Online Supplement

Methods

Animals

Male C57BL/6J mice were purchased from CLEA (Tokyo, Japan). Seven to 9-week-old mice were housed in individual cages in a temperature-, humidity-, and light-controlled room (12-h light and 12-h dark cycle) and allowed free access to water and a PUFA-free diet (fish meal-free F1; 362 kcal/100 g, 4.4% energy as fat; Funabashi Farm, Chiba, Japan) supplemented with or without 5% EPA (wt/wt) for 1 week (EPA-treated and control groups, respectively). All diets were changed every day to avoid oxidation. At the end of the experiment, mice were injected LPS (1 mg/kg) or saline (vehicle) after overnight fast and thereafter sacrificed under anesthesia by intraperitoneal (i.p.) injection of sodium pentobarbital (1 mg/kg; Nembutal, Abbott Laboratories, Abbott Park, IL).

New En face Method for Optimal Observation of Endothelial Surface (NEMOes)

Fixation and preparation of the mouse aorta were performed as described. Briefly, the samples were incubated with anti mouse Mac-2 monoclonal antibody (Dako Corporation, Carpinteria, CA) followed by incubation with biotinylated goat anti-mouse IgG and reaction with horseradish peroxidase-conjugated streptavidin (Dako). Staining was completed after incubation with a substrate-chromogen solution and counterstaining with hematoxylin. Six to 10 pictures of each field were captured at various focal lengths with an automatically regulated Z-stepper using an Image-Pro4.5J (Planetron Co., Tokyo, Japan) to select the clearest images automatically and produce a composite image of the entire thoracic aorta.

Measurement of Serum EPA and Triglyceride Concentrations
Serum Triglyceride (TG) concentrations were measured as described. Serum EPA concentrations were determined by gas chromatography.

**Cell Culture**

Human umbilical vein endothelial cells (HUVECs) and HL60, a promyelocytic human leukocyte cell line were obtained from Sanko Junyaku (Tokyo, Japan) and American Type Culture Collection (Rockville, MD), respectively, and cultured as described.

**Monocyte Adhesion Assay**

The protocols of monocyte adhesion assay under physiological flow conditions were described. In brief, HUVEC monolayers on coverslips were treated with 1% FBS-containing RPMI-1640 medium supplemented with or without EPA (50 μmol/L) for 1 h followed by stimulation with LPS (1 μg/ml), palmitate (200 μmol/L), or tumor necrosis factor-α (TNFα) (Research and Diagnostic Systems, Minneapolis, MN) (5 ng/ml) for the indicated period. The coverslips were positioned in a parallel-plate flow chamber and mounted on an IX70 inverted microscope (Olympus, Tokyo, Japan). The flow chamber was perfused for 2 to 3 min with perfusion medium (PBS containing 0.2% human serum albumin), over which HL60 cells were suspended to achieve a final concentration of 1 × 10^5 cells/ml and drawn through the chamber. The flow rate was controlled to generate a calculated shear stress of 1.0 dyne/cm² using a PHD2000 syringe pump (Harvard Apparatus Inc., Holliston, MA). The entire period of perfusion was recorded on videotape and captured images were analyzed to determine the number of rolling and adherent HL60 cells on HUVEC monolayers in 10 randomly selected 20 × microscopic fields. Cells were considered to be “adherent” after about 10 sec of stable contact with the monolayer.

**Fluorescent Immunobinding Assay**
Fluorescent immunobinding assay was carried out as described. In brief, HUVECs were incubated with the following primary antibodies; mouse anti-human E-selectin monoclonal antibody (mAb) (7A9), anti-human ICAM-1 mAb (Hu5/3), and anti-human VCAM-1 mAb (E1/6), at a concentration of 10 μg/ml for 1 h on ice, followed by a fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 1 h on ice. Cell surface-associated fluorescence was detected using a Cytofluor II fluorescent plate reader (Applied Biosystems Inc., Foster City, CA).

**Western Blot Analysis**

Total cell lysates and nuclear extracts were prepared as described previously. In brief, 10 μg of protein obtained from 1× 10⁷ HUVECs plated in a 10 cm dish was placed in each lane in 7.5~12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blot analysis using antibodies against NF-κB p65 (p65) (Santa Cruz Biotechnology), Inhibitor of κBα (IκB-α), phospho-p65 (Ser536) (93H1) (p-p65), p38 mitogen-activated protein kinase (MAPK) (p38), phospho-p38 MAPK (Thr180/Tyr182) (p-p38) (Cell Signaling Technology Inc., Danvers, MA), Lamin A/C (Millipore, Billerica, MA), and actin. Immunoblots were developed with horseradish peroxidase-conjugated secondary antibodies and detected using an ECL Advance Western Blotting Detection Kit (GE Healthcare Bio-Sciences Corp., NJ) and observed with a LAS-3000 mini system (Fuji Photo Film, Tokyo, Japan).

