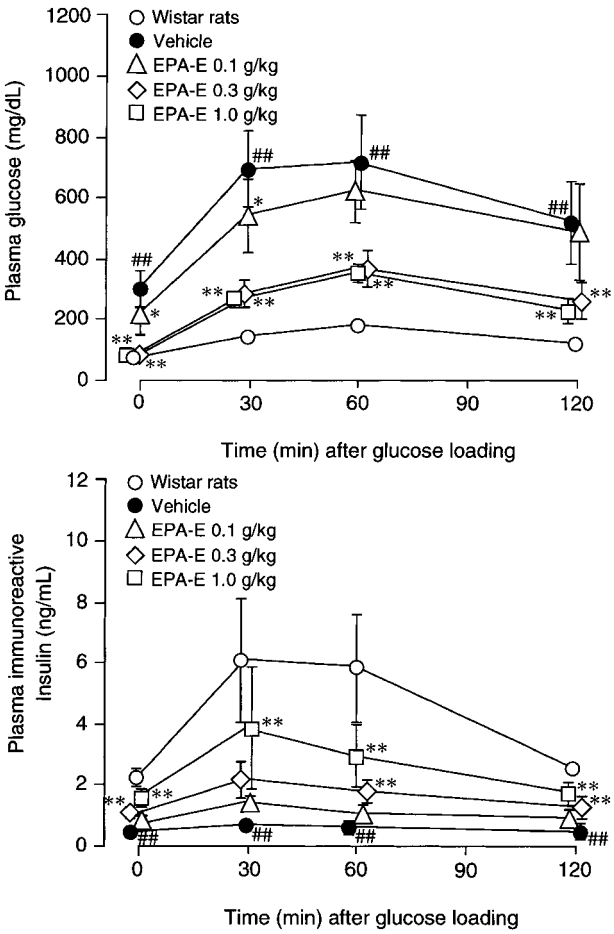

Fig 3. Plasma glucose and insulin after an oral glucose load in male WBN/Kob rats treated orally with EPA-E for 8 months. Data for each group represent the mean ± SD (n = 6). *$P < .05$, **$P < .01$ *v* vehicle (Dunnett's multiple test). ##$P < .01$ for the Wistar rats (Student's *t* test).

the selection of EPA-E dosage was as follows: the dose of 0.3 g/kg/d, previously found to be effective for suppressing the development of insulin resistance in OLETF rats and Dahl-S rats fed a high-sucrose diet,[5,6] was selected as a middle dose and 0.1 and 1.0 g/kg/d were used as a lower and higher dose, respectively. EPA-E was administered daily to the animals for 8 months via gavage using microsyringes (Gastight; Hamilton, Reno, NV). The remaining animals were treated with distilled water (1.0 mL/kg/d) for 8 months by gavage. Distilled water–treated WBN/Kob rats and Wistar rats served as the vehicle control and the normal control, respectively. Food consumption and body weight were recorded every 2 weeks throughout the experimental period.

Plasma Glucose, Insulin, and Lipid

Before treatment and after 2, 4, 6, and 8 months of treatment, heparinized blood samples were taken from a cervical vein under anesthesia with pentobarbital sodium (50 mg/kg body weight intraperitoneally) after a 4-hour fast (9 AM to 1 PM) and centrifuged at 2,200× *g* for 15 minutes at 4°C to isolate the plasma. Fasting plasma (9 AM to 1 PM) glucose was determined with an autoanalyzer (Cobas Fara; Roche, Basel, Switzerland) using the hexokinase method, and fasting plasma immunoreactive insulin was determined with a commercial enzyme-

