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TITLE PAGE

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

Takayuki Matsumoto, Naoaki Nakayama, Keiko Ishida, Tsuneo Kobayashi and Katsuo Kamata

Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan (T.M., N.N., K.I., T.K., K.K.)

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Corresponding author: Katsuo Kamata, Ph.D.

Department of Physiology and Morphology, Institute of Medicinal Chemistry,

Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan

Tel. and Fax.: +81-3-5498-5856

E-mail: kamata@hoshi.ac.jp

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ABBREVIATIONS: AA, arachidonic acid; ACh, acetylcholine; ANOVA, analysis of variance; BCA, bicinchoninic acid; COX, cyclooxygenase; EDCF, endothelium-derived contracting factor; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxing factor; EIA, enzyme immunoassay; ELISA, enzyme-linked immunosorbent assay; eNOS, endothelial nitric oxide synthase; EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; HDL, high-density lipoprotein; HRP, horseradish peroxidase; IP, prostacyclin receptor; JELIS, Japan EPA Lipid Intervention Study; K_{Ca}, calcium-activated potassium channel; KHS, Krebs-Henseleit solution; LETO, Long-Evans Tokushima Otsuka; L-NNA, N^{G}-nitro-L-arginine; NEFA, non-esterified fatty acid; NF-κB, nuclear factor-kappa B;

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NO, nitric oxide; NOS, nitric oxide synthase; PE, phenylephrine; p-ERK, phospho-extracellular signal-regulated kinase; PG, prostaglandin; PKA, protein kinase A; PUFAs, polyunsaturated fatty acids; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SK_{Ca}, small-conductance calcium-activated potassium channel; SNP, sodium nitroprusside; TRAM-34, [1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole]; TP, thromboxane receptor; TX, thromboxane

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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 rats (OLETF) exhibit endothelial dysfunction, leading to an imbalance between endothelium-derived vasodilators [viz. nitric oxide (NO) and hyperpolarizing factor (EDHF)] and vasoconstrictors (EDCF) [viz. cyclooxygenase (COX)-derived prostanoids] (*Am J 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 [as compared with age-matched control Long-Evans Tokushima Otsuka (LETO) rats]: 1) ACh-induced (endothelium-dependent) relaxation was impaired, 2) NO- and EDHF-mediated relaxations and nitrite production were reduced, 3) ACh-induced EDCF-mediated contraction and prostanoids production, and the protein expressions of COX-1 and COX-2 were all increased. When OLETF rats received chronic EPA treatment (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 prostanoids production, 5) reduced COX-2 expression, and 6) reduced phospho-extracellular signal-regulated kinase (p-ERK) expression. Moreover, EPA treatment reduced both ERK2 and nuclear factor-kappa B (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.

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INTRODUCTION

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; Feletou 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 as well as in type 1 diabetes (Kamata et al., 1989; Hattori et al., 1991; 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; Matsumoto et al., 2006a; Vanhoutte et al., 2005). 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 (n-3 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 (JELIS), has demonstrated a significant

reduction in the incidence of major coronary events following the addition of highly purified eicosapentaenoic acid (EPA), which is the only type of n-3 PUFA used clinically to treat the hyperlipidemia 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). Indeed, recent evidence indicates that n-3 PUFA may have an anti-inflammatory effect, an anti-atherosclerotic effect, an anti-arrhythmic effect, a blood pressure-lowering effect, a triglyceride-lowering effect, and an ameliorating effect on insulin resistance, and may also help to prevent endothelial dysfunction (Huang et al., 1997; Kusunoki et al., 2003; Din et al., 2004; von Schacky, 2006). However, the molecular mechanism(s) mediating such beneficial effects of n-3 PUFAs are not fully understood.

Otsuka Long-Evans Tokushima fatty (OLETF) rats manifest stable clinical and pathological features that resemble human type 2 diabetes (Kawano et al., 1992), and there are several reports of abnormalities of vascular function in this diabetic model (Kagota et al., 2000; Matsumoto et al., 2006b,c, 2007b). Moreover, we recently demonstrated (Matsumoto et al., 2007a): 1) that endothelial dysfunction is present in mesenteric arteries isolated from OLETF rats, 2) that this may be due to endothelium-derived factors developing an imbalance involving reduced EDRFs signaling and increased EDCFs signaling, and that the mechanisms underlying this abnormality may involve increments in the activities of both cyclooxygenase (COX)-1 and COX-2. We therefore proposed important roles for COX-derived constrictor prostaglandins (PGs) in the altered regulation of mesenteric arterial responsiveness seen in OLETF rats (Matsumoto et al., 2007a). Various studies on animal models of cardiovascular diseases have demonstrated disease prevention when treatment is started prior to the onset of complications (Huang et al., 1997; Din et al., 2004; von Schacky, 2006). However, treatment of diabetes patients does not begin until after the symptoms

have been diagnosed, a time at which in many instances complications are already present. Little information is available as to whether n-3 PUFAs can correct diabetic complications once the progression of the disease process has begun. The current study was therefore designed to examine the efficacy of EPA at reducing dysfunction in arteries from animals at the stage the type 2 diabetes when complications are already evident.

The aims of our study were to assess the effects of chronic 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.

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METHODS

Drugs and solutions

Apamin, arachidonic acid (AA), TRAM-34 [1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole], phenylephrine (PE), indomethacin, N^G-nitro-L-arginine (L-NNA), sodium nitroprusside (SNP), and monoclonal β-actin antibody were all purchased from Sigma Chemical Co. (St. Louis, MO), while acetylcholine chloride (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), while the antibodies against endothelial NOS (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 dimethylsulfoxide, while indomethacin was dissolved first in a small amount of 0.1 M Na_2CO_3 solution, 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 46-50 weeks old. For the chronic study, some OLETF and LETO rats were chronically given EPA (100 or 300 mg/kg/day, p.o.) for 4 weeks starting at 46-50 weeks old. Thus, we studied four groups: vehicle (5% gum arabic solution)-treated LETO and OLETF groups and EPA (100 mg/kg/day)- or EPA (300 mg/kg/day)-treated OLETF (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 *"Guide for the Care and Use of Laboratory Animals"* published by the US National Institutes of Health, and *"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). Briefly, plasma glucose, cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol, and serum non-esterified fatty acid (NEFA) levels were each determined by the use of a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan). Plasma insulin was measured by enzyme-immunoassay (EIA) (Shibayagi, 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 50-54 weeks of age, rats were anesthetized with diethyl ether, then euthanized by decapitation. The superior mesenteric artery was rapidly removed and immersed in oxygenated, modified Krebs-Henseleit solution (KHS). This solution consisted of (in mM) 118.0 NaCl, 4.7 KCl, 25.0 $NaHCO_3$, 1.8 $CaCl_2$, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, and 11.0 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% O_2- 5% CO_2) 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 g, 1.58 ± 0.03 g, 1.58 ± 0.04 g, 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 EDRF 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 (NOS inhibitor) (to investigate EDHF-type relaxation), 2) 10 μM indomethacin plus 10 μM TRAM-34 (specific inhibitor of the intermediate-conductance K_{Ca} channel) plus 100 nM apamin [specific inhibitor of the small-conductance K_{Ca} channel (SK_{Ca})] [co-treatment with these two K_{Ca}-channel inhibitors can block EDHF signaling according to a previous report (Busse et al., 2002) and our preliminary experiment] (to investigate NO-mediated relaxation), 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^+, these responses being taken as 100%. There was no significant difference in the response to 80 mM K^+ among the LETO (n = 11), OLETF (n = 12), EPA100 (n = 13), and EPA300 (n = 13) groups (1.63 ± 0.05 g, 1.60 ± 0.02 g, 1.68 ± 0.04 g, and 1.66 ± 0.03 g, respectively). To investigate the EDCF- or AA-mediated response, 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 (NO_2^-) and nitrate (NO_3^-)

