Eicosapentaenoic Acid Improves Imbalance between Vasodilator and Vasoconstrictor Actions of Endothelium-Derived Factors in Mesenteric Arteries from Rats at Chronic Stage of Type 2 Diabetes

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ABSTRACT

Accumulating evidence demonstrates that dietary intake of n-3 polyunsaturated fatty acids (PUFAs) is associated with a reduced incidence of several cardiovascular diseases that involve endothelial dysfunction. However, the molecular mechanism remains unclear. We previously reported that mesenteric arteries from type 2 diabetic Otsuka Long-Evans Tokushima fatty (OLETF) rats exhibit endothelial dysfunction, leading to an imbalance between endothelium-derived vaso-dilators [namely, nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF)] and vasoconstrictors [endothelium-derived contracting factors (EDCFs)] [Am J Physiol Heart Circ Physiol 293:H1480–H1490, 2007]. We hypothesized that treating OLETF rats with eicosapentaenoic acid (EPA), a major n-3 PUFA, may improve endothelial dysfunction by correcting this imbalance. In OLETF rats [compared with age-matched control Long-Evans Tokushima Otsuka (LETO) rats]: 1) acetylcholine (ACh)-induced (endothelium-dependent) relaxation was impaired, 2) NO- and EDHF-mediated relaxations and nitrite production were reduced, and 3) ACh-induced EDCF-mediated contraction, production of prostanoids, and the protein expressions of COX-1 and COX-2 were all increased. When OLETF rats received chronic EPA treatment long-term (300 mg/kg/day p.o. for 4 weeks), their isolated mesenteric arteries exhibited: 1) improvements in ACh-induced NO- and EDHF-mediated relaxations and COX-mediated contraction, 2) reduced EDCF- and arachidonic acid-induced contractions, 3) normalized NO metabolism, 4) suppressed production of prostanoids, 5) reduced COX-2 expression, and 6) reduced phosphoextracellular signal-regulated kinase (ERK) expression. Moreover, EPA treatment reduced both ERK2 and nuclear factor (NF)-κB activities in isolated OLETF aortas. We propose that EPA ameliorates endothelial dysfunction in OLETF rats by correcting the imbalance between endothelium-derived factors, at least partly, by inhibiting ERK, decreasing NF-κB activation, and reducing COX-2 expression.

Vascular tone is tightly regulated by endothelium-derived factors. These include relaxing factors (EDRFs) such as nitric oxide (NO), hyperpolarizing factors (EDHFs), and contracting factors (EDCFs) (Pieper, 1998; Busse et al., 2002; Féleétou and Vanhoutte, 2004; Cohen, 2005; Vanhoutte et al., 2005).

Alterations in these factors may be the cause of changes in resting blood pressure. Several lines of evidence suggest that endothelial dysfunction could play an important role in the development of both macro- and microangiopathy in animal models of diabetes and in diabetes patients (Pieper, 1998; De Vriese et al., 2000; Cohen, 2005; Matsumoto et al., 2006a). An accumulating body of evidence indicates that endothelium-dependent relaxation is impaired in several regions of the vasculature in animals and humans with type 2 diabetes and in type 1 diabetes (Kamata et al., 1989; Hattori et al., 1991; This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology, Japan; and the Open Research Center Project. Article, publication date, and citation information can be found at http://jpet.aspetjournals.org. doi:10.1124/jpet.108.148718.

ABBREVIATIONS: EDRF, endothelium-derived relaxing factor; NO, nitric oxide; EDHF, endothelium-derived hyperpolarizing factor; EDCF, endothelium-derived contracting factor; PUFAs, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; OLETF, Otsuka Long-Evans Tokushima fatty; COX, cyclooxygenase; PG, prostaglandin; AA, arachidonic acid; TRAM-34, [1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole]; PE, phenylephrine; L-NNA, N'-nitro-L-arginine; SNP, sodium nitroprusside; ACh, acetylcholine; HRP, horseradish peroxidase; eNOS, endothelial nitric-oxide synthase; ERK, extracellular signal-regulated kinase; LETO, Long-Evans Tokushima Otsuka; EPA100, EPA (100 mg/kg/day); EPA300, EPA (300 mg/kg/day); HDL, high-density lipoprotein; NEFA, nonesterified fatty acid; KHS, Krebs-Henseleit solution; KCa, calcium-activated potassium channel; TX, thromboxane; ELISA, enzyme-linked immunosorbent assay; NF, nuclear factor; TP, thromboxane receptor.
Pieper, 1998; De Vriese et al., 2000; Cohen, 2005; Matsumoto et al., 2006a, 2007c) and that a reduced production of EDRFs and/or defects in EDRF signaling may underlie this impairment in type 2 diabetic vessels (De Vriese et al., 2000; Matsumoto et al., 2006c, 2007b). Furthermore, this impairment of relaxation may be attributable not only to defective EDRF and/or EDHF signaling but also to increased EDCF signaling, leading to diabetic vasculopathy (De Vriese et al., 2000; Vanhoutte et al., 2005; Matsumoto et al., 2006a). Therefore, manipulation aimed at normalizing abnormal signaling by the above-mentioned endothelium-derived factors represents an important therapeutic target for diabetic vascular complications.