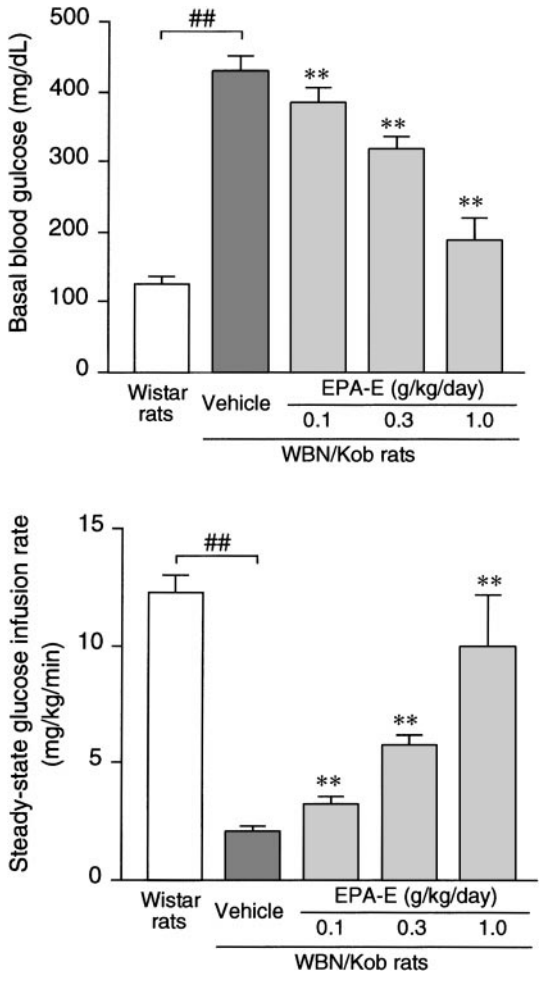

Fig 4. Insulin resistance in the euglycemic insulin-glucose clamp test in male WBN/Kob rats treated orally with EPA-E for 8 months. Each column represents the mean ± SD (n = 6). **$P < .01$ *v* vehicle (Dunnett's multiple test). ##$P < .01$ for the indicated groups (Student's *t* test).

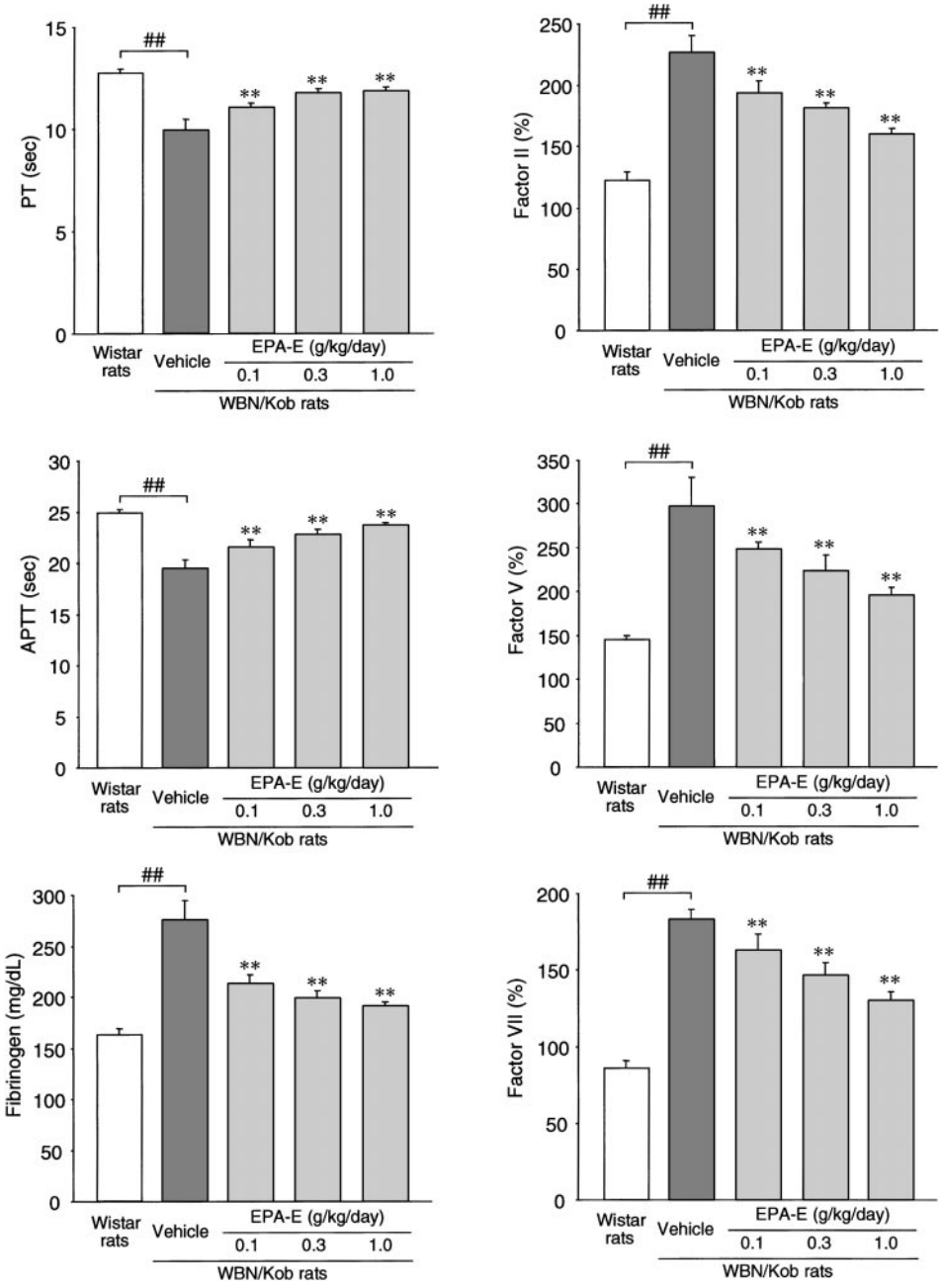

Fig 5. Coagulation time and coagulation factors in male WBN/Kob rats treated orally with EPA-E for 8 months. Each column represents the mean ± SD (n = 6). **$P < .01$ *v* vehicle (Dunnett's multiple test). ##$P < .01$ for the indicated groups (Student's *t* test).