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 5000 x g 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 nitrite and nitrate in plasma or KHS were measured using an automated NO detector/high-performance liquid chromatography system (ENO20; Eicom, Kyoto, Japan).

Release of prostaglandins

Prostanoid release was measured as in our previous papers (Matsumoto et al., 2007a, 2008). 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 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 EIA kit (Cayman Chemical, Ann Arbor, MI). Two-time diluted samples were used for measurements of PGE_2, thromboxane B_2 (TXB_2; a stable metabolite of TXA_2), and $PGF_{2\alpha}$, whereas one hundred-time diluted samples were used for the measurement of 6-keto $PGF_{1\alpha}$, 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 pg or ng/ mg 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 before (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 16000 x 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 bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford, IL). Samples (20 μg/lane) were resolved by electrophoresis on 10% SDS-PAGE gels, then transferred onto polyvinylidene difluoride (PVDF) membranes. Briefly, after blocking the residual protein sites on the

membrane with ImmunoBlock (Dainippon-pharm., Osaka, Japan) or PVDF blocking reagent (Toyobo, Osaka Japan), the membrane was incubated with anti-COX-1 (70 kDa; 1: 500), anti-COX-2 (72 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, anti-mouse or anti-rabbit antibody was used at a 1:10000 dilution in Tween PBS, followed by detection using SuperSignal (Pierce, Rockford, IL). 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 monoclonal primary antibody (42 kDa; 1: 5000), 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_2 and physically crushed to a fine powder in liquid N_2 using a Cryo-Press (Microtech Nichion, Chiba, Japan). Following lysation of these powder samples, phosphorylated ERK2 was lysed and its level quantified using a Human/Mouse/Rat Phospho-ERK2 (T185/Y187) Surveyor IC immunoassay system (R&D Systems Inc., 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, 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 (Carlsbad, CA) 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′-GGGACTTTCC-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 KCl. When appropriate, statistical differences were assessed by Dunnett's test for multiple comparisons after a one-way analysis of variance (ANOVA), a probability level of $P < 0.05$ being regarded as significant. Statistical comparisons between concentration-response curves were made using a two-way ANOVA, with Bonferroni's correction for multiple comparisons being performed post hoc ($P < 0.05$ again being considered significant).

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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, while 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, while HDL and insulin levels were similar between the two groups (Table 2). Treatment of OLETF rats with EPA at100 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 being at 100 – 300 nM, and responses then being progressively weaker up to 10 μM. This relaxation was significantly weaker in rings from OLETF rats than in those from LETO rats. Compared to 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 10 μM indomethacin, 100 nM apamin, and 10 μM TRAM-34. Under these conditions, the NO-mediated relaxation was slightly but significantly weaker in rings from OLETF rats than in those from 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 (a) significantly weaker in rings from OLETF rats than in those from LETO rats (Fig. 2B), and (b) 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).

Like 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 relaxating 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. 2C). To investigate the contractile component of the ACh-induced response, we added ACh (10 nM – 10 μM) cumulatively to rings in the 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 (a) significantly greater in the OLETF than in the LETO group (Fig. 3A), and (b) significantly weaker in the

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EPA100 group than in the OLETF group, and weaker still in the EPA300 group (Fig. 3A). Similarly, when we added AA (100 nM – 10 μM) cumulatively to rings in the presence of L-NNA (100 μM), a contractile response was seen in all groups (Fig. 3B). This contraction was (a) significantly greater in OLETF than in LETO, (b) unaffected by chronic EPA treatment at 100 mg/kg/day for 4 weeks, but (c) normalized by EPA at 300 mg/kg/day for 4 weeks to a level similar to that seen in the nondiabetic LETO group (Fig. 3B).

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-to-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 chronic EPA treatment, we measured the plasma levels of nitrite and nitrate as a measure of NO synthesis (Table 3). The plasma nitrite level was significantly lower in OLETF rats than in LETO rats, whereas the plasma nitrate level and nitrate-to-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 4 week's EPA (100 mg/kg/day) treatment; however, both the nitrite level and the nitrate-to-nitrite ratio in the plasma were normalized in OLETF rats treated with EPA at 300 mg/kg/day (Table 3).

Following stimulation with 10 μM ACh, the mesenteric artery nitrite level was significantly lower in the OLETF group than in LETO, and the nitrate-to-nitrite ratio tended to be higher in OLETF than in LETO (Table 3). Compared to 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-to-nitrite ratio versus the OLETF group (Table 3).

Effects of EPA on endothelium-stimulated release of prostanoids in OLETF rats.

Since 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 chronic EPA treatment on the endothelium-stimulated release of prostanoids in mesenteric arteries from all four groups (Fig. 4). ACh (10 μM) induced release of TXB_2 (stable metabolite of TXA_2; Fig. 4A), PGE_2 (Fig. 4B), $PGF_{2\alpha}$ (Fig. 4C), and 6-keto-$PGF_{1\alpha}$ (stable metabolite of PGI_2; Fig. 4D) in rings from all four groups, the endothelium-stimulated release of TXB_2 and that of PGE_2 being significantly greater in the OLETF group than in LETO. In contrast, the productions of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were not different between LETO and OLETF. Compared to those in the OLETF group, the productions of TXB_2, PGE_2, and 6-keto-$PGF_{1\alpha}$ tended to be decreased in the EPA100 group, but to our surprise release of all four prostanoids was significantly suppressed in the EPA 300 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 (viz. in EPA100 or EPA300 vs.

OLETF), but surprisingly the level of COX-2 expression tended to be reduced (EPA100) or was reduced (EPA300) by chronic EPA treatment ($P = 0.057$, EPA100 vs. OLETF; $P < 0.05$, EPA300 vs. OLETF) (Fig. 5B). The eNOS protein expression level was not different among the four groups (Fig. 5C).

Effects of EPA on aortic NF-κB activity in OLETF rats.

