Eicosapentaenoic Acid Suppresses Basal and Insulin-Stimulated Endothelin-1 Production in Human Endothelial Cells

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 cis-Polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) are the major fatty acids contained in fish oil, and are known to affect the various physiological properties of cell membranes in humans. The present study investigated the effects of polyunsaturated fatty acids on endothelin-1 (ET-1) production in human umbilical vein endothelial cells (HUVECs) and on insulin activity. After addition of various concentrations of EPA, docosahexaenoic acid, arachidonic acid, or linoleic acid to a culture medium, the concentration of ET-1 was measured using ELISA, and that of ET-1 mRNA was determined by RT-PCR. The results showed that EPA had the strongest inhibitory effect \((p < 0.05)\) on both basal ET-1 production and ET-1 mRNA levels. In addition, insulin \((10^{-7} \text{mol/l})\) markedly increased ET-1 production, and EPA also significantly decreased the effect induced by insulin. Pretreatment with \(\text{Ca}^{2+}\) chelator EGTA \((1 \text{mmol/l})\), NOS inhibitor L-NAME \((300 \text{μmol/l})\), or calmodulin antagonist W-7 \((300 \text{μmol/l})\) inhibited NO production by EPA \((100 \text{μmol/l})\), but these pretreatments had no effect on ET-1 production by EPA. These findings suggest that EPA reduces basal and insulin-enhanced ET-1 production by inhibiting ET-1 mRNA production. These effects of EPA may contribute to its vasorelaxant and anti-atherosclerotic effects. (Hypertens Res 2003; 26: 655–661)

Key Words: eicosapentaenoic acid, endothelin-1, insulin, human endothelial cells

Introduction

Fatty acids affect various cellular events, such as neutrophil activation (1), platelet aggregation (2, 3), and cell growth and differentiation (4). Polyunsaturated fatty acids are major components of phospholipids, the fundamental structural unit of biological membranes, and play a key role in membrane function (5). Omega-3 polyunsaturated fatty acids, such as the eicosapentaenoic acid (EPA, C20:5 \(\omega 3\)) and docosahexaenoic acid (DHA, C22:6 \(\omega 3\)) contained in fish oil, are \(\text{cis}\)-form and modulate the composition of the fatty acid moiety of membrane phospholipids in tissues; in addition, they may influence the activity of membrane-bound proteins.

Dietary supplementation with fish oil reduces the incidence of coronary heart disease and atherosclerosis (6, 7), and also exerts antihypertensive (8, 9), hypolipidemic (10, 11) and cardioprotective effects (12). Several mechanisms have been postulated to explain the antihypertensive effects of \(\omega 3\) fatty acids. Diets enriched with fish oil facilitate endothelium-dependent relaxation and reduce endothelium-dependent contractions in atherosclerotic, hypercholesterolemic, and normal arteries (13, 14). Moreover, fish oil shifts the balance of constrictor/dilator prostaglandins to favor vasodilation (15).

Omega 3 fatty acids have been reported to inhibit the contraction of rat aortic rings induced by agonists such as \(\alpha\)-adrenoceptor agonists and angiotensin II (16), and a diet rich in EPA can decrease systemic vascular reactivity to nor-
adrenaline and lower systemic blood pressure in humans (17). Long term treatment with EPA improves exercise-induced vasodilation in patients with coronary artery disease (18), and EPA may lower blood pressure by altering the activities of the membrane sodium transport systems (19).