In epidemiological and clinical trials, treatment with fish oil rich in n-3 polyunsaturated fatty acids (PUFAs) or with the n-3 PUFAs themselves has been shown to reduce the incidence of cardiovascular diseases, including type 2 diabetes (Din et al., 2004; von Schacky, 2006). A large-scale, prospective, randomized clinical trial, the Japan EPA Lipid Intervention Study, has demonstrated a significant reduction in the incidence of major coronary events after the addition of highly purified eicosapentaenoic acid (EPA), which is the only type of n-3 PUFA used clinically to treat the hyperliipidemia occurring during low-dose statin therapy (Yokoyama et al., 2007). This suggests that EPA has pleiotropic effects in addition to its well-known lipid-lowering effect (Din et al., 2004; von Schacky, 2006). Recent evidence indicates that n-3 PUFA may have an anti-inflammatory effect, an antiatherosclerotic effect, an antiarrhythmic effect, a blood pressure-lowering effect, a triglyceride-lowering effect, and an antiatherosclerotic effect, an antiarrhythmic effect, a blood "long-term EPA treatment on the impaired actions of endothelium-derived factors seen in mesenteric arteries isolated from OLETF rats at the established chronic stage of type 2 diabetes and to identify some of the molecular mechanisms involved.

Materials and Methods

Drugs and Solutions. Apanarin, arachidonic acid (AA), TRAM-34, phentylphrine (PE), indomethacin, Nω-nitro-L-arginine (L-NNA), sodium nitroprusside (SNP), and monoclonal β-actin antibody were all purchased from Sigma-Aldrich (St. Louis, MO), and acetylcholine (ACH) was from Daiichi-sankyo Pharmaceuticals (Tokyo, Japan). Antibodies against COX-1 and COX-2 were from Cayman Chemical (Ann Arbor, MI). Horseradish peroxidase (HRP)-linked secondary anti-mouse and anti-rabbit antibodies were purchased from Promega (Madison, WI), and the antibodies against endothelial nitric-oxide synthase (eNOS), ERK1/2, and phosphorylated ERK1/2 (pT202/pY204) were from BD Biosciences (San Jose, CA). Drugs were dissolved in saline, except where otherwise noted. TRAM-34 was dissolved in dimethyl sulfoxide, whereas indomethacin was dissolved first in a small amount of 0.1 M Na2CO3 solution and then made up to the final volume with distilled water. Highly purified EPA ethyl ester (purity, 98%; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) was suspended in 5% gum arabic solution.

Animals and Experimental Design. Five-week-old male OLETF rats and LETO rats, a genetic control for OLETF, were supplied by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). Food and water were given ad libitum in a controlled environment (room temperature, 21–22°C; room humidity, 50 ± 5%) until the rats were 46 to 50 weeks old. For the long-term study, some OLETF and LETO rats were given EPA (100 mg/kg/day) p.o.) for 4 weeks starting at 46 to 50 weeks old. Thus, we studied four groups: vehicle (5% gum arabic solution)-treated LETO and OLETF groups and EPA (100 mg/kg/day) and EPA (300 mg/kg/day) 5% gum arabic solution-treated LETO and OLETF groups and EPA (100 mg/kg/day) and -3 PUFA used

Animals and Experimental Design. Five-week-old male OLETF rats and LETO rats, a genetic control for OLETF, were supplied by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). Food and water were given ad libitum in a controlled environment (room temperature, 21–22°C; room humidity, 50 ± 5%) until the rats were 46 to 50 weeks old. For the long-term study, some OLETF and LETO rats were given EPA (100 mg/kg/day) p.o.) for 4 weeks starting at 46 to 50 weeks old. Thus, we studied four groups: vehicle (5% gum arabic solution)-treated LETO and OLETF groups and EPA (100 mg/kg/day) and EPA (300 mg/kg/day)-treated LETO (hereafter termed EPA100 or EPA300) groups. This study was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan).

Assessment of Blood Parameters and Blood Pressure. Plasma parameters and systemic blood pressure were measured as described previously (Matsumoto et al., 2006b, 2007a,b, 2008). In brief, plasma glucose, cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol, and serum nonesterified fatty acid (NEFA) levels were each determined by the use of a commercially available enzyme kit (Wako Pure Chemicals, Osaka, Japan). Plasma insulin was measured by enzyme immunoassay (Shibayagi Co. Ltd., Gunma, Japan). After a given rat had been in a constant-temperature box at 37°C for a few minutes, its blood pressure was measured by the tail-cuff method using a blood pressure analyzer (BP-98A; Softron, Tokyo, Japan).

