In Vivo and In Vitro Inhibition of Monocyte Adhesion to Endothelial Cells and Endothelial Adhesion Molecules by Eicosapentaenoic Acid

Hideto Yamada, Masayuki Yoshida, Yasutaka Nakano, Takayoshi Suganami, Noriko Satoh, Tomoya Mita, Kosuke Azuma, Michiko Itoh, Yukio Yamamoto, Yasutomi Kamei, Minoru Horige, Hirotaka Watada, Yoshihiro Ogawa

Objective—A large-scale, prospective, randomized clinical trial has recently revealed that the addition of highly purified eicosapentaenoic acid (EPA) to low-dose statin therapy significantly reduces the incidence of major coronary events. Here we investigated in vivo and in vitro effect of EPA on monocyte adhesion to endothelial cells and adhesion molecules.

Methods and Results—A new en face immunohistochemistry of endothelial surface in combination with confocal microscopy revealed marked reduction of lipopolysaccharide (LPS)-induced monocyte adhesion to the aortic endothelium in parallel with the suppression of vascular cell adhesion molecule 1 (VCAM-1) and nuclear translocation of nuclear factor-κB p65 in EPA-treated mice relative to vehicle-treated groups. In an in vitro adhesion assay system under physiological flow conditions, EPA inhibited LPS-induced monocyte adhesion and endothelial adhesion molecules. We found significant decrease in plasma concentrations of soluble intercellular adhesion molecule 1 (sICAM-1) and sVCAM-1 in patients with the metabolic syndrome after a 3-month administration of highly purified EPA (1.8 g daily). Multivariate regression analysis revealed that EPA administration is the only independent determinant of sICAM-1 and sVCAM-1.

Conclusions—This study provides evidence that EPA inhibits monocyte adhesion to endothelial cells in parallel with the suppression of endothelial adhesion molecules in vivo and in vitro. (Arterioscler Thromb Vasc Biol. 2008;28:2173-2179.)

Key Words: adhesion molecules ■ endothelial cells ■ EPA ■ monocytes ■ TLR4

Obesity may be viewed as a state of chronic low-grade inflammation and confers a higher risk of atherosclerotic diseases.¹ There is considerable evidence that obese adipose tissue is markedly infiltrated by macrophages,²,³ suggesting that they may participate in the inflammatory pathways that are activated in obese adipose tissue. Using an in vitro coculture system composed of adipocytes and macrophages, we have demonstrated that saturated fatty acids released from adipocytes via the macrophage-induced lipolysis serve as a naturally occurring ligand for Toll-like receptor 4 (TLR4) to induce the inflammatory changes in macrophages through nuclear factor-κB (NF-κB) activation.⁴,⁵ Notably, n-3 polyunsaturated fatty acids (n-3 PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid are unable to activate macrophages and can even antagonize the proinflammatory effect of saturated fatty acids or LPS,⁶ a well-defined exogenous ligand for TLR4,⁷ thereby highlighting the anti-inflammatory effect of n-3 PUFAs.

Atherosclerosis is a complex pathological process that is associated with vascular wall dysfunction and inflammation.⁸ The monocyte-endothelial cell interaction may play a crucial role in atherosclerotic plaque formation.⁹ Indeed, the adhesion of circulating monocytes to the intimal endothelial cell monolayer is thought to be one of the earliest events, which is mediated through complex interactions among multiple adhesion molecules and their counterreceptors expressed by both endothelial cells and monocytes, such as selectins, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and β1 and β2-integrins.¹⁰ Several previous reports indicated that the TLR4/NF-κB signaling pathway in both endothelial cells and monocytes/macrophages is involved in the development of vascular dysfunction and atherosclerosis.¹¹-¹³ and expression of adhesion molecules is regulated through NF-κB activation.¹⁴ Therefore, modulation of monocyte adhesion to the vascular endothelium may represent an attractive therapeutic target for atherosclerosis.