It is known that activation of NF-κB 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-κB activation. Nuclear extracts prepared from aortic tissue were subjected to ELISA using a Trans-AM kit (Fig. 6A). NF-κB activity tended to be greater in OLETF than in LETO (Fig. 6A). Compared to its level in the OLETF group, this activity was not altered in the EPA100 group, while in the EPA300 group it was significantly reduced, to a level similar to that seen in the LETO group (Fig. 6A).

Effects of EPA on ERK activity in OLETF rats.

We (Matsumoto et al., 2006b) and others (Jiang et al., 1999) have reported that activation of ERK is associated with vascular dysfunction in diabetic states. We therefore sought to test the hypothesis that EPA that reduce vascular ERK activity in OLETF rats (Fig. 6B and Fig. 7). Phosphorylated ERK2 activity in the thoracic aorta tended to be greater in OLETF than in LETO. In the EPA300 group, p-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, while 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, since 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). Briefly, OLETF rats are characterized by 1) increasing 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) a hyperinsulinemia that is present at 24 weeks of age and declines after 55 weeks of age, and conversion to insulin-dependent diabetes after ~40 weeks of age (Kawano et al., 1992). In the present study, we used 50- to 54-week-old OLETF rats, which are have established diabetes, their blood glucose levels being around 500 mg/dl. Moreover, we and others have demonstrated that abnormalities of vascular function are present in several arteries in OLETF rats (Kagota et al., 2000; Matsumoto et al., 2006b,c, 2007b), and there is recent evidence of an imbalance between endothelium-derived factors, such as decreased EDRFs signaling and increased EDCFs signaling, in mesenteric arteries from OLETF rats at the chronic stage of diabetes (Matsumoto et al., 2007a, 2008b). 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 chronic treatment with EPA.

In the present OLETF rats, the plasma glucose, cholesterol, and triglyceride, and serum NEFA concentrations were all raised versus those of LETO rats. When we chronically 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 retain a degree of hyperglycemia, hypercholesterolemia, and hypertriglycemia. 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 lipid-lowering one (Mita et al., 2007).

A novel, intriguing, and potentially important finding made in this study was that EPA enhances the actions of opposing endothelium-derived factors: viz. not only EDRFs signaling, but also EDCF signaling. Chronic 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 favour of this idea is that the diminished endothelium-dependent relaxation seen in chronic diabetic OLETF rat mesenteric arteries 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 since the contribution made by EDHF-mediated responses appears significantly greater in small than in large arteries (Busse et al., 2002; Feletou 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; Feletou 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; Feletou 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/PKA signaling and/or reduced endothelial K_{Ca} activity (Matsumoto et al., 2006c). There are several reports suggesting that EPA may affect putative molecules involved in EDHF signaling. For instance, EPA (a) prevents the impairment of gap-junctional intercellular communication that is induced by hypoxia/reoxygenation in endothelial cells (Zhang et al., 2002), and (b) exerts an antiarrhythmic effect via activation of PKA (Szentandrassy et al., 2007). Moreover, the present EPA treatment reduced the production of prostanoids, substances that cause TP-receptor activation. This effect of EPA treatment would be expected to increase EDHF signaling since activation of the TP receptor leads to a reduction in SK_{Ca}-channel activity (Crane and Garland, 2004), channel activity that is of crucial importance in the initiation of the EDHF signal after endothelial stimulation (Busse et al., 2002; Feletou and Vanhoutte, 2004). On the basis of the previously published evidence and our data, we suggest that the improvement in EDHF signaling seen in chronically 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-receptor antagonist. 3) The protein expressions of COX-1 and COX-2 were augmented in mesenteric arteries from OLETF rats as 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 attributed, at least in part, to an enhanced production of EDCF via COX-1 and COX-2, with this EDCF then activating TP receptors. Indeed, 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 PGI_2 is generally described as acting as an EDRF by stimulating the IP receptor, PGI_2 can also act as an EDCF by activating TP receptors in conditions such as hypertension and aging (Feletou and Vanhoutte, 2006; Vanhoutte et al., 2005). In the present study, the ACh-induced COX-mediated response was vasoconstriction rather than vasodilation. Moreover, the stable PGI_2 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 PGI_2 acting as an EDCF in our model because IP receptors are no longer functional, as previously reported for a hypertensive model (Feletou and Vanhoutte, 2006; Vanhoutte et al., 2005).

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, 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, but on the basis of the above results and the 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 2-series PGs and increase the production of 3-series PGs, including TXA_3, PGE_3, and PGI_3 (Din et al., 2004; Deeb et al., 2008). The 2-series PGs derived from AA are pro-inflammatory and pro-aggregatory, whereas the 3-series PGs derived from n-3 PUFAs are anti-inflammatory and inhibit platelet aggregation. Moreover, TXA_3 is a much less potent vasoconstrictor than TXA_2 (Din et al., 2004; Deeb et al., 2008). In the present study, although we did not determine the amount of 3-series PGs produced by EPA-treated OLETF rat mesenteric arteries, we did demonstrate that in OLETF rats: (a) the enhanced production of 2-series PGs was significantly suppressed by EPA, and (b) the enhanced AA-mediated contraction was significantly reduced by EPA, as were EDCF-mediated responses. To judge from these results and the 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. Further, 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 since such responses may contribute to blood-pressure homeostasis (Feletou and Vanhoutte, 2004, 2006; Vanhoutte et al., 2005). However, another possibility is that the improvement of endothelial function brought about by chronic EPA treatment is secondary to the reduction in blood pressure. Indeed, several reports have demonstrated that improvements in EDHF signaling or EDCF signaling can result from blood pressure-lowering therapy (Feletou and Vanhoutte, 2004, 2006). At present, which of the above EPA-mediated events (viz. improvement in endothelial function or lowering of blood pressure) is the primary effect of such treatment remains unclear. Future research will therefore 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 chronic 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, a 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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FOOTNOTES

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Address for correspondence:

Dr Katsuo Kamata, Department of Physiology and Morphology, Institute of Medicinal Chemistry,Hoshi University, Shinagawa-ku, Tokyo 142-8501, Japan. E-mail: kamata@hoshi.ac.jp

LEGENDS FOR FIGURES

Figure 1: EPA improves endothelium-dependent relaxation in OLETF rats.

Concentration-response curves for ACh (A)- and SNP (B)-induced responses in isolated rings of 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 METHODS. Data are means ± S.E.M. from n = 8 (A) or n = 5 (B) experiments. *P < 0.05, **P < 0.01, ***P < 0.001, LETO vs. OLETF group. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, OLETF vs. EPA300 group.

Figure 2: EPA affects various endothelium-derived relaxing factors [viz. NO (A), EDHF (B), and COX-derived factor (C)] in OLETF rats.