linked immunosorbent assay ([ELISA] Lbis insulin kit; Shibayagi, Gunma, Japan). Plasma levels of total cholesterol, phospholipids, triglycerides, and free fatty acids were measured enzymatically with the autoanalyzer.

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 (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 plasma glucose by the method already described. Samples were also taken at 0, 30, 60, and 120 minutes for measurement of plasma immunoreactive insulin with the ELISA kit (Lbis insulin kit).

Euglycemic Insulin-Glucose Clamp Test

After the 8-month treatment, the hyperinsulinemic-euglycemic clamp test was performed with the method of Kergoat and Portha[10] on the other half of the rats in each group to evaluate insulin-mediated whole-body glucose uptake. After an overnight fast, the surgical treatment required for the test was performed on the animals anesthetized with an intraperitoneal injection of urethane (640 mg/kg) and α-chloralose (52 mg/kg). Then, porcine insulin (Actrapid; Novo, Copenhagen, Denmark) diluted in 1% bovine serum albumin (fatty acid–free, 97% pure; Sigma, St Louis, MO) was infused at a constant rate (10 mU/min/kg) for about 2 hours following a loading infusion at 20 mU/min/kg for 10 minutes. Five minutes after the start of the insulin infusion, an infusion of 10% glucose solution commenced, and the arterial blood glucose concentration was clamped at about 110 mg/dL