Measurement of Isometric Force. Vascular isometric force was recorded as in our previous papers (Matsumoto et al., 2003, 2004, 2005, 2007a, 2008). At 50 to 54 weeks of age, rats were anesthetized with diethyl ether and then euthanized by decapitation. The suprerior mesenteric artery was rapidly removed and immersed in oxygenated, modified Krebs-Henseleit solution (KHS). This solution consisted of 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM NaHCO3, 1.8 mM CaCl2, 1.2 mM NaH2PO4, 1.2 mM MgSO4, and 11.0 mM dextrose. The artery was carefully cleaned of all fat and connective tissue, and ring segments 2 mm in length were suspended by a pair of stainless steel pins in a well oxygenated (95% O2/5% CO2) bath containing 10 ml of KHS at 37°C. The rings were stretched until an optimal resting tension of 1.0 g was loaded and then allowed to equilibrate for at least 60 min. Force generation was monitored by means of an isometric transducer (model TB-611T; Nihon Kohden, Tokyo, Japan).
For the relaxation studies, mesenteric rings were precontracted with an equally effective concentration of PE (0.1–1 μM) (i.e., so that the tension developed in response to PE was similar among all groups). There was no significant difference in the response to PE among the LETO (n = 36), OLETF (n = 36), EPA100 (n = 36), and EPA300 (n = 36) groups (1.51 ± 0.03, 1.58 ± 0.03, 1.58 ± 0.04, and 1.59 ± 0.04 g, respectively). When the PE-induced contraction had reached a plateau level, ACh (1 nM–10 μM) was added in a cumulative manner. After the addition of sufficient aliquots of the agonist to produce the chosen concentration, a plateau response was allowed to develop before the addition of the next dose of the same agonist. To investigate the influences of the various factors that might constitute EDHF in the present preparations, we examined ACh-induced relaxation in the absence or presence of various inhibitors, as follows: 1) 10 μM indomethacin (COX inhibitor) plus 100 μM L-NNA (nitric-oxide synthase inhibitor) (to investigate EDHF-type relaxation), 2) 10 μM indomethacin plus 10 μM TRAM-34 (specific inhibitor of the intermediate-conductance K<sub>nc</sub> channel) plus 100 nM apamin (specific inhibitor of the small-conductance K<sub>ca</sub> channel) [cotreatment with these two K<sub>nc</sub>-channel inhibitors can block EDHF signaling according to a previous report (Busse et al., 2002) and our preliminary experiment] (to investigate NO-mediated relaxation), and 3) 100 μM L-NNA plus 10 μM TRAM-34 plus 100 nM apamin (to investigate COX-mediated relaxation). To assess endothelium-independent relaxation, we examined SNP (0.1 nM–10 μM)-induced relaxation in the presence of 10 μM indomethacin plus 100 μM L-NNA. Rings were incubated with the appropriate inhibitor(s) for 30 min before administration of PE.

For the contraction studies, mesenteric rings were first contracted using 80 mM K<sup>+</sup>, with these responses being taken as 100%. There was no significant difference in the response to 80 mM K<sup>+</sup> among the LETO (n = 11), OLETF (n = 12), EPA100 (n = 13), and EPA300 (n = 13) groups (1.63 ± 0.05, 1.60 ± 0.02, 1.68 ± 0.04, and 1.66 ± 0.03 g, respectively). To investigate the EDHC- or AA-mediated response, endothelium-intact mesenteric rings were treated with 100 μM L-NNA for 30 min. After this incubation period, ACh (10 nM–10 μM) or AA (100 nM–10 μM) was cumulatively applied. After the addition of sufficient aliquots of the agonist to produce the chosen concentration, a plateau response was allowed to develop before the addition of the next dose of the same agonist.

**Measurement of Nitrite and Nitrate.** The concentrations of nitrite and nitrate in the effluent from each tissue were measured by the method described previously (Matsumoto et al., 2007a,c). For the determination of plasma NO metabolites, 0.3 ml of 100% methanol was added to 0.3 ml of each plasma sample, and the sample was then centrifuged at 5000g for 10 min at 4°C. To evaluate the release of NO metabolites in mesenteric arteries, each mesenteric ring was placed in KHS at 37°C and then treated with ACh (10 μM) for 15 min. The concentrations of nitrate and nitrite in plasma or KHS were measured using a automated NO detector/high-performance liquid chromatography system (ENO20; Eicom, Kyoto, Japan).

**Release of Prostaglandins.** Prostanoid release was measured as in our previous articles (Matsumoto et al., 2007a, 2007b). To allow us to measure this release, mesenteric arteries from each of the four groups were cut into transverse rings 4 mm in length. These were placed for 30 min in siliconized tubes containing 1.0 ml of KHS in the presence of 100 μM L-NNA at 37°C, and then 10 μM ACh was applied for 15 min. Next, after the mesenteric rings had been removed, the tubes were freeze-clamped in liquid nitrogen and stored at −80°C for later analysis. The prostaglandins were measured using a commercially available enzyme immunoassay kit (Cayman Chemical). Two-time diluted samples were used for measurements of PG<sub>E2</sub>, thromboxane (TX) B<sub>2</sub> (a stable metabolite of TXA<sub>2</sub>), and PGP<sub>2α</sub>, whereas 100× diluted samples were used for the measurement of 6-keto PGF<sub>1α</sub>, a stable metabolite of prostacyclin. The various assays were performed as described in the manufacturer’s procedure booklet. The amounts of prostaglandins released are expressed as picograms or nanograms per milligram of wet weight of mesenteric artery.