Concentration-response curves for ACh-induced responses in isolated rings of mesenteric arteries obtained from LETO, OLETF, and EPA (100 or 300 mg/kg/day for 4 weeks)-treated OLETF (EPA100 or EPA300) rats. Data were obtained in the presence of the following drugs: 10 μM indomethacin plus 100 nM apamin plus 10 μM TRAM-34 (A), 10 μM indomethacin plus 100 μM L-NNA (B), or 100 μM L-NNA plus100 nM apamin plus 10 μM TRAM-34 (C). Details are given under METHODS. Data are means ± S.E.M. from n = 8 (A), n = 7 (B), or n = 8 (C) experiments. *P < 0.05, **P < 0.01, ***P < 0.001, LETO group vs. OLETF group. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, OLETF group vs. EPA300 group.

Figure 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 L-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 METHODS. Data are means ± S.E.M. from n = 7 (A) or n = 4-6 (B) experiments. *P < 0.05, ***P < 0.001 LETO group vs. OLETF group. †P < 0.05, OLETF group vs. EPA100 group. $^{\#}$P < 0.05, $^{\#\#\#}$P < 0.001, OLETF group vs. EPA300 group.

Figure 4: EPA suppresses endothelium-stimulated prostanoid release in OLETF rats.

Release of prostanoids [TXB_2 (stable metabolite of TXA_2) (A), PGE_2 (B), $PGF_{2\alpha}$ (C), and 6-keto-$PGF_{1\alpha}$ (stable metabolite of prostacyclin) (D)] evoked by 10 μM ACh in mesenteric artery rings isolated from LETO, OLETF, and EPA (100 or 300 mg/kg/day for 4 weeks)-treated OLETF (EPA100 or EPA300) rats. Details are given under METHODS. Data are means ± S.E.M. from n = 8 experiments. **P < 0.01, ***P < 0.001, LETO group vs. OLETF group. $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, $^{\#\#\#}$P < 0.001, OLETF group vs. EPA300 group.

Figure 5: EPA suppresses COX-2 expression in OLETF mesenteric arteries.

Analysis of COX-1 (A), COX-2 (B), and eNOS (C) protein expressions in mesenteric arteries obtained from LETO, OLETF, and EPA (100 or 300 mg/kg/day for 4 weeks)-treated OLETF (EPA100 or EPA300) rats. *Upper panels*: representative Western blots for COX-1, COX-2, eNOS, and β-actin. *Lower panels*: bands were quantified as described in METHODS. Ratios were calculated for the optical density of each COX or eNOS over that of β-actin. Details are given under METHODS. Data are means ± S.E.M. from n = 6 experiments. *P < 0.05, **P < 0.01, LETO group vs. OLETF group. $^{\#}$P < 0.05, OLETF group vs. EPA300 group.

Figure 6: Effects of EPA on NF-κB and ERK2 activities in aortae from OLETF rats.

Quantification of NF-κB activity (A) and phosphorylated ERK2 (B) in aortae from LETO, OLETF, and EPA (100 or 300 mg/kg/day for 4 weeks)-treated OLETF (EPA100 or EPA300) rats. Details are given under METHODS. Data are means ± S.E.M. from n = 10 (A) or n = 5 (B) experiments. *P < 0.05, **P < 0.01, OLETF vs. EPA300 group.

Figure 7: Effects of EPA on mesenteric ERK activities in OLETF rats.

Analysis of phosphorylated ERK1/2 and ERK1/2 protein expressions in mesenteric arteries obtained from the four groups. *Left panels*: representative Western blots for phosphorylated ERK1/2 and ERK1/2. The lanes are from the same gel and were noncontiguous. *Right panels*: bands were quantified as described in METHODS. Ratios were calculated for the optical density of phosphorylated ERK1/2 over that of the corresponding ERK1/2. Details are given under METHODS. Data are means ± S.E.M. from n = 6 experiments. *P < 0.05, **P < 0.01, OLETF vs. EPA300 group.

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Table 1. Body weight, blood pressure, and heart rate of the four experimental groups

	Body weight (g)	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Heart rate (beats/min)
LETO (n = 10)	542.2 ± 8.7	112.0 ± 2.3	89.3 ± 2.0	393.2 ± 10.7
OLETF (n = 10)	596.9 ± 19.8*	145.5 ± 3.7***	118.2 ± 3.2***	381.6 ± 14.6
OLETF EPA100 (n = 10)	573.4 ± 31.0	136.5 ± 2.7***	112.6 ± 2.5***	388.3 ± 16.6
OLETF EPA300 (n = 10)	556.2 ± 29.1	128.9 ± 4.4**,##	103.4 ± 4.9*,#	391.6 ± 9.5

Values are means ± S.E.M. LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty; EPA100, EPA (100 mg/kg/day)-treated OLETF group; EPA300, EPA (300 mg/kg/day)-treated OLETF group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. LETO. #P < 0.05, ##P < 0.01 vs. OLETF.

Table 2. Values of blood parameters in the four experimental groups

	LETO (n = 10)	OLETF (n = 10)	OLETF EPA100 (n = 10)	OLETF EPA300 (n = 10)
Glucose (mg/dl)	145.0 ± 5.5	517.0 ± 32.9***	468.0 ± 58.4***	507.5 ± 45.5***
Insulin (ng/ml)	2.71 ± 0.3	3.18 ± 0.5	2.60 ± 0.7	3.20 ± 0.9
Cholesterol (mg/dl)	129.0 ± 4.8	208.0 ± 14.2***	204.8 ± 17.3***	203.7 ± 18.1***
Triglyceride (mg/dl)	142.0 ± 14.2	640.6 ± 57.4***	494.4 ± 107.8**	505.5 ± 47.2***
HDL (mg/dl)	78.3 ± 3.5	78.6 ± 11.1	95.5 ± 10.1	117.2 ± 8.9***, #
NEFA (mEq/l)	0.32 ± 0.02	0.57 ± 0.04***	0.55 ± 0.03***	0.54 ± 0.05***

Values are means ± S.E.M. LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty; EPA100, EPA (100 mg/kg/day)-treated OLETF group; EPA300, EPA (300 mg/kg/day)-treated OLETF group. **P < 0.01, ***P < 0.001 vs. LETO. #P < 0.05 vs. OLETF.