In epidemiological and clinical trials, fish oil rich in n-3 PUFAs or n-3 PUFAs have reduced the incidence of coronary...
heart disease. A large-scale, prospective, randomized clinical trial, or the Japan EPA Lipid Intervention Study (JELIS), has demonstrated that the addition of highly purified EPA, the only class of n-3 PUFAs used clinically to treat hyperlipidemia, to low-dose statin therapy significantly reduces the incidence of major coronary events, suggesting the pleiotropic effect of EPA in addition to its well-known lipid lowering effect. It is, therefore, tempting to speculate the beneficial effect of n-3 PUFAs on monocyte-endothelial cell interactions during the course of atherosclerosis. Indeed, a couple of reports showed that n-3 PUFAs inhibit monocyte–endothelial cell interactions in vitro; however, there is no direct evidence for the inhibitory effect of EPA on monocyte-endothelial cell adhesion and endothelial adhesion molecules in vivo. Moreover, there has been no report showing the inhibitory effect of EPA on endothelial adhesion molecule expression in patients with the metabolic syndrome, a major risk of atherosclerotic diseases.

Using a new en face immunohistochemistry of endothelial surface in combination with confocal microscopy, we demonstrate for the first time that EPA markedly inhibits LPS-induced monocyte adhesion to the mouse aortic endothelium in parallel with the suppression of endothelial adhesion molecules and nuclear translocation of NF-κB p65 in vivo. The inhibitory effect is also confirmed under physiological flow conditions in vitro. We also show that highly purified EPA significantly reduces soluble ICAM-1 (sICAM-1) and soluble VCAM-1 (sVCAM-1) in patients with the metabolic syndrome. This study provides in vivo and in vitro evidence that EPA inhibits monocyte adhesion to endothelial cells in parallel with the suppression of endothelial adhesion molecules.

Materials and Methods

Materials
Preparation and characterization of EPA used in vivo and in vitro were reported elsewhere. All other reagents were purchased from Sigma and Nacalai Tesque unless otherwise noted.

Animals
All animal experiments were conducted in accordance to the guidelines of Tokyo Medical and Dental University Committee on Animal Research (No. 0060026). Details are described in supplemental Methods (available online at http://atvb.ahajournals.org/).

New En Face Method for Optimal Observation of Endothelial Surface
Details are described in supplemental Methods.

En Face Analysis of Adhesion Molecule and NF-κB Activation on Aortic Endothelial Surface
After fixation, the aorta was dissected carefully from the aortic arch to lower thoracic region and was immersed in 10% buffered formalin for 1 hour at 4°C. The thoracic aorta was removed of fat and cut open longitudinally along the ventral side with scissors and rinsed 3 times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBS-T). The samples were incubated overnight at 4°C with PBS-T containing antiamouse VCAM-1 and NF-κB p65 (p65) polyclonal antibodies (Santa Cruz Biotechnology Inc; 1:100) after 30 minutes blocking with PBS-T containing 10% donkey serum, and then rinsed 3 times with PBS-T. It was followed by incubation with PBS-T containing the secondary donkey antibody labeled with Alexa Fluor 488 and 594 fluorophore molecules (Molecular Probes Inc; 1:600) for 1 hour at room temperature. The samples were rinsed 3 times with PBS-T, placed on a slide glass with the intimal side up, and covered with VECTORSHIELD Mounting Medium with DAPI (Vector Laboratories Inc). Each sample was viewed immediately with a FV1000 confocal system (Olympus). Three to 6 pictures of each field were captured at various focal lengths and counted as positive cells per 1000 endothelial cells.

Details regarding cell culture, monocyte adhesion assay, fluorescent immunobinding assay, western blot analysis, human study, statistical analysis are described in supplemental Methods.

Results

Effect of EPA on LPS-Induced Monocyte Adhesion to Aortic Endothelium and Endothelial Expression of Adhesion Molecules In Vivo
Monocyte accumulation and atherosclerotic lesion formation are known to occur reproducibly at specific sites in the arterial tree, such as arterial branches. Using a newly developed new en face method for optimal observation of endothelial surface (NEMOes), we examined the effect of EPA on LPS-induced monocyte adhesion to the endothelial surface surrounding the orifice of intercostal arteries of thoracic aorta in C57BL/6J mice. The number of monocytes adhering to the aortic endothelium was significantly increased in LPS-treated mice relative to vehicle-treated mice (P<0.01; Figure 1 and supplemental Figure I). The inhibitory effect tended to be observed up to 24 hours after LPS treatment (supplemental Figure I). In this study, serum EPA concentrations in EPA-treated and control groups were consistent with previous reports (supplemental Figure II). Serum TG concentrations tended to be decreased by EPA treatment, although not statistically significant (supplemental Figure IIB and IIC).