**Western Blotting.** The protein levels of the COXs, eNOS, ERK1/2, and phosphorylated ERK1/2 were quantified using immunoblotting procedures, essentially as described previously (Matsumoto et al., 2004, 2007a, 2008). Aortic or mesenteric arterial tissues were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS containing protease and phosphatase inhibitor cocktails (Complete Protease Inhibitor Cocktail and PhosSTOP; Roche Diagnostics, Indianapolis, IN). The lysate was cleared by centrifugation at 16,000 g for 10 min at 4°C. The supernatant was collected, and the proteins were solubilized in Laemmli’s buffer containing mercaptoethanol. Protein concentrations were determined by means of a bichinchoninic acid protein assay reagent kit (Pierce Chemical, Rockford, IL). Samples (20 μl/gl) were resolved by electrophoresis on 10% SDS-polyacrylamide electrophoresis gels and then transferred onto polyvinylidene difluoride membranes. In brief, after blocking the residual protein sites on the membrane with ImmunoBlock (Dainippon-pharm., Osaka, Japan) or polyvinylidene difluoride blocking reagent (Toyobo Engineering, Osaka, Japan), the membrane was incubated with anti-COX-1 (70 kDa; 1:500), anti-COX-2 (70 kDa; 1:500), anti-eNOS (140 kDa; 1:1000), anti-ERK1/2 (44 and 42 kDa; 1:1000), or anti-phospho-ERK1/2 (pT202/pY204) (44 and 42 kDa; 1:1000) in blocking solution. HRP-conjugated, antimouse, or anti-rabbit antibody was used at a 1:1,000 dilution in Tween 20-phosphate-buffered saline, followed by detection using SuperSignal (Pierce Chemical). To normalize the data, we used β-actin as a housekeeping protein. The β-actin protein levels were determined after stripping the membrane and probing with β-actin monomolecular primary antibody (42 kDa; 1:5,000), with HRP-conjugated anti-mouse IgG as the secondary antibody. The optical densities of the bands on the film were quantified using densitometry, with correction for the optical density of the corresponding β-actin band.

**Quantification of Phosphorylated ERK2 (Using ELISA).** Aortic tissues were rapidly removed and carefully cleaned of all fat and connective tissue. They were then frozen in liquid N<sub>2</sub> and physically crushed to a fine powder in liquid N<sub>2</sub> using a Cryo-Press (Microtech Nichion, Chiba, Japan). After lyisation of these powder samples, phosphorylated ERK2 was lysed and its level quantified using a Human/Mouse/Rat Phospho-ERK2 (Thr185/Tyr187) Surveyor IC immunoassay system (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Trans-AM NF-κB Transcriptional Factor Assay.** Nuclear protein extracts were isolated from aortic tissues using a Nuclear Extract Kit (Active Motif Inc., Carlsbad, CA) according to the manufacturer’s instructions, and aliquots of nuclear protein were stored at −80°C. NF-κB activation was assayed using the protocol supplied with Active Motif’s ELISA-based transactivation Trans-AM kit. The NF-κB Trans-AM kit contains a 96-well plate with immobilized oligonucleotides encoding an NF-κB consensus site (5′-GGGACTT-3′). The active form of NF-κB contained in the nuclear extract from aortic tissue binds specifically to this oligonucleotide. The primary antibody used to detect NF-κB recognizes an epitope on p65 that is accessible only when NF-κB is activated and bound to its target DNA. An HRP-conjugated secondary antibody provides a sensitive colorimetric readout easily quantified by spectrophotometry.

**Data Analysis and Statistics.** Data are expressed as means ± S.E.M. Each relaxation response is expressed as a percentage of the contraction induced by PE. Contractile responses are expressed as a percentage of the response to 80 mM K<sup>+</sup>. When appropriate, statistical differences were assessed by Dunnett’s test for multiple comparisons after a one-way analysis of variance, a probability level of P < 0.05 being regarded as significant. Statistical comparisons between concentration-response curves were made using a two-way analysis of variance, with Bonferroni’s correction for multiple comparisons being performed post hoc (P < 0.05 again being considered significant).
Results

Body Weight, Blood Pressure, and Blood Chemistry. At the time of the experiment (50–54 weeks old), the body weight of the OLETF rats was greater than that of the age-matched nondiabetic control LETO rats (Table 1). The systolic and diastolic blood pressure of OLETF rats was higher than that of LETO rats, whereas heart rate was similar between the two groups (Table 1). As shown in Table 2, at the time of the experiment, nonfasted OLETF rats exhibited hyperglycemia, their blood glucose levels being significantly higher than those of nonfasted LETO rats. Plasma cholesterol, triglyceride, and NEFA levels were all significantly higher in OLETF rats than in LETO rats, whereas HDL and insulin levels were similar between the two groups (Table 2). Treatment of OLETF rats with EPA at 100 mg/kg/day, for 4 weeks, did not alter the above parameters (compared with the OLETF group), but treatment at 300 mg/kg/day, for 4 weeks, significantly lowered blood pressure (Table 1) and significantly increased HDL (compared with the OLETF group) (Table 2).

Effects of EPA on Endothelium-Dependent Relaxation in OLETF Rats. We previously demonstrated (Matsumoto et al., 2007a, 2008) that mesenteric arteries from OLETF rats at the chronic stage of diabetes exhibit endothelial dysfunction and that this results from an imbalance of endothelium-derived factors such as reduced EDRF signaling and increased EDCF signaling. As shown in Fig. 1A, ACh induced a concentration-dependent relaxation, with the maximum response 100 to 300 nM and responses then progressively weaker up to 10 μM. This relaxation was significantly weaker in rings from OLETF rats than in those from LETO rats. Compared with that in the OLETF group, the ACh-induced relaxation was not different in the EPA100 group but significantly improved in the EPA300 group (Fig. 1A). On the other hand, the SNP-induced endothelium-independent relaxation was not different among the four groups (Fig. 1B).

To investigate which (if any) endothelium-derived factors might be improved in mesenteric arteries from EPA-treated OLETF rats, we examined ACh-induced relaxation in the presence of various inhibitors (Fig. 2). To investigate the component of the ACh-induced endothelium-dependent relaxation that is mediated by NO, we performed a series of experiments in which ACh was added cumulatively to rings precontracted by PE in the combined presence of 100 μM L-NNa plus 10 μM indomethacin (Fig. 2B). This EDHF-mediated relaxation was: 1) significantly weaker in rings from OLETF rats than in those from LETO rats (Fig. 2B) and 2) slightly but not significantly greater in the EPA100 group than in the OLETF group but significantly greater in the EPA300 group than in both the OLETF group and the nondiabetic LETO group (Fig. 2B).