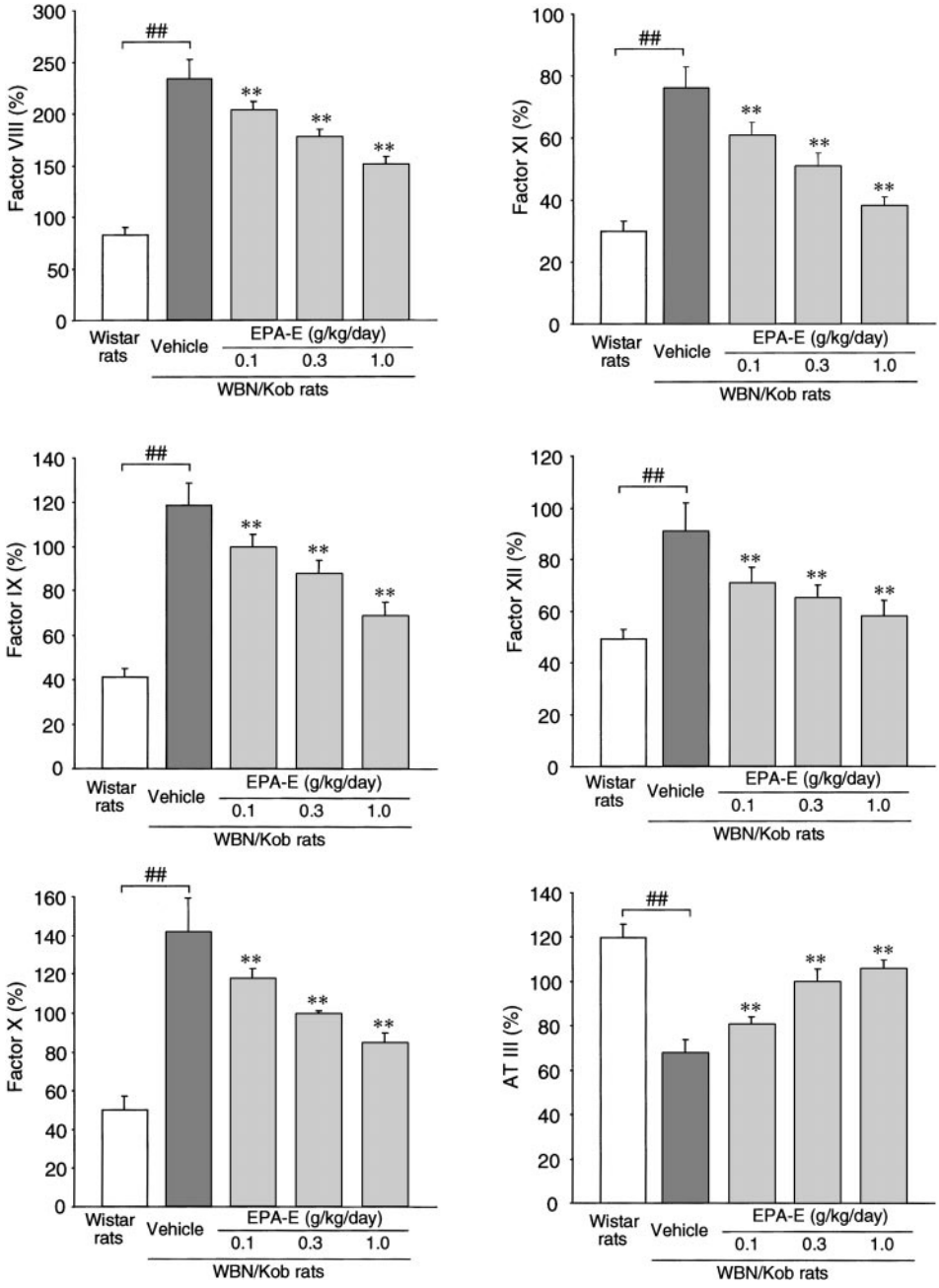

Fig 6. Coagulation factors and coagulation inhibitory factor in male WBN/Kob rats treated orally with EPA-E for 8 months. Each column represents the mean ± SD (n = 6). **$P < .01$ *v* vehicle (Dunnett's multiple test). ##$P < .01$ for the indicated groups (Student's *t* test).

by varying the rate of glucose infusion. Blood samples for determination of glucose were obtained at 5-minute intervals throughout the study and analyzed with an electrode-type glucose analyzer (ANTSENSE; Miles-Sankyo, Tokyo, Japan). Total body glucose uptake was determined as the mean glucose infusion rate (GIR) during the last 20 minutes of the study. Plasma insulin during the clamp was comparable in all groups, with a mean level of 123 ± 4 to 125 ± 5 μU/mL.

Blood Coagulation

At the end of the 8-month treatment, citrated blood samples were obtained under anesthesia with pentobarbital sodium (50 mg/kg body weight intraperitoneally) with a disposable polypropylene syringe from the abdominal aorta of the rats used for OGTT and centrifuged at 2,200× *g* for 15 minutes at 4°C to isolate the plasma. The prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen level, and plasma activity of factors II, V, VII, VIII, IX, X, XI, and XII were measured by the viscosity method using an automatic coagulation analyzer (KC-40; Heinrich Amelung, Lieme, Germany). Antithrombin III (AT III) activity was measured with the autoanalyzer with a synthetic substrate (acetyl-D-arginyl-glycyl-L-arginyl-*p*-nitroanilide dihydrochloride).

Fibrinolysis

The level of tissue-type plasminogen activator (t-PA) and activity of plasminogen, α_2-plasmin inhibitor (α_2-PI), and plasminogen activator inhibitor (PAI) in plasma samples 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 minutes at 25°C to prepare platelet-rich plasma (PRP), and an additional centrifugation at 1,600× *g* for 10 minutes at 25°C was