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Table 3. Levels of NO metabolites (nitrite and nitrate) in plasma and in ACh-stimulated mesenteric arteries from the four experimental groups

	LETO	OLETF	OLETF EPA100	OLETF EPA300
Plasma				
Nitrite (nM)	342.5 ± 61.5 (8)	200.1 ± 21.3 (8)*	221.8 ± 33.9 (7)	331.1 ± 49.9 (8)#
Nitrate (μM)	17.6 ± 0.4 (8)	25.9 ± 2.5 (8)**	26.1 ± 3.4 (7)*	24.1 ± 2.6 (8)*
Nitrate-to-nitrite ratio	60.3 ± 7.8 (8)	136.5 ± 15.9 (8)***	139.1 ± 33.5 (7)*	79.4 ± 8.6 (8)##
Mesenteric arteries (ACh stimulation)				
Nitrite (nmol/min/g tissue)	3.4 ± 0.2 (4)	2.0 ± 0.2 (4)**	3.0 ± 0.5 (6)	4.3 ± 0.7 (6)#
Nitrate (nmol/min/g tissue)	45.4 ± 8.3 (4)	41.4 ± 2.1 (4)	36.6 ± 4.0 (6)	38.7 ± 2.8 (6)
Nitrate-to-nitrite ratio	13.8 ± 2.9 (4)	21.5 ± 1.7 (4)	13.8 ± 2.5 (6)	9.6 ± 1.3 (6)###

Values are means ± S.E.M. (no. of determinations within parentheses). LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty; EPA100, EPA (100 mg/kg/day)-treated OLETF group; EPA300, EPA (300 mg/kg/day)-treated OLETF group. *P < 0.05, **P < 0.01, ***P < 0.001 vs. LETO. #P < 0.05, ##P < 0.01, ###P < 0.001 vs. OLETF.

Figure 1

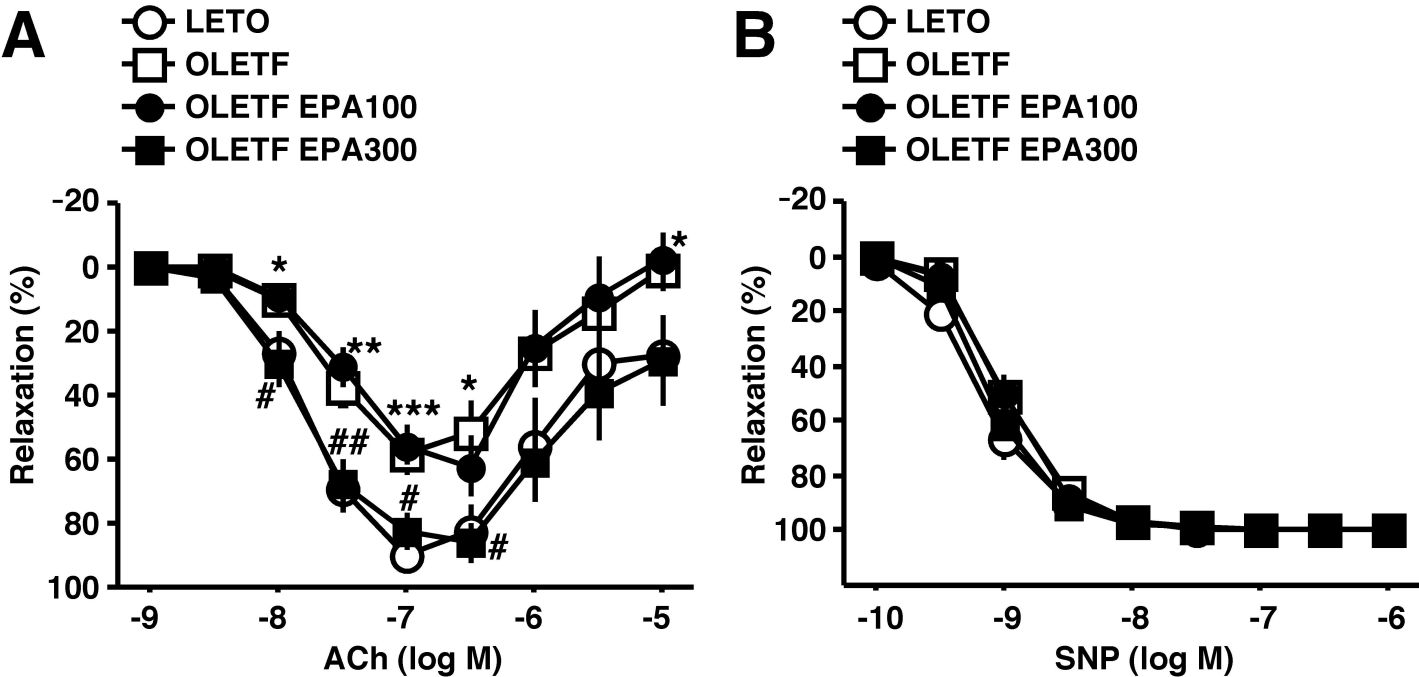

A
LETO
OLETF
OLETF EPA100
OLETF EPA300
Relaxation (%)
-20
0
20
40
60
80
100
-9
-8
-7
-6
-5
ACh (log M)
*
**
***
*
*
#
##
#
#
B
LETO
OLETF
OLETF EPA100
OLETF EPA300
Relaxation (%)
-20
0
20
40
60
80
100
-10
-9
-8
-7
-6
SNP (log M)