Similar to NO and EDHF, COX-derived factors such as prostacyclin are known to be endothelium-derived vasodilators (Wise and Jones, 1996). To assess the endothelium-dependent relaxing effects of COX-derived factors and their modulation by EPA treatment, we added ACh cumulatively to rings precontracted by PE in the combined presence of 100 μM L-NNa, 100 nM apamin, and 10 μM TRAM-34 (Fig. 2C). Under these conditions, tension developed as the concentration of ACh was increased (0.3–10 μM) in all groups (Fig. 2C), although it was significantly greater in rings from OLETF rats than in those from LETO rats. This ACh-induced contractile response in the combined presence of 100 μM L-NNa, 100 nM apamin, and 10 μM TRAM-34 was completely blocked by 10 μM indomethacin treatment in both LETO and OLETF groups (data not shown). This COX-mediated response did not differ between the OLETF and EPA100 groups, but it was significantly weaker in the EPA300 group than in the OLETF group at the higher end of the ACh concentration range used (Fig. 2C). Inspection of the curves in Fig. 2C indicates that in these preparations, COX-derived factor(s) may make a prominent contribution to the weakening of the endothelium-dependent relaxation observed at higher ACh concentrations (see Fig. 1A).

Effects of EPA on Endothelium-Dependent Contraction in OLETF Rats. As described above (Fig. 1A), at higher concentrations of ACh (i.e., 1–10 μM), a reduced ACh-induced relaxation was observed, with the relaxation being more nearly abolished in rings from OLETF rats than in those from LETO rats. Moreover, the ACh-induced COX-mediated response was a contraction that was greater in OLETF rats than in LETO rats (Fig. 2A). Relative to that in the OLETF group, this NO-mediated relaxation was not altered in the EPA100 group, but it was slightly but significantly increased in the EPA300 group (Fig. 2A).

To investigate the component of the ACh-induced endothelium-dependent relaxation that is mediated by EDHF, we performed a series of experiments in which ACh was added cumulatively to rings precontracted by PE in the presence of 100 μM L-NNa plus 10 μM indomethacin (Fig. 2B). This EDHF-mediated relaxation was: 1) significantly weaker in rings from OLETF rats than in those from LETO rats (Fig. 2B) and 2) slightly but not significantly greater in the EPA100 group than in the OLETF group but significantly greater in the EPA300 group than in both the OLETF group and the nondiabetic LETO group (Fig. 2B).

TABLE 1

<table>
<thead>
<tr>
<th>Body weight, blood pressure, and heart rate of the four experimental groups</th>
<th>Body Weight</th>
<th>Systolic Blood Pressure</th>
<th>Diastolic Blood Pressure</th>
<th>Heart Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>LETO (n = 10)</td>
<td>542.2 ± 8.7</td>
<td>112.0 ± 2.3</td>
<td>89.3 ± 2.0</td>
<td>393.2 ± 10.7</td>
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<tr>
<td>OLETF (n = 10)</td>
<td>596.9 ± 19.8*</td>
<td>145.5 ± 3.7***</td>
<td>118.2 ± 3.2***</td>
<td>381.6 ± 14.6</td>
</tr>
<tr>
<td>OLETF EPA100 (n = 10)</td>
<td>573.4 ± 31.0</td>
<td>136.5 ± 2.7***</td>
<td>112.6 ± 2.5***</td>
<td>388.3 ± 16.6</td>
</tr>
<tr>
<td>OLETF EPA300 (n = 10)</td>
<td>556.2 ± 29.1</td>
<td>128.9 ± 4.4***</td>
<td>103.4 ± 4.9**</td>
<td>391.6 ± 9.5</td>
</tr>
</tbody>
</table>

EPA100, EPA (100 mg/kg/day)-treated OLETF group; EPA300, EPA (300 mg/kg/day)-treated OLETF group.
* P < 0.05 vs. OLETF.
** P < 0.01 vs. OLETF.
*** P < 0.001 vs. OLETF.
presence of L-NNA (100 μM), conditions in which ACh-induced contractions in mesenteric arteries from LETO and OLETF rats have been reported to be completely blocked by endothelial denudation or by preincubation with indomethacin (Matsumoto et al., 2007a). As shown in Fig. 3A, in the presence of L-NNA (100 μM), an ACh-induced contraction was observed at higher ACh concentrations (i.e., 0.3–10 μM) in rings from all four groups. This contractile response was 1) significantly greater in the OLETF than in the LETO group (Fig. 3A), 2) significantly weaker in the EPA100 group than in the OLETF group, and 3) still weaker in the EPA300 group (Fig. 3A). Likewise, when we added AA (100 nM–10 μM)
cumulatively to rings in the presence of 1-NNa (100 µM), a contractile response was seen in all groups (Fig. 3B). This contraction was 1) significantly greater in OLETF than in LETO and 2) unaffected by long-term EPA treatment at 100 mg/kg/day for 4 weeks but 3) normalized by EPA at 300 mg/kg/day for 4 weeks.