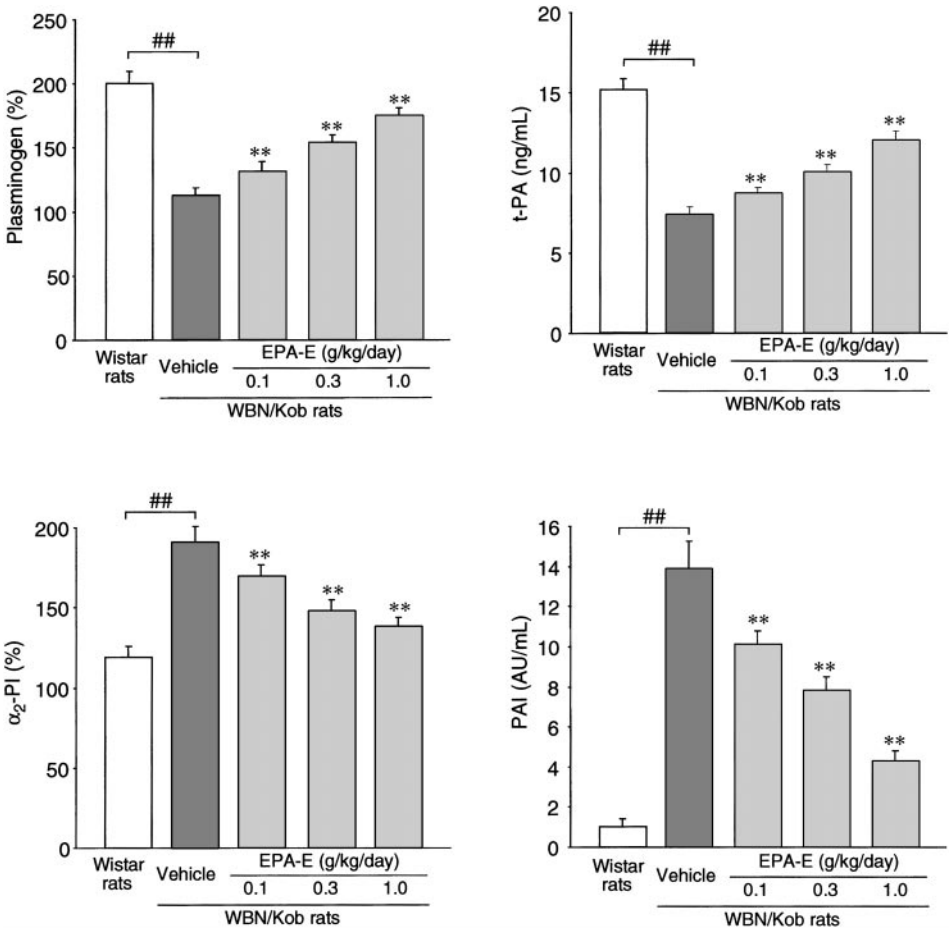

Fig 7. Fibrinolysis factors and fibrinolysis inhibitory factors in male WBN/Kob rats treated orally with EPA-E for 8 months. Each column represents the mean ± SD (n = 6). **$P < .01$ *v* vehicle (Dunnett's multiple test). ##$P < .01$ for the indicated groups (Student's *t* test).

used 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 platelet count to 2.5×10^5/μL. Aggregation in response to adenosine diphosphate ([ADP] 1 and 5 μmol/L) or collagen (0.5 and 1 μg/mL) was recorded with a platelet aggregometer (PAC-801; MC Medical, Tokyo, Japan) using a nephelometric method with PRP. Then, the maximum aggregation rate of the samples induced by ADP or collagen was calculated.

Platelet Lipid (cholesterol and phospholipid) Analysis

Two milliliters of a citrated blood sample was added to a test tube containing 160 μL 77-mmol/L EDTA-2K. The samples were then centrifuged at 400× *g* for 10 minutes at 4°C to prepare PRP, and an additional centrifugation at 2,200× *g* for 10 minutes at 4°C was used to obtain platelets. These platelets were suspended in an ice-cold buffer consisting of 0.154 mol/L NaCl, 0.154 mol/L Trishydrochloride, pH 7.4, and 77 mmol/L EDTA-2K, pH 7.4 (45:4:1 vol/vol/vol), and the suspension was centrifuged at 2,200× *g* for 10 minutes 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 lipid extraction was performed. The washed platelets were disrupted with an ultrasonicator, after which 1 mL sonicated platelet suspension was incubated with 12 mL chloroform: methanol (2:1 vol/vol) at room temperature for 60 minutes. Then, the treated suspension was incubated with 2.5 mL 0.05% sulfuric acid at room temperature for 10 minutes to extract the lipids. The solvent fraction containing lipids was isolated by centrifugation at 1,100× *g* for 15 minutes at 25°C and then dried by evaporation under vacuum. Using the extracted lipids, we determined the total cholesterol (enzymatic CES-COD-POD method) and phospholipid (permanganate salt method) content and calculated the cholesterol to phospholipid (C/P) molar ratio.

Statistical Analysis

All data are expressed as the mean ± SD. Dunnett's test[11] was used to analyze the significance of differences between the vehicle control and EPA-E–treated groups. A difference was regarded as statistically significant at a *P* level less than .05.

RESULTS

Incidence of Diabetes in Male WBN/Kob Rats

At the end of the 8-month treatment period, the incidence of diabetes in the vehicle control group was 100% (12 of 12 animals), as all animals in this group exceeded 200 mg/dL for the 4-hour fasting glucose level. On the other hand, the incidence in EPA-E groups at 0.1, 0.3, and 1.0 g/kg was 92% (11 of 12 rats), 50% (6 of 12 rats), and 17% (2 of 12 rats), respectively. Male WBN/Kob rats with a 4-hour fasting glucose greater than 200 mg/dL were characterized by a gradual increase in glucose without a reduction with aging. EPA-E significantly and dose-dependently suppressed the incidence of diabetes and the 4-hour fasting plasma glucose at a dose of 0.3 g/kg or higher compared with the vehicle control. All Wistar rats used as normal controls were nondiabetic (Fig 1).