Endothelial cells in vascular walls are known to regulate vascular tone under normal and various pathophysiological conditions. The normal endothelium produces relaxing factors that play an important role in maintaining homeostasis of the blood vessel wall (20, 21). One of these important endothelium-derived relaxing factors has been identified as nitric oxide (NO) (22). On the other hand, endothelin-1 (ET-1), a 21 amino acid peptide, is a potent vasoconstrictor and pressor substance released into the supernatant of cultured endothelial cells (23), and exerts a wide variety of biological effects in different tissues. Although ET-1 is produced by various types of cells, including vascular smooth muscle cells, vascular endothelial cells are the major sources of ET-1. Increased circulating concentrations of ET-1 have been found in hypertensive and renal disorders (23). Various substances, such as insulin (24), lipopolysaccharides (25), tumor necrosis factor (26), interleukin 1-γ, and transforming growth factor-β (TGF-β) (27), are known to stimulate ET-1 production in endothelial cells. On the other hand, bendipine inhibits the expression of ET-1 and TGF-β1 in Dahl salt-sensitive hypertensive rats (28). In cultured bovine mesangial cells (29), EPA has been reported to inhibit mitogen-induced ET-1 production, but the effects of EPA on human endothelial cells and its underlying mechanism remain to be clarified.

Therefore, the purpose of the present study was to examine the effects of EPA on the production of ET-1 by vascular endothelial cells, and compare them with the effects of other polyunsaturated fatty acids. We also investigated the influence of EPA on the effects of insulin on ET-1 production. Our results showed that EPA inhibits ET-1 production, and also modulates the effects of insulin on ET-1 production in human vascular endothelial cells.

**Methods**

**Materials**

HEPES buffered saline solution, trypsin-EDTA, and trypsin-neutralizing solution were purchased from BioWhittaker, Inc. (Walkersville, USA). cis-5,8,11,14,17-Eicosapentaenoic acid (sodium salt, EPA), cis-4,7,10,13,16,19-docosahexaenoic acid (sodium salt, DHA), arachidonic acid (sodium salt, AA), linoleic acid (sodium salt, LNA), oleic acid (sodium salt, OA), ethyleneeglycol bis(2-aminoethylether) tetraacetic acid (EGTA), and Nω-nitro-ω-arginine methyl ester (L-NAME) were purchased from Sigma (St. Louis, USA). N-(6-aminoxhexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) was purchased from Seikagaku Corp. (Tokyo, Japan). Human endothelin-1 (ET-1) was purchased from Peptide Research Inc. (Osaka, Japan). Human insulin (Humalin R, 100 IU/ml) was obtained from Eli Lilly Japan (Tokyo, Japan).

**Cell Preparation**

Human umbilical vascular endothelial cells (HUVECs) were purchased from BioWhittaker, Inc. Cultured cells (HUVECs) were maintained in EGM (endothelial growth medium) supplemented with 0.1% hEGF (human epidermal growth factor recombinant), 0.1% hydrocortisone, 0.1% GA-1000 (gentamicin sulfate, amphotericin-B), 0.4% BBE (bovine brain extract) and 2.0% fetal bovine serum in an atmosphere of 5% CO2 and 95% air at 37°C in 25-cm² flasks. At confluence, cells were split 1:3 after they were detached using 0.25% trypsin in 0.02% ethylenediamine tetraacetic acid (EDTA). Media were changed twice weekly. All cultures were used within 3 weeks of establishing primary cultures and at the third to fifth passage.

**Measurement of ET-1 Concentration**

The amount of ET-1 produced by HUVECs was determined by measuring the concentration of ET-1 in cultured medium using an ELISA Kit (TECHNE, Minneapolis, USA). Confluent monolayers cultured in 35 mm dishes were washed twice with phosphate buffered saline (pH 7.4), and then 2 ml of the above-described EGM medium supplemented with various concentrations of EPA, DHA, AA, LNA or OA was added. Over the first hour after the addition of each of the above fatty acids, the ET-1 production and NO production induced by fatty acids increased in a time-dependent manner. Then, at 1 h, both productions reached a near-plateau. Therefore, cultured medium at 1 h after the addition was used for the measurement of ET-1 and NO. Aliquots (1.5 ml) of cultured medium were collected and centrifuged for 2 min at 12,000 g. Diluted conjugate (100 µl; antibody to ET-1 conjugated to horseradish peroxidase) was added to each ET-1 antibody pre-coated well. Then, 100 µl of collected supernatant or standard solution was added to each well with sufficient force to ensure mixing, and incubated at room temperature for 30 min. Thereafter, the contents of each well were aspirated and washed with wash buffer (buffered surfactant), supplemented with 100 µl substrate (tetrathymethylbenzidine), and incubated at room temperature for 30 min. Then, 100 µl stop solution (acid solution) was added to each well. The optical density of each well was determined using a biolumin 960 microplate reader (Molecular Dynamics Japan, Tokyo, Japan) set at 450 nm, with the correction wavelength set at 620 nm. The concentration of ET-1 was calculated by comparison with standard solutions containing 0–150 pg/ml ET-1 prepared using the EGM medium described above. In the same way, at 1 h after the addition of one of the above fatty acids to cultured medium pretreated with 1 µmol/l insulin for 2 h, ET-1 concentrations in the medium were measured. ET-1
concentrations in the medium at 1 h after the addition of EPA (100 µmol/l) to cultured medium pretreated with EGTA (1 mmol/l), L-NAME (300 µmol/l), or W-7 (300 µmol/l) for 2 h were also measured.