Effect of EPA on NO Synthesis (as Nitrite and Nitrate) in Plasma and Mesenteric Arteries in OLETF Rats. The plasma level of nitrite, an oxidation product of NO, correlates with the level of NO biosynthesis (Kleinbongard et al., 2006), and the nitrate/nitrite ratio is often used as an indirect marker of oxidative stress (Oelze et al., 2006). To investigate whether these plasma parameters were altered by long-term EPA treatment, we measured the plasma levels of nitrite and nitrate as a measure of NO synthesis (Table 3). The plasma nitrate level was significantly lower in OLETF rats than in LETO rats, whereas the plasma nitrate level and nitrate/nitrite ratio were each significantly greater in OLETF than in LETO (Table 3). Compared with the OLETF group, no significant differences in these plasma NO metabolites were noted in OLETF rats given EPA for 4 weeks (100 mg/kg/day) treatment; however, both the nitrite level and the nitrate/nitrite ratio in the plasma were normalized in OLETF rats treated with EPA at 300 mg/kg/day (Table 3).

After stimulation with 10 µM ACh, the mesenteric artery nitrite level was significantly lower in the OLETF group than in LETO, and the nitrate/nitrite ratio tended to be higher in OLETF than in LETO (Table 3). Compared with their levels in mesenteric arteries from the OLETF group, NO metabolites were not altered in the EPA100 group. However, in the EPA300 group, there was a significantly increased nitrite level and a significantly decreased nitrate/nitrite ratio versus the OLETF group (Table 3).

Effects of EPA on Endothelium-Stimulated Release of Prostanoids in OLETF Rats. Because both the published evidence and the present findings indicate that overproduction of prostanoids contributes to endothelial dysfunction in diabetic arteries (Bagi et al., 2005; Pannirselvam et al., 2005; Matsumoto et al., 2007a), we examined the effects of long-term EPA treatment on the endothelium-stimulated release of prostanoids in mesenteric arteries from all four groups (Fig. 4). ACh (10 µM) induced the release of TXB2 (stable metabolite of TXA2; Fig. 4A), PGE2 (Fig. 4B), PGF2α (Fig. 4C), and 6-keto-PGF1α (stable metabolite of PGI2; Fig. 4D) in rings from all four groups, with the endothelium-stimulated release of TXB2 and that of PGF2α significantly greater in the OLETF group than in LETO. In contrast, the productions of PGF2α and 6-keto-PGF1α were not different between LETO and OLETF. Compared with those in the OLETF group, the productions of TXB2, PGE2, and 6-keto-PGF1α tended to be decreased in the EPA100 group, but to

![Fig. 3. EPA suppresses EDCF (A)- and AA (B)-induced contractions in OLETF rats. Concentration-response curves for ACh (A)- and AA (B)-induced contractions (in the presence of 100 µM 1-NNa) in mesenteric arteries obtained from LETO, OLETF, and EPA (100 or 300 mg/kg/day for 4 weeks)-treated OLETF (EPA100 or EPA300) rats. Details are given under Materials and Methods. Data are means ± S.E.M. from n = 7 (A) or n = 4–6 (B) experiments. * P < 0.05; and ***, P < 0.001 LETO group versus OLETF group; †, P < 0.05; and ###, P < 0.001, OLETF group versus EPA300 group.](image-url)
our surprise, release of all four prostanoids was significantly suppressed in the EPA300 group (Fig. 4).

Effects of EPA on COX and eNOS Protein Expressions in OLETF Rats. To investigate the possible mechanisms underlying the beneficial effects of EPA on endothelial function in OLETF rats, we examined whether the expressions of the proteins for COX and eNOS might be altered in mesenteric arteries (Fig. 5). Western blotting analysis of mesenteric arteries from the LETO, OLETF, EPA100, and EPA300 groups allowed detection of immunoreactive proteins (Fig. 5). The protein expressions for COX-1 (Fig. 5A) and COX-2 (Fig. 5B) were significantly greater in OLETF than in LETO. EPA treatment did not alter COX-1 expression (namely in EPA100 or EPA300 versus OLETF), but it was surprising that the level of COX-2 expression tended to be reduced (EPA100) or was reduced (EPA300) by long-term EPA treatment (\(P < 0.05 \pm P < 0.001\), LETO group versus OLETF group; \(\#\), \(P < 0.05\); \(\#\#\), \(P < 0.01\); and \(\#\#\#\), \(P < 0.001\), OLETF group versus EPA300 group).

Effects of EPA on Aortic NF-\(\kappa B\) Activity in OLETF Rats. It is known that activation of NF-\(\kappa B\) in the vasculature plays an important role in the development of endothelial dysfunction in diabetes (Bierhaus et al., 2001). We sought to test the hypothesis that the beneficial effects of EPA in OLETF rats might be associated with reduced NF-\(\kappa B\) activation. Nuclear extracts prepared from aortic tissue were subjected to ELISA using a Trans-AM kit (Fig. 6A). NF-\(\kappa B\) activity tended to be greater in OLETF than in LETO (Fig. 6A). In the EPA300 group, phospho-ERK2 activity was significantly suppressed compared with the OLETF group (Fig. 6B). We also compared phosphorylated ERK1/2 protein expression levels in mesenteric arteries among the four groups using Western blotting. The levels tended to be higher in OLETF than in LETO, whereas in the EPA300 group, each level was significantly decreased versus that in the OLETF group (Fig. 7).

Discussion

EPA seems likely to assume an increasingly important role in the realm of therapeutics because it has several beneficial effects in diabetes and other diseases. However, little is known about its abilities to correct endothelial functions once the progression of the disease process has begun, and the detailed mechanism(s) underlying its effects are still poorly understood.