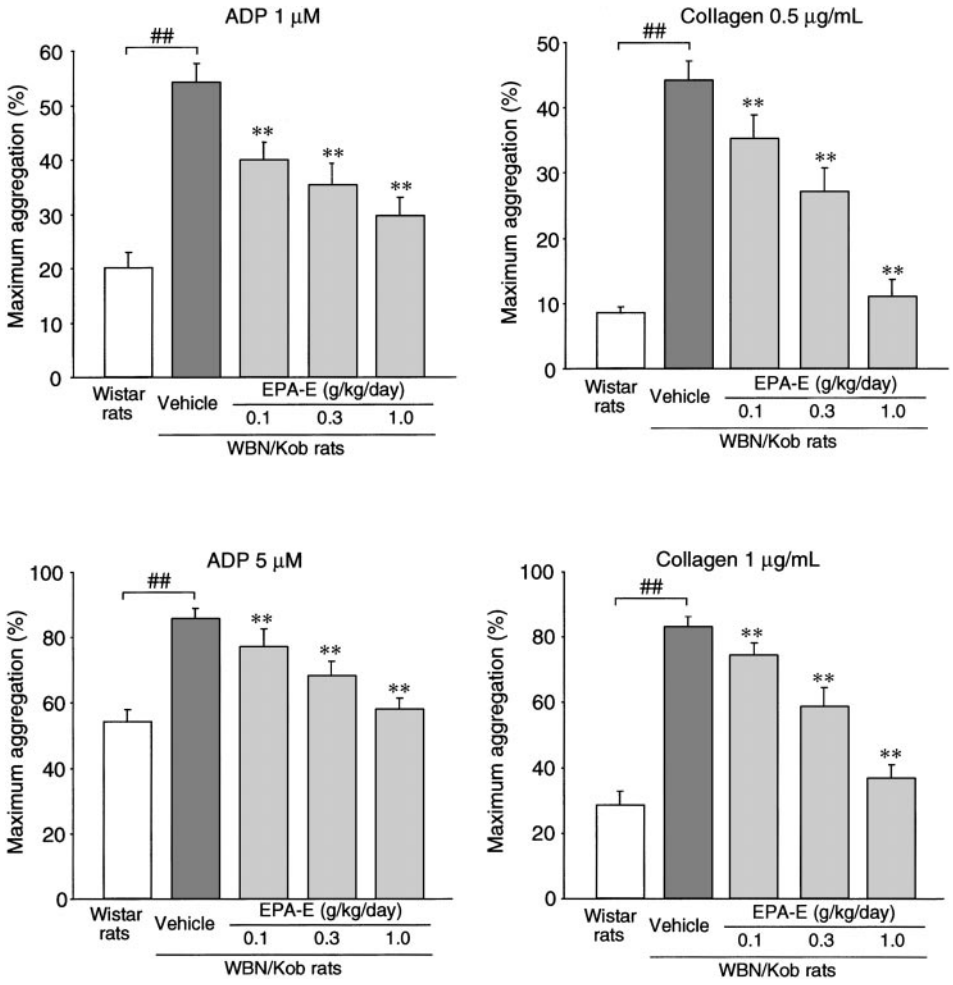

Fig 8. ADP- or collagen-induced platelet aggregation in male WBN/Kob rats treated orally with EPA-E for 8 months. Each column represents the mean ± SD (n = 6). **$P < .01$ *v* vehicle (Dunnett's multiple test). ##$P < .01$ for the indicated groups (Student's *t* test).

Plasma Insulin

Vehicle-treated WBN/Kob rats showed a significant decrease in insulin compared with Wistar rats at the end of the treatment period. EPA-E groups treated with 0.3 and 1.0 g/kg showed a significant and dose-dependent increase in insulin compared with the vehicle group (Fig 2).

Plasma Lipids

There were no significant changes in plasma lipid levels at the end of the 8-month treatment with EPA-E.

OGTT

After the 8-month treatment period, 6 of 12 diabetic rats arbitrarily selected from the vehicle control group and 6 of 11 diabetic rats, all 6 diabetic rats, and 2 diabetic plus 4 nondiabetic rats, selected from the EPA-E 0.1-, 0.3-, and 1.0-g/kg groups, respectively, were subjected to the OGTT. Also, 6 of the 12 Wistar rats were used as normal controls.

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. The Wistar rats used as normal controls had plasma glucose less than 200 mg/dL at each determination. In contrast, the EPA-E groups on 0.3 and 1.0 g/kg showed a significant and dose-dependent increase of plasma immunoreactive insulin at each point compared with the vehicle control group. There was no apparent difference between the low-dose and vehicle control groups. Wistar rats showed a prominent secretion pattern for insulin (Fig 3).

Hyperinsulinemic-Euglycemic Clamp

EPA-E groups showed a significant and dose-dependent decrease of basal blood glucose compared with the vehicle control group, and the level in Wistar rats was also significantly lower. In addition, the GIR in the vehicle control and EPA-E 0.1-, 0.3-, and 1.0-g/kg groups was 2.1 ± 0.2, 3.2 ± 0.4, 5.8 ± 0.4, and 10.0 ± 2.2 mg/kg/min, respectively. The EPA-E groups had significantly and dose-dependently higher values than the vehicle control group. The GIR in Wistar rats was 12.3 ± 0.7 mg/kg/min (Fig 4).