Determination of NO

The amount of NO released from HUVECs was determined by measuring the concentration of NO\textsubscript{x} in cultured medium using the Griess reaction (30). One milliliter of Griess reagent (0.5% naphthylethylenediamine dihydrochloride and 5% sulphanilamide in 25% H\textsubscript{3}PO\textsubscript{4}) was added to 1 ml of the supernatant described above, and the mixture was incubated at room temperature for 10 min. The absorbance at 540 nm was measured in a Beckman DU-70 spectrophotometer (Fullerton, USA). The concentration of [NO\textsubscript{2}⁻] was calculated by comparison with the absorbance at 540 nm of a standard solution of 0–50 µmol/l NaNO\textsubscript{2} prepared using the EGM medium described above.

Protein Assay

After HUVECs proliferated to confluence in 35 mm dishes, the cells were washed three times in a HEPES buffered saline solution and then lysed in 0.5 mol/l NaOH. The protein content of the cytolysate in total cells was measured by Bradford’s protein assay. The ET-1 concentration and NO concentration were corrected by the protein content.

RNA Isolation and RT-PCR

After HUVECs proliferated to confluence in 25 mm\textsuperscript{2} flasks, the cells were washed twice in a HEPES buffered saline solution and were then trypsinized and centrifuged for 2 min at 12,000 × g. HUVECs were washed twice in HEPES buffered saline solution. Total cellular RNA was extracted from the cell pellet using Trizol Reagent (Life Technology, Tokyo, Japan). The RNA concentration was estimated from the optical density at 260 and 280 nm in a Beckman DU-640 spectrophotometer. The RNA (1 µg) obtained was subjected to enforced reverse transcription using an RNA PCR Kit (AMV) (Takara, Shiga, Japan). PCR was carried out using the same kit. The primers used were as follows: ET-1-S, AAACAGCAGTCTTAGGCGC; and ET-1-AS, TGGGTCACATAACGCTCTC. The synthesized total DNA was size-fractionated in 1.5% agarose gel containing ethidium bromide.

Statistical Analysis

Data are expressed as the mean ± SD. Statistical analysis was performed using ANOVA and Fisher’s multiple range test, and values of \( p < 0.05 \) were considered to indicate statistical significance.

Results

EPA Reduced ET-1 Production in Vascular Endothelial Cells

Figure 1 shows the effects of five fatty acids—EPA, DHA, AA, LNA, and OA (100 µmol/l each)—on ET-1 production in HUVECs. Compared with the control solution, EPA, DHA, and AA decreased the concentration of ET-1 in the culture medium. Compared with the control solution, EPA markedly decreased the concentration of ET-1 in the culture medium (\( p < 0.05 \)), DHA and AA decreased the concentration to a lesser, but still significant degree (\( p < 0.05 \)), and LNA and OA decreased the concentration only slightly (\( p < 0.05 \)). Figure 2 illustrates the effects of various concentrations of EPA on ET-1 production. As can be seen, EPA attenuated ET-1 production in a concentration-dependent manner in HUVECs.
Interaction of EPA and Insulin on ET-1 Production

To elucidate the effects of EPA under abnormal conditions, we treated HUVECs with human insulin. It is known that insulin enhances ET-1 production in endothelial cells (31).