Confocal microscopic analysis of the aortic endothelium revealed that endothelial surface expression of VCAM-1 is significantly increased in LPS-treated mice relative to vehicle-treated mice (P<0.01; Figure 2), which is significantly inhibited by EPA treatment (P<0.01; Figure 2).

Effect of EPA on LPS-Induced Monocyte Adhesion to Endothelial Cells and Endothelial Expression of Adhesion Molecules In Vitro
Using an in vitro adhesion assay system under physiological flow conditions, we examined the effect of EPA on monocyte-endothelial interaction; monocyte rolling and adhesion on the HUVEC monolayers (Figure 3A and supplemental Figure III). The monocyte rolling and adhesion were significantly increased in LPS-treated HUVECs relative to vehicle-treated HUVECs (P<0.01; Figure 3A), which were significantly inhibited in HUVECs treated with EPA (P<0.01; Figure 3A). Palmitate, a major saturated fatty acid which can activate the NF-κB pathway via TLR4, was also used to induce monocyte-endothelial interaction. Interestingly, monocyte adhesion was significantly increased in palmitate-treated HUVECs relative to vehicle-treated HUVECs 4 hours after palmitate treatment (P<0.01; supplemental Figure IV), which was significantly inhibited by EPA treatment (P<0.01; supplemental Figure IV). The tendency was observed up to 24 hours after palmitate treatment.
We also found that the monocyte rolling and adhesion are significantly increased in tumor necrosis factor (TNF) \( \alpha / \beta \)-treated HUVECs relative to vehicle-treated HUVECs \( P<0.01; \) supplemental Figure VA), which is significantly inhibited in HUVECs treated with EPA \( P<0.01; \) supplemental Figure VA).

Fluorescent immunobinding assay revealed that expression of ICAM-1 and VCAM-1 is significantly increased in LPS-treated HUVECs relative to vehicle-treated HUVECs \( P<0.01; \) supplemental Figure VA), which is significantly inhibited in HUVECs treated with EPA \( P<0.01; \) supplemental Figure VA).

Effect of EPA on LPS-Induced Intracellular Signal Transduction In Vitro and In Vivo

Western blot analysis showed that the amount of Inhibitor of \( \kappa \)B (I\( \kappa \)B-\( \alpha \)) protein is reduced in total cell lysates in parallel with the increase in phospho-p65 (p-p65) and p65 in nuclear extracts from LPS-treated HUVECs relative to vehicle-treated HUVECs (Figure 4A and 4B). The LPS-induced decrease in I\( \kappa \)B-\( \alpha \) and increase in nuclear p-p65 and p65 was reversed in HUVECs treated with EPA (Figure 4A and 4B). We also found that phospho-p38 mitogen-activated protein kinase (p-p38) is increased in total cell lysates from LPS-treated HUVECs relative to vehicle-treated HUVECs, which is inhibited by EPA treatment (Figure 4C).

We also examined whether EPA reduces LPS-induced increase in nuclear translocation of p65 in vivo. Confocal microscopic analysis revealed that nuclear translocation of p65 in endothelial cells is significantly increased in parallel with the increase in VCAM-1 expression in LPS-treated group relative to vehicle-treated group \( P<0.05; \) Figure 5A and 5B). The LPS-induced increase in nuclear translocation of p65 was significantly inhibited in parallel with VCAM-1 expression in EPA-treated group relative to vehicle-treated group \( P<0.01; \) Figure 5A and 5B).
A

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EPA (-)  EPA (+)

Vehicle LPS Vehicle LPS

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Vehicle LPS Vehicle LPS

Figure 3. Effect of EPA on LPS-induced monocyte adhesion and expression of endothelial adhesion molecules in HUVECs. A, The number of rolling and adhesive monocytes on HUVECs 24 hours after LPS 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 hours after LPS treatment with EPA treatment. **P<0.01. n=3. Data are representative of 3 separate experiments.