OLETF rats manifest stable clinical and pathological features that resemble human type 2 diabetes (Kawano et al., 1992). In brief, OLETF rats are characterized by: 1) increas...
ing body weight just after weaning, 2) a late onset of hyperglycemia after 18 weeks of age and diagnosable diabetes after 24 weeks of age, and 3) hyperinsulinemia that is present at 24 weeks of age and declines after 55 weeks of age (Kawano et al., 1992). In the present study, we used 50-to 54-week-old OLETF rats, which have established diabetes, their blood glucose levels being around 500 mg/dl. Moreover, we and others (Kagota et al., 2000; Matsumoto et al., 2006b,c, 2007b) have demonstrated that abnormalities of vascular
function are present in several arteries in OLETF rats, and there are recent evidence of an imbalance between endothelium-derived factors, such as decreased EDRF signaling and increased EDCF signaling, in mesenteric arteries from OLETF rats at the chronic stage of diabetes (Matsumoto et al., 2007a, 2008). Therefore, we used such rats because long-term diabetic conditions entail severe diabetic complications associated with cardiovascular dysfunction and because no previous study has investigated whether the endothelial dysfunction seen in mesenteric arteries in the established phase of diabetes might be improved by long-term treatment with EPA.

In the present OLETF rats, plasma glucose, cholesterol, triglyceride, and serum NEFA concentrations were all raised versus those of LETO rats. When we administered 300 mg/kg/day EPA for 4 weeks to such established OLETF rats, the EPA did not affect the blood glucose, insulin, cholesterol, or NEFA levels, although it did slightly lower blood triglyceride and significantly elevate the HDL level. EPA has beneficial effects on lipid metabolism (Din et al., 2004; von Schacky, 2006) yet apparently exerts vasculoprotective influences, even though the animals concerned retained a degree of hyperglycemia, hypercholesterolemia, and hypertriglyceremia. This is supported by evidence that EPA has such vasculoprotective effects as an ability to delay or prevent the progression of atherosclerosis by an action that is beyond its lipolowering one (Mita et al., 2007).

A novel, intriguing, and potentially important finding of this study was that EPA enhances the actions of opposing endothelium-derived factors, in particular, EDCF signaling in addition to EDRF signaling. Long-term treatment of OLETF rats with EPA led to an increase in the NO-mediated relaxation that seemed to be due to a normalization of NO metabolism, rather than to increased eNOS expression. However, the beneficial effects of EPA on endothelial function in diabetic mesenteric arteries could be due to a normalization of endothelium-derived factors other than NO. The evidence in favor of this idea is that the diminished endothelium-dependent relaxation seen in diabetic OLETF rat mesenteric arteries long-term was associated with a marked attenuation of EDHF-mediated responses and increased production of COX-derived contractile prostanoids (EDCF) but little alteration in the NO-mediated responses.

EDHF is thought to play important roles in the regulation of blood pressure and in the development of diabetic microvascular complications because the contribution made by EDHF-mediated responses seems significantly greater in small arteries than in large arteries (Busse et al., 2002; Félotou and Vanhoutte, 2004; Matsumoto et al., 2006a). Although several reports have suggested that treatment with n-3 PUFAs leads to improvements in EDHF-mediated responses (Nagao et al., 1995; Félotou and Vanhoutte, 2004), to our knowledge, the present study is the first to find that EPA normalizes EDHF-mediated signaling in diabetic arteries. Despite numerous studies aimed at identifying a specific factor responsible for endothelium-dependent hyperpolarization, the putative mediator of the EDHF response has not been firmly identified; it may actually be one, or a combination, of a number of candidates (Busse et al., 2002; Félotou and Vanhoutte, 2004; Matsumoto et al., 2006a). We previously demonstrated that ACh-induced EDHF-mediated relaxation, which proved to be sensitive to a gap-junction inhibitor, is impaired in the mesenteric arteries of type 1 (Matsumoto et al., 2003) and type 2 (Matsumoto et al., 2006c) diabetic rats. Furthermore, in OLETF mesenteric arteries, this impairment may be due to defective cAMP/protein kinase A signaling and/or reduced endothelial KCa activity (Matsumoto et al., 2006c). There are several reports suggesting that EPA may affect putative molecules involved in EDHF signaling. For instance, EPA prevents the impairment of gap-junctional intercellular communication that is induced by hypoxia/reoxygenation in endothelial cells (Zhang et al., 2002) and exerts an antiarrhythmic effect via activation of protein kinase A (Szentandrásy et al., 2007). Moreover, the present EPA treatment reduced the production of prostanooids, substances that cause TP activation. This effect of EPA treatment would be expected to increase EDHF signaling because activation of the TP leads to a reduction in small-conductance KCa channel activity (Crane and Garland, 2004), which is of crucial importance in the initiation of the EDHF signal after endothelial stimulation (Busse et al., 2002; Félotou and Vanhoutte, 2004). On the basis of the previously published evidence and our data, we suggest that the improvement in EDHF signaling seen in long-term EPA-treated OLETF rats may be due to changes in these signalings.