Blood Coagulation

The vehicle control group showed a significant decrease in PT and APTT, a significant increase in fibrinogen and factors II, V, VII, VIII, IX, X, XI, and XII, and a significant decrease in AT III activity compared with the normal control group of male Wistar rats. The EPA-E groups had significant and dose-dependent increases in PT, APTT, and AT III activity and

decreases in the other coagulation parameters compared with the vehicle control group (Figs 5 and 6).

Fibrinolysis

The vehicle control group showed a significant decrease in plasminogen and t-PA and a significant increase in α_2-PI and PAI compared with the Wistar rats. The EPA-E groups showed a significant increase in plasminogen and t-PA and a significant decrease in α_2-PI and PAI in a dose-dependent manner compared with the vehicle control group (Fig 7).

Platelet Aggregation

The vehicle control group showed a significant increase of platelet aggregation induced by ADP or collagen as compared with the Wistar rats. The EPA-E groups had a significant and dose-dependent decrease of platelet aggregation induced by ADP or collagen compared with the vehicle control group (Fig 8).

Platelet Lipid Analysis

The vehicle control group showed a significant increase of cholesterol and a significant decrease of phospholipid in platelets compared with the Wistar rats. As a result, the C/P ratio was significantly elevated in the vehicle control group. The EPA-E groups showed a significant and dose-dependent improvement in these parameters compared with the vehicle control group (Fig 9).

DISCUSSION

Highly purified EPA-E has diverse pharmacological activities that include a lipid (especially triglyceride)-lowering effect,[2-4] an antithrombotic effect (inhibitory effect on platelet aggregation[1] and thromboxane A_2 production[1]), an antiinflammatory effect (inhibitory effect on inflammatory cytokine production),[12-14] and an inhibitory effect on the proliferation of vascular smooth muscle cells.[15-17] Recently, we demonstrated that EPA-E is useful for preventing insulin resistance in OLETF rats[5] and in Dahl-S rats fed a high-sucrose diet[6] and that it improves the function of vascular endothelial cells in terms of stimulating endothelial nitric oxide synthetase activity in Dahl-S rats fed a high-sucrose diet containing 1.00% salt.[7] Thus, in the present study, we investigated the preventive effect of long-term treatment with EPA-E on the development of diabetes, insulin resistance, and abnormalities of blood coagulation in male WBN/Kob rats.

At the end of the 8-month oral treatment period, plasma glucose in the vehicle control group was 361 ± 72 mg/dL, and in the EPA-E dosage groups 0.1, 0.3, and 1.0 g/kg, it was 297 ± 72, 201 ± 98, and 136 ± 53 mg/dL, respectively. EPA-E significantly suppressed plasma glucose at a dose of 0.3 g/kg or greater, and the value at this middle dose was roughly similar to the 4-hour fasting glucose level (200 mg/dL) that is considered to indicate the diabetic state. The high-dose group was nondiabetic on the basis of the mean glucose value. In terms of individual data, all 12 WBN/Kob rats that received vehicle alone were completely diabetic. In contrast, of the 12 animals per group on oral EPA-E 0.1, 0.3, and 1.0 g/kg, 11 (92%), 6 (50%), and 2 (10%), respectively, were diabetic. The incidence of diabetes in the middle- and high-dose groups was significantly suppressed. Our data suggest first that EPA-E inhibits the