Effect of EPA on Soluble Adhesion Molecules in Humans

To obtain insight into the effect of EPA on monocyte adhesion to endothelial cells in humans, we examined whether highly purified EPA decreases plasma concentrations of sICAM-1 and sVCAM-1 in patients with the metabolic syndrome. There was no significant difference in the measured variables tested between EPA and control groups before EPA administration (Table). After a 3-month EPA administration, plasma EPA concentrations were significantly increased in EPA group relative to control group (P<0.01). In EPA group, plasma triglyceride (TG) concentrations were significantly decreased relative to control group (P<0.01), although there were no difference in body mass index (BMI), waist circumference (WC), systolic blood pressure (SBP), fasting plasma glucose (FPG), plasma concentrations of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C). Both plasma sICAM-1 and sVCAM-1 concentrations were significantly reduced after EPA administration (P<0.01), but unchanged in control group (Table). To determine the factors independently influencing the changes in plasma sICAM-1 and sVCAM-1 concentrations, multivariate regression analysis was performed (supplemental Tables I and II). Plasma sICAM-1 and sVCAM-1 concentrations were inversely correlated only with EPA treatment.

Discussion

Using NEMOes in combination with confocal microscopic analysis of en face aortic endothelium, we demonstrated for the first time that EPA markedly inhibits LPS-induced monocyte adhesion to the aortic endothelium, nuclear translocation of NF-κB p65, and endothelial surface expression of VCAM-1 in mice. The new technique, originally described by Verna et al., will provide a unique opportunity to study monocyte adhesion to and intracellular signal transduction in endothelial cells in vivo. There is a report by Sethi et al that oxidized EPA is able to inhibit LPS-induced monocyte rolling and adhesion in the mouse mesenteric venules in vivo. However, whether EPA inhibits monocyte adhesion to the aortic endothelium under higher shear stress has never been addressed. To our knowledge, this study is the first in vivo observation that EPA inhibits monocyte adhesion to the aortic endothelial cells and reduces aortic endothelial expression of adhesion molecules. These observations are consistent with a previous report by Matsumoto et al that a long-term EPA treatment reduces atherosclerotic plaque formation during the late phase of atherosclerosis in ApoE-deficient mice. Furthermore, we found that LPS-induced monocyte adhesion tends to be reduced with coadministration of EPA and statins relative to administration of statins alone in vitro and in vivo (unpublished data Yamada H, Yoshida M, Watada H, Ogawa Y, 2008). These observations support the concept that EPA is able to delay and prevent the progression of atherosclerosis at both the early and late phases of atherosclerosis beyond its lipid lowering effect. Using the in vitro adhesion assay system, we previously demonstrated that pharmacological inhibition of endothelial adhesion molecules results in the reduction of monocyte-endothelial cell adhesion. In this study, we found that EPA results in marked inhibition of LPS-induced increase in monocyte adhesion in parallel with the
suppression of endothelial adhesion molecules and NF-κB activation in HUVECs. The LPS-induced increase in nuclear translocation of NF-κB p65 is also inhibited in mice treated with EPA in vivo. Although EPA may also act on circulating monocytes and thus modulate their adhesion to endothelial cells in vivo, it is likely that EPA inhibition of monocyte adhesion to endothelial cells is mediated at least in part through the suppression of endothelial adhesion molecules.

The molecular mechanism by which EPA reduces expression of endothelial adhesion molecules is unclear at present. In this study, we demonstrated that EPA inhibits LPS-induced nuclear translocation of p65 in endothelial cells both in vivo and in vitro, which is consistent with our previous observation that EPA suppresses NF-κB activity in macrophages treated with LPS or saturated fatty acids.6 We also found that EPA inhibits LPS-induced increase in p-p38, which is known to mediate NF-κB activation.14 It is, therefore, conceivable that the upstream signaling of IκB-α and p38 is important for the antiinflammatory effect of EPA. There is a report suggesting that peroxisome proliferator-activated receptor (PPAR) α interacts with p65 and interferes with NF-κB activation.28 Indeed, Sethi et al reported that an i.p. injection of oxidized EPA suppresses monocyte rolling and adhesion to the venous endothelium via a PPARα-dependent pathway.23