**Human Study**

A total of 60 Japanese obese patients with dyslipidemia (23 men and 37 women, mean age 51.6 ± 1.8 years, mean BMI 29.8 ± 0.6 kg/m², mean hemoglobin A1c (HbA1c) 6.0 ± 0.2 %) were recruited in our clinics. They all met the metabolic syndrome (MS) criteria by the modified National Cholesterol Education Program-Adult treatment Panel III (NCEP-ATP III) definition. The study protocol was approved by the ethical
committee on human research of Kyoto Medical Center and Medical Research Institute, Tokyo Medical and Dental University, and all participants gave written informed consent.

Patients were assigned to one of the following treatment groups (a single-blind and run-in period randomization, which patients received); those treated for 3 months with either diet alone (control group) or diet plus EPA (1.8 g daily) (EPA group). At the beginning and the end of the study, we measured body mass index (BMI), waist circumference (WC), systolic blood pressure (SBP), fasting plasma glucose (FPG), plasma concentrations of total cholesterol (TC), triglyceride (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), and EPA according to the standard procedures. Plasma concentrations of sICAM-1 and sVCAM-1 were measured using commercially available immunoassays (Research and Diagnostic Systems, Minneapolis, MN). Lipid lowering medications such as statins and fibrates were excluded. Several patients in both groups had medication with oral antidiabetic agents and antihypertensive agents and there were no changes in medication during the study.

**Statistical Analysis**

Data are presented as mean ± SEM, and \( P < 0.05 \) was considered statistically significant. In cell culture and animal studies, Bartlett test was used to verify the homogeneity of variances followed by one-way ANOVA to determine the overall difference between groups. When the variance was not homogenous, the data were analyzed with Kruskal-Wallis test. If one-way ANOVA or Kruskal-Wallis test was significant, differences between individual groups were estimated using Scheffe test.
In human study, repeated measures ANOVA (control and EPA groups × before and after the treatment) was used to access the comparative effect of EPA treatment on the measured variables. A two-tailed, paired \( t \)-test was applied for the evaluation of changes from baseline conditions to those at 3 months in the same group. Comparisons of the means between the two groups at baseline or post-treatment (abbreviated as \( \Delta \)) were performed by Student’s \( t \)-test (Table 1). Changes from baseline conditions to those at 3 months were abbreviated as \( \Delta \). Multivariate regression analysis was performed to elucidate factors related to \( \Delta sICAM-1 \) and \( \Delta sVCAM-1 \) in all subjects. The following variables were assessed: age, \( \Delta WC \), \( \Delta SBP \), \( \Delta FPG \), \( \Delta TG \), \( \Delta TC \), and EPA treatment (supplemental Table I and II). All statistical analyses were performed using the Stat View program version 5.0 for Windows (SAS Institute, Cary, NC) and Excel 2003 (Microsoft, Redmond, WA) with the add-in software Statcel 2.10

References


Legends to Supplemental Figures

**Figure I. Effect of EPA on monocytes adhering to the aortic endothelium of mice 24 h after LPS treatment**

(A) Representative *en face* views of immunohistochemical staining with Mac-2 antibody for monocytes adhering to aortic endothelium of mice 24 h after i.p. injection of LPS with EPA treatment. Arrowheads denote Mac-2-positive cells. Scale bar, 100 μm.

(B) Quantification of Mac-2-positive cells. **\( P < 0.01 \). \( n = 5 \) to 7.

**Figure II. Serum concentrations of TG and EPA in mice 6 h and 24 h after LPS treatment**

(A) Serum EPA concentrations in mice 6 h and 24 h after i.p. injection of LPS with EPA treatment. \( n = 4 \) to 5. (B) Serum TG concentrations in mice 6 h after i.p. injection of LPS with EPA treatment. \( n = 6 \) to 7. (C) Serum TG concentrations in mice 24 h after i.p. injection of LPS with EPA treatment. \( n = 4 \) to 9. *\( P < 0.05 \) and **\( P < 0.01 \).

**Figure III. Representative movies of LPS-induced monocyte rolling and adhesion in vitro**


**Figure IV. Effect of EPA on palmitate-induced monocyte adhesion in HUVECs**

The number of adhesive monocytes on HUVECs 4 h and 24 h after palmitate treatment with EPA treatment under physiological flow conditions. **\( P < 0.01 \). \( n = 10 \) high power fields. Data are representative of 3 separate experiments.

**Figure V. Effect of EPA on TNFα-induced monocyte adhesion and expression of**
endothelial adhesion molecules in HUVECs

(A) The number of rolling and adhesive monocytes on HUVECs 24 h after TNFα treatment with EPA treatment under physiological flow conditions. ** $P < 0.01$. $n = 10$ high power fields. Data are representative of 3 separate experiments. (B) Endothelial surface expression of ICAM-1, VCAM-1, and E-selectin in HUVECs 24 h after TNFα treatment with EPA treatment. ** $P < 0.01$. $n = 3$. Data are representative of 3 separate experiments.
Table I. Multivariate regression analysis for ΔsICAM-1 with the metabolic variables as independent variables

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<th>Partial correlation coefficient</th>
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R2 = 0.224  F = 2.15  R2 = 0.107  F = 6.97
Table II. Multivariate regression analysis for ΔsVCAM-1 with the metabolic variables as independent variables

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R2 = 0.177    F = 1.60    R2 = 0.129    F = 8.61