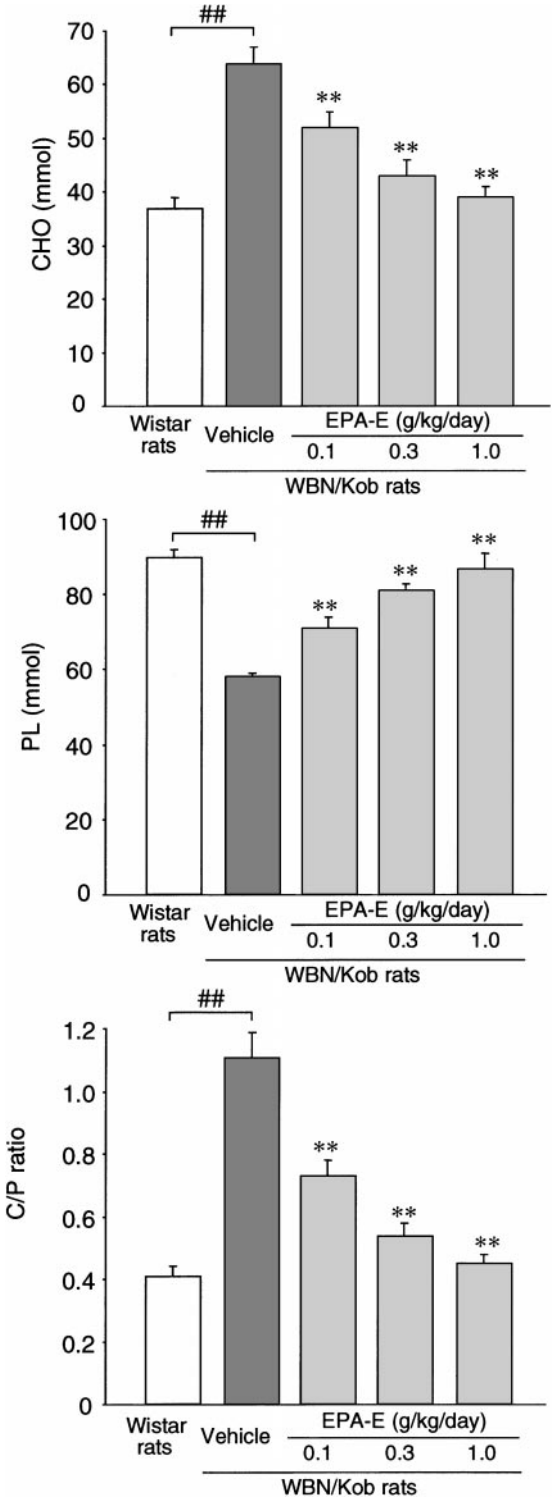

Fig 9. Cholesterol (CHO), phospholipid (PL), and C/P molar ratio in male WBN/Kob rats treated orally with EPA-E for 8 months. Each column represents the mean ± SD (n = 6). **$P < .01$ *v* vehicle (Dunnett's multiple test). ##$P < .01$ for the indicated groups (Student's *t* test).

development of diabetes in WBN/Kob rats. After the 8-month oral treatment, EPA-E caused a significant decrease in basal blood glucose and an increase in the GIR in a dose-dependent manner in the euglycemic insulin-glucose clamp test. This finding suggests that EPA-E caused an improvement of insulin resistance in WBN/Kob rats. This result agrees with the results obtained in OLETF rats,[5] Dahl-S rats fed a high-sucrose diet,[6] and Dahl-S rats fed a high-sucrose diet containing 1.00% salt.[7] In addition, there was a significant relationship ($r = -.975$, $P < .001$) between the 4-hour fasting glucose and the GIR (insulin resistance) in the euglycemic insulin-glucose clamp test. In the OGTT after the 8-month oral treatment, EPA-E at a dose of 0.3 g/kg or higher elicited a significant decrease in plasma glucose and an increase in plasma immunoreactive insulin before and 30, 60, and 120 minutes after glucose loading. This finding supports the result of the euglycemic insulin-glucose clamp test already described.

In this study, EPA-E caused a significant and dose-dependent increase in PT and APTT, decrease in fibrinogen and factor II, V, VII, VIII, IX, X, XI, and XII activity, and increase in AT III activity. These results suggest that EPA-E causes an improvement of hypercoagulation due to the diabetic state at a dose of 0.1 g/kg or higher. In addition, EPA-E treatment also caused a significant increase in plasminogen and t-PA and, conversely, a decrease in α_2-PI and PAI in a dose-dependent manner. These findings suggest that EPA-E ameliorates 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 in a dose-dependent fashion.

The above-mentioned findings suggest that EPA-E has a suppressive effect on the onset of diabetes and insulin resistance with long-term administration of 0.3 g/kg and 0.1 g/kg or higher, respectively, and a beneficial effect on blood coagulation abnormalities at 0.1 g/kg or higher in WBN/Kob rats. Also, there were significant correlations (1 or 5 μmol/L ADP, $r = .936$ or .876, respectively, $P < .001$; 0.5 or 1 μg/mL collagen, $r = .875$ or .848, respectively, $P < .001$) between the level of fibrinogen, as a representative procoagulant, and ADP- or collagen-induced platelet aggregation (1 or 5 μmol/L ADP, $r = -.911$ or $-.914$, respectively, $P < .001$; 0.5 or 1 μg/mL collagen, $r = -.916$ or $-.928$, respectively, $P < .001$) and between the level of AT III and ADP- or collagen-induced platelet aggregation. However, further studies are needed to clarify the mechanism of EPA-E action against diabetes mellitus.

In conclusion, our data demonstrate an amelioration of hyperglycemia and insulin resistance and a beneficial alteration of certain factors known to promote thrombosis and atherosclerosis in rats treated with EPA-E.

ACKNOWLEDGMENT

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