In this regard, PPARγ has been shown to export NF-κB p65 from the nucleus via direct protein–protein interaction, thus attenuating NF-κB activity.29 Moreover, a PPARγ antagonist GW9662 has reversed EPA-induced inhibition of ICAM-1 mRNA expression in endothelial cells in vitro.30 However, we found that GW9662 fails to reverse the EPA-induced inhibition of monocyte adhesion in the in vitro adhesion assay system (unpublished data Yamada H, Yoshida M, Ogawa Y, 2008). On the other hand, Arita et al demonstrated that resolvinE1, an endogenous lipid mediator derived from EPA, exerts an antiinflammatory effect through interaction with leukotriene B4 receptor BLT1 and chemerin receptor ChemR23.30 Further studies are required to elucidate how EPA inhibits the monocyte-endothelial interaction.

Evidence has accumulated indicating that elevated levels of circulating free fatty acids, especially saturated free fatty acids derived from dietary animal fat or via the macrophage-induced adipocyte lipolysis in obese adipose tissue, may potentially contribute to the development of the inflammatory changes and dysfunction in the vascular wall.31–33 For instance, Kim et al recently reported that a high-fat diet induces vascular insulin resistance and inflammatory responses by signaling through the TLR4/NF-κB pathway and suggested that such effect is mediated at least in part by saturated free fatty acids.34 In this study, we also found that palmitate induces monocyte adhesion to endothelial cells in the in vitro adhesion assay system, which is inhibited by EPA treatment. These observations, taken together, suggest that the TLR4/NF-κB pathway plays a role in the deleterious effect of saturated free fatty acids on monocyte-endothelial cell interaction in the vasculature. The above discussion supports the concept that antagonism of the TLR4/NF-κB pathway in vascular wall offers a novel therapeutic strategy to prevent or treat obesity-related atherosclerotic diseases. Using the in vitro coculture of adipocytes and macrophages, we have demonstrated that EPA reverses the coculture-induced reduc-

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**Table. Clinical Characteristics and Metabolic Parameters Before and After the Follow-Up Period**

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>EPA Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>11/19</td>
<td>12/18</td>
</tr>
<tr>
<td>Age</td>
<td>52.9±2.75</td>
<td>50.3±2.48</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>29.7±0.79</td>
<td>29.7±0.79</td>
</tr>
<tr>
<td>WC, cm</td>
<td>94.7±2.02</td>
<td>95.2±2.04</td>
</tr>
<tr>
<td>SBP, mm Hg</td>
<td>136±3.42</td>
<td>133±3.26</td>
</tr>
<tr>
<td>FPG, mg/dl</td>
<td>115±4.94</td>
<td>120±7.43</td>
</tr>
<tr>
<td>TC, mg/dl</td>
<td>203±5.52</td>
<td>206±5.66</td>
</tr>
<tr>
<td>TG, mg/dl</td>
<td>164±13.1</td>
<td>159±12.4</td>
</tr>
<tr>
<td>HDL-C, mg/dl</td>
<td>57.2±2.57</td>
<td>54.2±2.97</td>
</tr>
<tr>
<td>LDL-C, mg/dl</td>
<td>125±5.31</td>
<td>128±5.31</td>
</tr>
<tr>
<td>EPA, µg/ml</td>
<td>73.0±10.1</td>
<td>69.3±7.76</td>
</tr>
<tr>
<td>sICAM-1, ng/ml</td>
<td>210±9.66</td>
<td>210±10.2</td>
</tr>
<tr>
<td>sVCAM-1, ng/ml</td>
<td>622±38.3</td>
<td>624±38.8</td>
</tr>
</tbody>
</table>

Data are expressed as the mean±SEM. **P<0.01 vs before determined by 2-tailed, paired t test. †P<0.01 vs control determined by Student t test.
tion of adiponectin secretion at least in part by suppressing the saturated fatty acids/TLR4/NF-κB pathway in macrophages and suggested that the beneficial effect of EPA is attributable at least partly to the improvement of obesity-induced adipose tissue inflammation. It is, therefore, conceivable that EPA reduces the incidence of obesity-induced atherosclerotic diseases through the suppression of adipocyte-macrophage interaction in the adipose tissue and monocyte-endothelial interaction in the vascular tissue.