Figure 3 shows the effects of the five fatty acids studied—EPA, DHA, AA, LNA, and OA (100 µmol/l each)—on ET-1 production in HUVECs pretreated for 2 h with 1 µmol/l insulin. Compared with the control, EPA, DHA and AA decreased the concentration of ET-1 stimulated by insulin in the culture medium. EPA induced a particularly marked decrease in ET-1. However, LNA and OA showed little effect on the concentration of ET-1. Figure 4 shows the effects of various concentrations of EPA on ET-1 production in HUVECs pretreated for 2 h with 1 µmol/l insulin. In the absence of EPA, insulin increased the concentration of ET-1. EPA inhibited the insulin-evoked increase of ET-1 in a concentration-dependent manner.

EGTA, L-NAME, and W-7 Reduced the NO Production Enhanced by EPA

Figure 5 shows the effects of the Ca$^{2+}$ chelator EGTA, NOS inhibitor L-NAME, and calmodulin antagonist W-7 on NO production in HUVECs. These inhibitors did not change the concentration of [NO$^\cdot$] compared with the control for the first 60 min of culturing. On the other hand, 100 µmol/l EPA significantly increased the concentration of [NO$^\cdot$] in the culture medium. However, EPA scarcely increased NO production in the presence of EGTA, L-NAME, or W-7.

EPA Reduced ET-1 Production Even in the Presence of EGTA, L-NAME, or W-7

Figure 6 shows the effects of EPA on ET-1 production in the presence or absence of EGTA, L-NAME, and W-7. In the absence of these inhibitors, 100 µmol/l EPA significantly decreased ET-1 production in HUVECs. In the presence of these inhibitors, 100 µmol/l EPA decreased ET-1 production to the same degree as the absence of these inhibitors for the first 60 min of culturing.

Effects of EPA on ET-1 mRNA Expression

Figure 7 shows the electrophoretogram of amplified products of ET-1 mRNAs obtained from HUVECs incubated with or without 30, 100, or 300 µmol/l EPA. In this protocol, the 542 bp band corresponded to ET-1 mRNA. In the control cells and the cells treated with 30–100 µmol/l EPA,
HUVECs expressed the ET-1 mRNA-band. On the other hand, incubation with 300 µmol/l EPA resulted in only a very weak expression of the ET-1 mRNA-band. In RT-PCR with low quantification potential, the brightness of the band under incubation with 300 µmol/l EPA became equivalent to that under incubation of 30–100 µmol/l EPA under an increased number of PCR amplification cycles. Under insulin stimulation, the band was detectable under incubation with 300 µmol/l EPA, but the brightness decreased when the number of amplification cycles was reduced (data not shown).

Discussion

The major findings of the present study were as follows. 1) In HUVECs, EPA decreased the production of ET-1 in a dose-dependent manner, and EPA had the most potent effect among the fatty acids examined. 2) One µmol/l insulin increased the production of ET-1 about 1.5-fold compared with the baseline values, and EPA suppressed these effects of insulin in a dose-dependent manner. 3) The Ca²⁺ chelator EGTA, NOS inhibitor L-NNAME, and calmodulin antagonist W-7 inhibited the increase in NO production induced by EPA, but they did not inhibit the effects of EPA on ET-1 production. 4) The expression of ET-1 mRNA was inhibited after addition of EPA. Thus, EPA inhibited the production of ET-1 induced by insulin as well as the production of ET-1 under basal conditions via the inhibition of ET-1 mRNA production. It is therefore very likely that these effects of EPA on ET-1 in HUVECs contributed to the vasorelaxant and antiatherosclerotic effects of EPA.