In our previous experiments on mesenteric arteries (Matsumoto et al., 2007a), we found the following: 1) In the OLETF group, endothelium-dependent contraction was abolished by selective COX-1 and/or COX-2 inhibitors. In contrast, in the LETO group, the selective COX-2 inhibitor caused no reduction in this contraction. 2) In both OLETF and LETO, the EDCF-mediated response was completely blocked by a TP antagonist. 3) The protein expressions of COX-1 and COX-2 were augmented in mesenteric arteries from OLETF rats compared with those from LETO rats. These results suggest that in a disease such as type 2 diabetes, the observed reduction in the relaxation induced by an endothelium-dependent dilator can be attribu-
uted, at least in part, to an enhanced production of EDCF via COX-1 and COX-2, with EDCF activating TPs. This is supported by evidence from some hypertensive or diabetic models (Garcia-Cohen et al., 2000; Bagi et al., 2005; Alvarez et al., 2007). Moreover, although PGL2 is generally described as acting as an EDRF by stimulating the prostacyclin receptor receptor, PGL2 can also act as an EDCF by activating TPs in conditions such as hypertension and aging (Vanhoutte et al., 2005; Féletou and Vanhoutte, 2006). In the present study, the ACH-induced COX-mediated response was vasoconstriction rather than vasodilation. Moreover, the stable PGL2 analog beraprost induced vasoconstriction rather than vasodilation in mesenteric arteries isolated from 50- to 54-week-old LETO and OLETF rats (unpublished observation). These results are consistent with PGL2 acting as an EDCF in our model because prostacyclin receptors are no longer functional, as previously reported for a hypertensive model (Vanhoutte et al., 2005; Féletou and Vanhoutte, 2006).

Although COX-1 is constitutively expressed, COX-2 is induced by various inflammatory factors. Enhanced COX-2 expression is found in inflammation-associated cardiovascular diseases such as hypertension and type 2 diabetes (Garcia-Cohen et al., 2000; Bagi et al., 2005; Alvarez et al., 2007). Massaro et al. (2006) demonstrated that n-3 PUFA could directly suppress COX-2 induction through decreased NF-κB activation and decreased binding of NF-κB to the COX-2 promoter. In that study, n-3 PUFA decreased NF-κB activation by decreasing both the generation of cytokine-stimulated reactive oxygen species and ERK activation (Massaro et al., 2006). Furthermore, Diaz Encarnacion et al. (2008) recently reported that fish oil containing a high content of n-3 PUFAs ameliorates renal injury in hypertensive rats through an inhibition of ERK, decreased NF-κB activation, and inhibition of COX-2 expression. In the present study, we found that in OLETF rats, 1) the elevated levels of NF-κB activity and ERK activity in the aorta were decreased by EPA and that 2) the elevated levels of COX-2 and ERK activity in mesenteric arteries were also suppressed by EPA. Because of the difficulty of obtaining a sufficient weight of tissue, we did not directly assess NF-κB activity in mesenteric arteries; however, based on the above results and relevant previous evidence, we speculate that EPA may suppress COX-2 expression via reductions in the activities of NF-κB and ERK.

Another possible explanation for the beneficial effect of EPA on AA homeostasis is that it inhibits COX activity by competing with AA. Elevated EPA/AA ratios reduce the production of two-series PGs and increase the production of three-series PGs, including TXA3, PGE3, and PGL3 (Din et al., 2004; Deeb et al., 2008). The two-series PGs derived from AA are proinflammatory and proaggregatory, whereas the three-series PGs derived from n-3 PUFAs are anti-inflammatory and inhibit platelet aggregation. Moreover, TXA3 is a much less potent vasoconstrictor than TXA2 (Din et al., 2004; Deeb et al., 2008). In the present study, although we did not determine the amount of three-series PGs produced by EPA-treated OLETF rat mesenteric arteries, we did demonstrate in OLETF rats that 1) the enhanced production of 2-series PGs was significantly suppressed by EPA and 2) the enhanced AA-mediated contraction was significantly reduced by EPA, as were EDCF-mediated responses. To judge from these results and relevant previous evidence, EPA may reduce EDCF signalings through a normalization of AA homeostasis via the above multiple signaling pathways.

Our results provide the first evidence of the potential of EPA as a therapeutic drug for the improvement of EDHF- and/or EDCF-mediated responses in type 2 diabetes. Furthermore, we suggest that EPA has a blood pressure-lowering effect in our diabetic rats, as it does in several hypertensive models (Din et al., 2004; von Schacky, 2006; Diaz Encarnacion et al., 2008). This may be attributable to improvements in EDHF- and/or EDCF-mediated responses because such responses may contribute to blood pressure homeostasis (Féletou and Vanhoutte, 2004, 2006; Vanhoutte et al., 2005). However, another possibility is that the improvement of endothelial function brought about by long-term EPA treatment is secondary to the reduction in blood pressure. Several reports have demonstrated that improvements in EDHF signaling or EDCF signaling can result from blood pressure-lowering therapy (Féletou and Vanhoutte, 2004, 2006). At present, which of the above EPA-mediated events (namely, improvement in endothelial function or lowering of blood pressure) is the primary effect of such treatment remains unclear. Therefore, future research will need to focus, for example, on the time course of changes in blood pressure and endothelial functions in animals in a diabetic state.

In conclusion, our study suggests that long-term EPA treatment of OLETF rats improves endothelial functions by correcting the existing imbalance between signalings by endothelium-derived factors and that the beneficial effects of EPA may be due to inhibition of ERK activation, decrease in NF-κB activation, and inhibition of COX-2 expression. These findings not only support the beneficial effects of EPA previously demonstrated in large intervention studies of cardiovascular disease but also offer a credible explanation for the beneficial effects that EPA has on the vascular system in type 2 diabetes.

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References


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