Figure 2

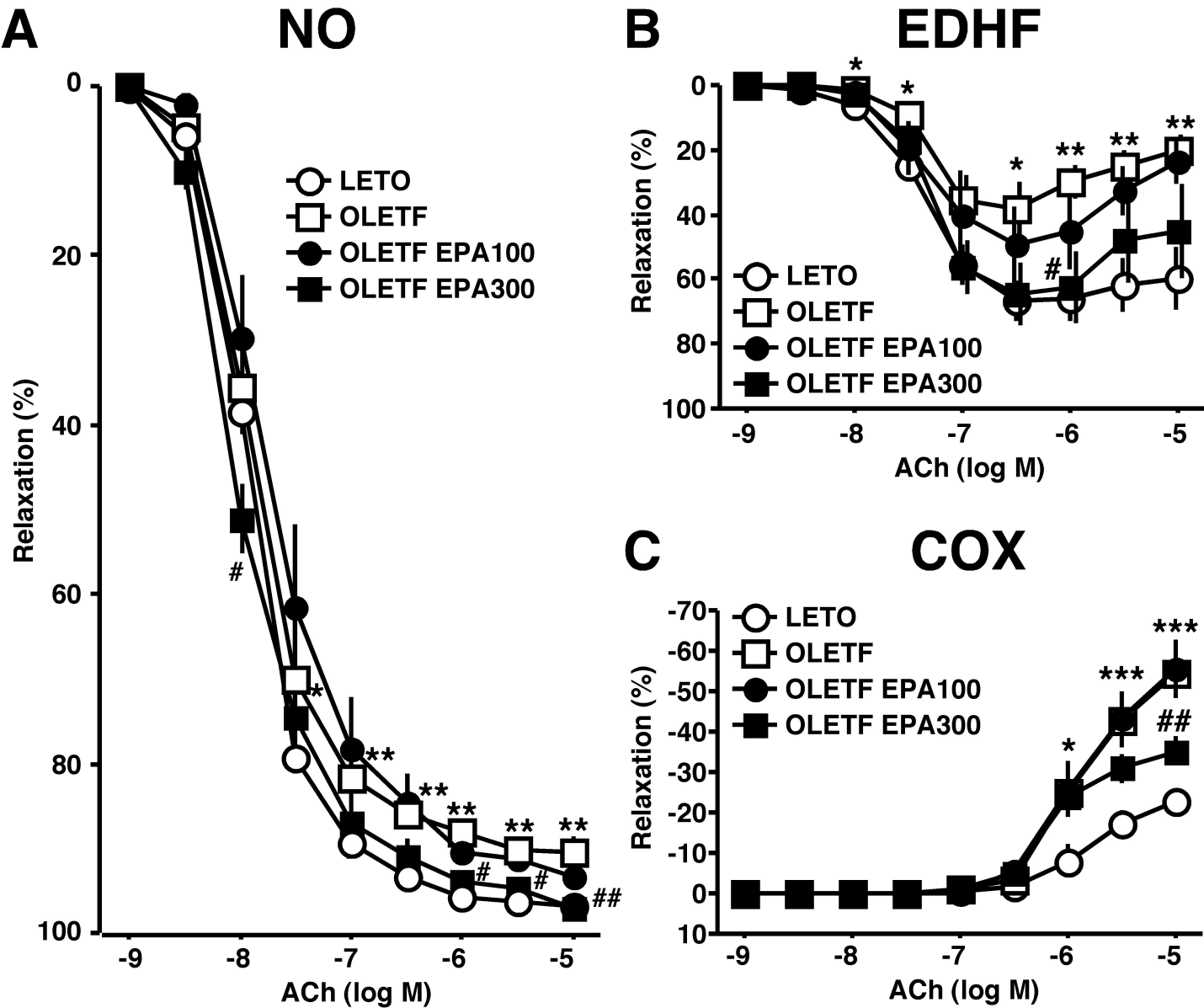

A
NO
B
EDHF
C
COX
LETO
OLETF
OLETF EPA100
OLETF EPA300
Relaxation (%)
ACh (log M)
0
20
40
60
80
100
-9
-8
-7
-6
-5
-70
-60
-50
-40
-30
-20
-10
10
*
**
***
#
##

Figure 3

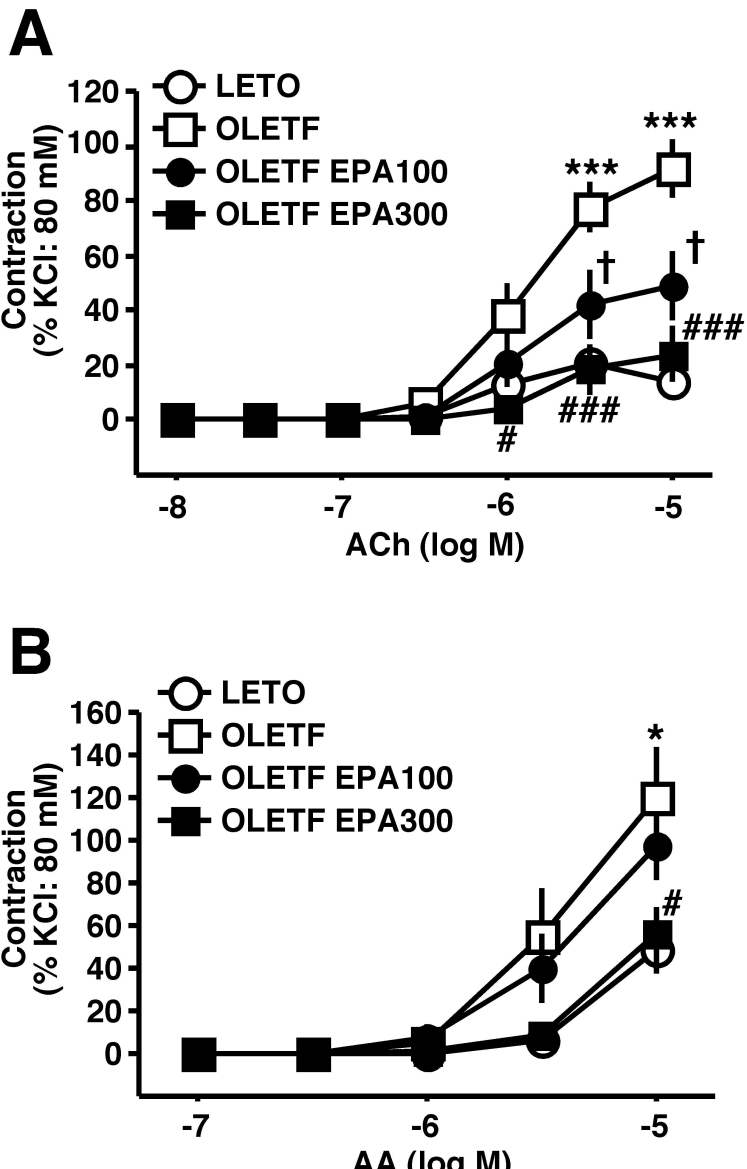

A
LETO
OLETF
OLETF EPA100
OLETF EPA300
Contraction (% KCl: 80 mM)
120
100
80
60
40
20
0
***
***
†
†
###
###
#
-8
-7
-6
-5
ACh (log M)
B
LETO
OLETF
OLETF EPA100
OLETF EPA300
Contraction (% KCl: 80 mM)
160
140
120
100
80
60
40
20
0
*
#
-7
-6
-5
AA (log M)