In cultured bovine mesangial cells (29), EPA has been reported to inhibit the production of ET-1. To date, however, there have been no reports of the effects of EPA on the production of ET-1 in human vascular endothelial cells. In this study, we also showed that EPA reduced ET-1 production in HUVECs. ET-1 is a peptide consisting of 21 amino acids. Its mRNA is transcribed from the ET-1 gene in the nucleus, translated to prepro-ET-1 in the rough endoplasmic reticulum, and then enzymatically cut, producing big ET-1. Then, the 21 amino acid ET-1 is generated. Using RT-PCR, EPA was found to inhibit the expression of ET-1 mRNA, leading us to hypothesize that EPA inhibited ET-1 production at the transcriptional level. It is known that the production of ET-1 is stimulated by thrombus formation factors, such as thrombin (32, 33) and TGF-β (34), as well as by vasoactive substances such as angiotensin II (35) and insulin (24, 36), whereas it is inhibited by NO (37–40). In this study, EPA significantly increased NO production in HUVECs, and EGTA, L-NNAME and W-7 reduced the EPA-induced NO production. In terms of the mechanism by which EPA induces NO production in HUVECs, our results suggested that EPA induced an inflow of Ca²⁺ through the Ca²⁺ channel in the cellular membrane, and produced NO by a calcium-calmodulin-dependent activation of eNOS, in agreement with the findings of a previous study Okuda et al. (41). It has also been suggested that EPA produces NO by translocating eNOS localized in the cellular membrane into the cytoplasm (42). On the other hand, in the present study, EPA significantly decreased ET-1 production, but the EPA-induced de-
crease of ET-1 production in the presence of these inhibitors was equal to that in the absence of inhibitors. Therefore, the inhibitory effects of EPA on ET-1 production may not have been due to an increase in the production of NO.

In the present study, EPA showed the most potent effects on ET-1 production among the polyunsaturated fatty acids examined. This may have been due to the structure of EPA and to the higher affinity of EPA for the cell membrane. Generally, long-chain fatty acids such as EPA are absorbed from the small intestines, and incorporated into triglycerides (TG), which circulate as chylomicrons in the lymph system (43). In plasma, fatty acids are incorporated mainly into TG, phospholipids, and cholesterol esters in lipoproteins (43). Fatty acids in these lipoproteins are incorporated into the cells after being separated from the proteinic component by lipase present on the cellular surface. In the culture experiment on endothelial cells, after incubation with EPA the concentration of AA in the endothelial cells gradually decreased, while the concentration of docosapentaenoic acid (DPA) biosynthesized from EPA by the elongase increased with the increase in the concentration of EPA in endothelial cells (41). This indicates that EPA is metabolized by some metabolic enzyme in endothelial cells. Furthermore, based on the immediate increase in intracellular Ca\(^{2+}\) concentration in endothelial cells after incubation with EPA (41), EPA seemed to be achieving this effect independently.

In a previous study, when 1.8 g/day EPA-ester was orally administered to Japanese patients with type II diabetes, the EPA levels as total lipids in plasma increased from 280 \(\pm\) 34 \(\mu\)mol/l (before administration) to 581 \(\pm\) 65 \(\mu\)mol/l (mean \(\pm\) SE) (after administration) (44). In an earlier study, when 9 g/day of fish oil (containing 1.6 g of EPA) was orally administered to German patients with hyperlipoproteinemia, the EPA levels as total lipids in plasma increased from 119 \(\pm\) 70 to 619 \(\pm\) 172 \(\mu\)mol/l (mean \(\pm\) SD), and the EPA levels as free fatty acids in plasma increased from 7 \(\pm\) 3 to 40 \(\pm\) 10 \(\mu\)mol/l (mean \(\pm\) SD) (45). Thus, the concentrations of EPA (30–300 \(\mu\)mol/l) used in this study seemed to be within clinical and physiological ranges.

In conclusion, EPA decreased the production of ET-1, a vasopresor substance, both under basal conditions and after administration of insulin. These effects are considered to play an important role in the hypotensive and antiatherosclerotic effects of EPA. In addition, blood pressure is often elevated after administration of insulin to patients with diabetes, and thus it is very likely that EPA also contributes to the vasorelaxant and antiatherosclerotic effects in these patients.

**References**

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