Soluble forms of endothelial adhesion molecules have been clinically used as surrogate markers to reflect endothelial surface expression of adhesion molecules. There are previous reports showing that treatment with n-3 PUFAs reduces significantly sICAM-1 in patients with dyslipidemia. However, whether n-3 PUFAs have an inhibitory effect on endothelial expression of adhesion molecules in the metabolic syndrome has not been addressed so far. In this study, we demonstrated for the first time that highly purified EPA decreases both plasma sICAM-1 and sVCAM-1 concentrations in patients who met the criteria of the metabolic syndrome or a higher risk group of atherosclerosis. Indeed, we also confirmed that cardiac-ankle vascular index, a new index of arterial stiffness independent of blood pressure, and other biomarkers such as adiponectin and high-sensitivity C-reactive protein are significantly improved relative to control group as short as 3-month EPA administration (unpublished data Satoh N, Ogawa Y, 2008). These observations, taken together, suggest that the inhibitory effect of EPA on monocyte-endothelial interaction may also contribute to the marked antiatherogenic effect of EPA in patients at higher risk of atherosclerosis. In this study, multivariate regression analysis revealed that EPA reduces both sICAM-1 and sVCAM-1 independently of other metabolic parameters. These observations support the concept that EPA acts directly on endothelial cells to reduce levels of endothelial adhesion molecules and prevents the progression of atherosclerosis and major coronary events. However, this study does not exclude the possibility that the EPA-induced reduction in TG influences the reduction in soluble adhesion molecules.

In conclusion, this study provides in vivo and in vitro evidence that EPA inhibits monocyte adhesion to endothelial cells in parallel with the suppression of endothelial adhesion molecules, thereby highlighting the pleiotropic effect of EPA. Given the potentially important role of monocyte adhesion in the pathogenesis of atherosclerosis, the data of this study could be relevant to the protective effects of EPA in patients at risk for cardiovascular disease.

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**Disclosures**

None.

**References**


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Figure I

(A) LPS (-) LPS (+)

EPA (-) EPA (+)

(B) **

- **EPA (-)**
- **EPA (+)**

Vehicle LPS

(Cell / mm²)
Figure II

(A) EPA

Vehicle  
LPS

0  100  200  300  (µg/ml)

EPA (-)  EPA (+)

(B) TG

Vehicle  
LPS

0  40  80  120 (mg/dl)

EPA (-)  EPA (+)

(C) TG

Vehicle  
LPS

0  40  80  120 (mg/dl)

EPA (-)  EPA (+)
Figure III

Flow assay (Movie)

LPS (-)                      LPS (+)

EPA (-)                      EPA (+)

Movie I                      Movie III

Movie II                     Movie IV
Figure IV

Adhesion (HL60 cells / HPF)

4 h

** **

24 h

EPA (-) (-) (+) Vehicle Palmitate

EPA (-) (-) (+) Vehicle Palmitate
Figure V

(A)

![Graph showing Rolling and Adhesion](image)

(B)

![Graph showing ICAM-1, VCAM-1, and E-selectin](image)
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 $1 \times 10^5$ cells/ml and drawn through the chamber. The flow rate was controlled to generate a calculated shear stress of 1.0 dyne/cm$^2$ 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.\textsuperscript{5,7} 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.\textsuperscript{7} In brief, 10 μg of protein obtained from 1× 10\textsuperscript{7} 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/m\textsuperscript{2}, 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.\textsuperscript{8} 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 Δ) were performed by Student’s t-test (Table 1). Changes from baseline conditions to those at 3 months were abbreviated as Δ. Multivariate regression analysis was performed to elucidate factors related to ΔsICAM-1 and ΔsVCAM-1 in all subjects. The following variables were assessed: age, ΔWC, ΔSBP, ΔFPG, ΔTG, ΔTC, 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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$R^2 = 0.177$  $F = 1.60$  $R^2 = 0.129$  $F = 8.61$