Figure 4

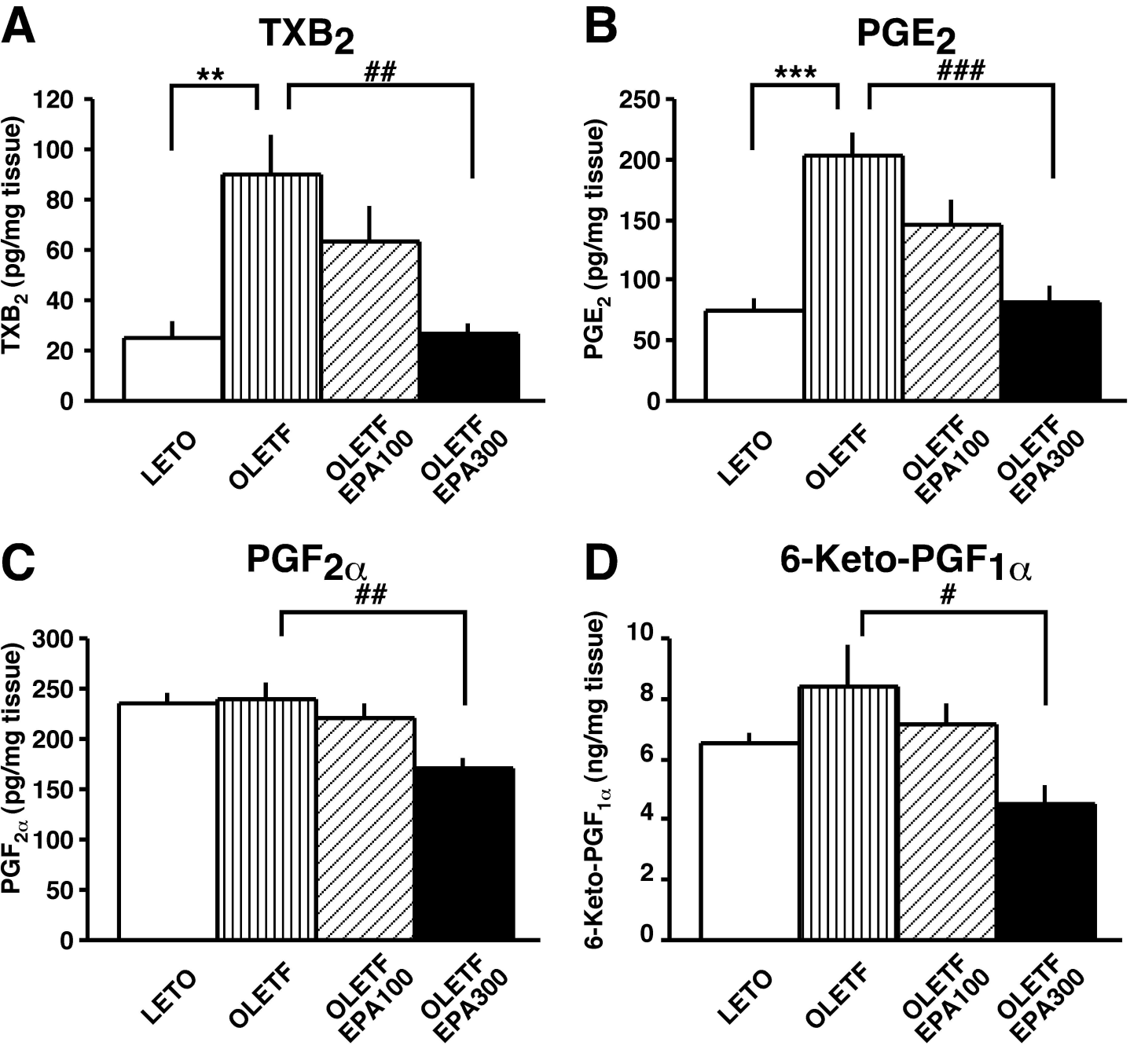

A
TXB2
**
##
TXB2 (pg/mg tissue)
120
100
80
60
40
20
0
LETO
OLETF
OLETF EPA100
OLETF EPA300
B
PGE2
***
###
PGE2 (pg/mg tissue)
250
200
150
100
50
0
LETO
OLETF
OLETF EPA100
OLETF EPA300
C
PGF2α
##
PGF2α (pg/mg tissue)
300
250
200
150
100
50
0
LETO
OLETF
OLETF EPA100
OLETF EPA300
D
6-Keto-PGF1α
#
6-Keto-PGF1α (ng/mg tissue)
10
8
6
4
2
0
LETO
OLETF
OLETF EPA100
OLETF EPA300

Figure 5

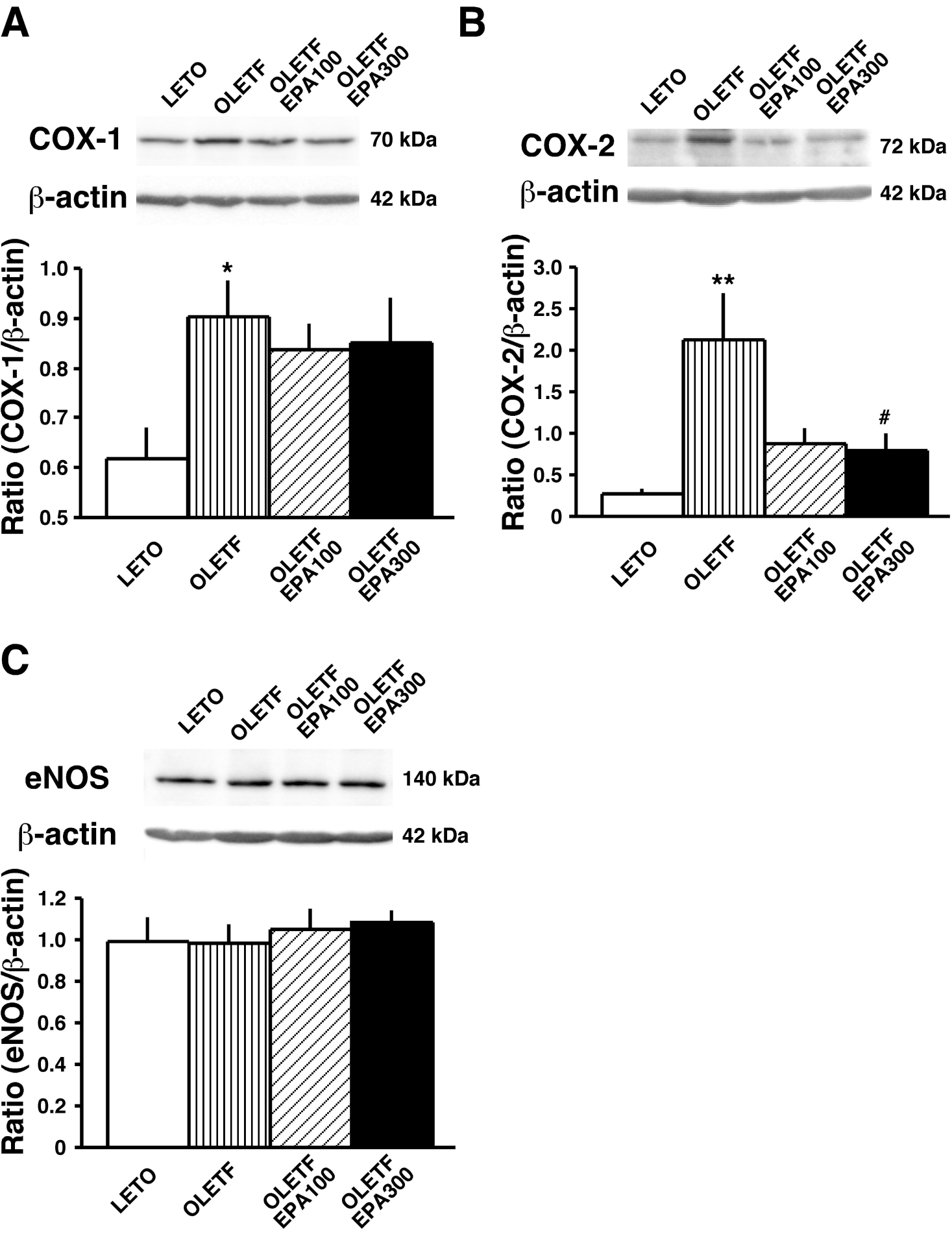

A
LETO
OLETF
OLETF EPA100
OLETF EPA300
COX-1
70 kDa
β-actin
42 kDa
Ratio (COX-1/β-actin)
1.0
0.9
0.8
0.7
0.6
0.5
*
LETO
OLETF
OLETF EPA100
OLETF EPA300
B
LETO
OLETF
OLETF EPA100
OLETF EPA300
COX-2
72 kDa
β-actin
42 kDa
Ratio (COX-2/β-actin)
3.0
2.5
2.0
1.5
1.0
0.5
0
**
#
LETO
OLETF
OLETF EPA100
OLETF EPA300
C
LETO
OLETF
OLETF EPA100
OLETF EPA300
eNOS
140 kDa
β-actin
42 kDa
Ratio (eNOS/β-actin)
1.2
1.0
0.8
0.6
0.4
0.2
0
LETO
OLETF
OLETF EPA100
OLETF EPA300

Figure 6

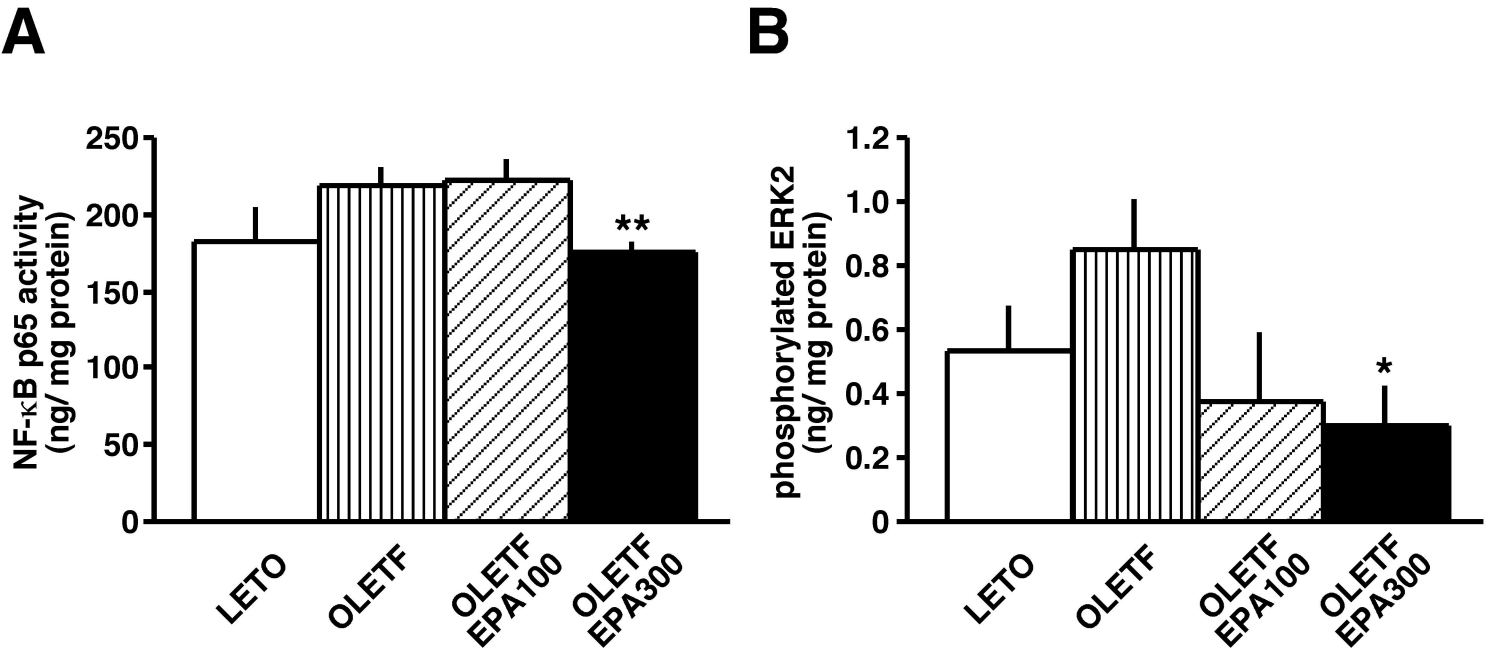
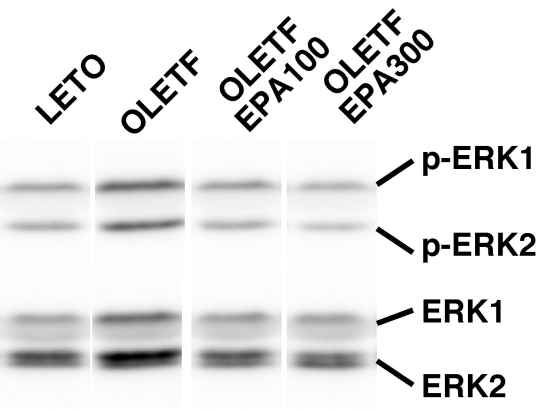
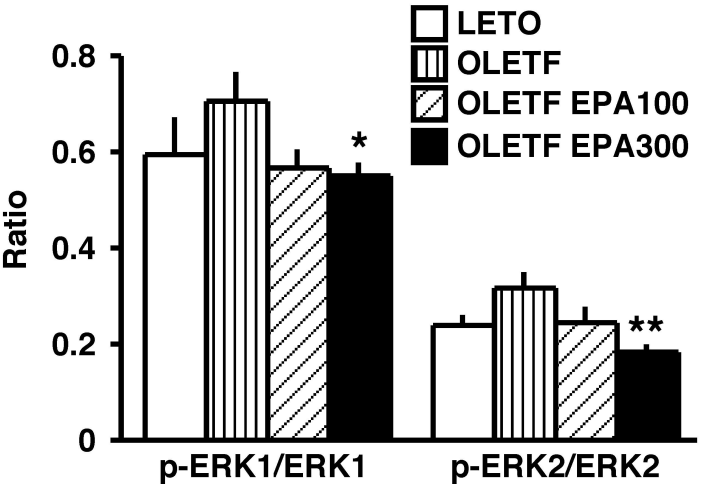

A
B
NF-κB p65 activity
(ng/ mg protein)
250
200
150
100
50
0
**
LETO
OLETF
OLETF EPA100
OLETF EPA300
phosphorylated ERK2
(ng/ mg protein)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
LETO
OLETF
OLETF EPA100
OLETF EPA300

Figure 7


LETO
OLETF
OLETF EPA100
OLETF EPA300
p-ERK1
p-ERK2
ERK1
ERK2


LETO
OLETF
OLETF EPA100
OLETF EPA300
Ratio
0.8
0.6
0.4
0.2
0
*
**
p-ERK1/ERK1
p-ERK2/ERK2