

Imbalance between endothelium-derived relaxing and contracting factors in mesenteric arteries from aged OLETF rats, a model of Type 2 diabetes

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Matsumoto T, Kakami M, Noguchi E, Kobayashi T, Kamata K. Imbalance between endothelium-derived relaxing and contracting factors in mesenteric arteries from aged OLETF rats, a model of Type 2 diabetes. *Am J Physiol Heart Circ Physiol* 293: H1480–H1490, 2007. First published May 18, 2007; doi:10.1152/ajpheart.00229.2007.—We investigated whether the balance between endothelium-derived relaxing factors (EDRFs) and endothelium-derived contracting factors (EDCFs) might be altered in mesenteric arteries from aged Otsuka Long-Evans Tokushima Fatty (OLETF) rats (a Type 2 diabetic model) [vs. age-matched control Long-Evans Tokushima Otsuka (LETO) rats]. ACh-induced relaxation was impaired in the OLETF group, and a tendency for the relaxation to reverse at high ACh concentrations was observed in both groups. This tendency was abolished by indomethacin. Nitric oxide- and/or endothelium-derived hyperpolarizing factor-mediated relaxation and the protein expressions of phospho-endothelial nitric oxide synthase (Ser1177) and extracellular superoxide dismutase were also reduced in OLETF. An ACh-induced contraction was observed at higher ACh concentrations in the presence of N^G -nitro-L-arginine (L-NNA) but was greater in OLETF rats. This contraction in OLETF rats was reduced by cyclooxygenase (COX) inhibitors and by prostanoid-receptor antagonists. The ACh-induced productions of thromboxane A_2 and PGE₂ were greater in OLETF than LETO rats, as were the mesenteric artery COX-1 and COX-2 protein expressions. Moreover, *tert*-butyl hydroperoxide (*t*-BOOH) (membrane-permeant oxidant) induced a concentration-dependent contraction that was greater in OLETF rats. The *t*-BOOH-mediated contraction was increased both by L-NNA and by endothelium removal in LETO but not OLETF rats, suggesting that a negative modulatory role of the endothelium was lost in OLETF rats. These results suggest that an imbalance between EDRFs and EDCFs may be implicated in the endothelial dysfunction seen in aged OLETF mesenteric arteries, and may be attributable to increased oxidative stress.

cyclooxygenase; oxidative stress; prostanoids

TYPE 2 DIABETES MELLITUS, which has reached epidemic proportions in Western countries, is associated with a markedly increased incidence of cardiovascular diseases. However, the exact relations among Type 2 diabetes, obesity, and cardiovascular disease are not completely understood and have been the subject of some dispute. Type 2 diabetes is often part of an array of complex abnormalities referred to as “metabolic syndrome,” which is frequently accompanied by an elevated blood pressure (47).

Vascular tone is controlled by endothelium-derived factors such as relaxing factors [EDRFs; in particular, nitric oxide (NO) and hyperpolarizing factors (EDHF)] and contracting factors (EDCFs) (3, 9, 10, 43, 50). Changes in these factors

may be the cause of changes in resting blood pressure. Several lines of evidence suggest that endothelial dysfunction could play a key role in the development of both macro- and microangiopathy in diabetes patients and in animal models of diabetes (6, 9, 30, 43). An accumulating body of evidence indicates that endothelium-dependent relaxation is impaired in several blood vessels in Type 2 diabetes, both in animal models and in patients (6, 22, 27), as well as in Type 1 diabetes (6, 11, 23, 29, 30, 34, 36, 39). There are several reports suggesting that a decreased production of EDRFs and/or defects in EDRF signaling underlie the impairment of endothelium-dependent relaxation seen in Type 2 diabetic vessels (6, 27, 32, 33). Moreover, this impairment of relaxation may be attributable not only to reduced amounts of EDRF and/or EDHF but also to increased amounts of EDCF, leading to diabetic vasculopathy (6, 27, 30, 50).

Endothelium-dependent contractions are mediated by the products of cyclooxygenases (COXs) (10, 14, 15, 50). The COXs metabolize arachidonic acid to endoperoxides, which can be further broken down by enzymes to form prostaglandin (PG)E₂, PGD₂, PGF_{2 α} , prostacyclin (PGI₂), and thromboxane (Tx)A₂ (4). The precise identity of the prostaglandins accounting for EDCF remains uncertain. One or several of these prostanoids may diffuse across to the smooth muscle and activate thromboxane-prostanoid (TP) receptors, which then leads to an influx of calcium into the smooth muscle cells and consequent contraction (50). EDCF-mediated responses are observed not only in hypertension but also in diabetes, and they probably reflect premature aging of blood vessel walls subjected to exaggerated oxidative stress (10, 50). The available information confirms that in humans EDCF-mediated responses contribute to the blunting of endothelium-dependent relaxation in aged subjects and in essential hypertensive patients (50). Identification of EDCF could therefore provide new insights into the mechanism responsible for endothelial dysfunction, and could potentially reveal new therapeutic targets. Although various candidates have been proposed for the identity of EDCF in animal models of hypertension (50), the identity of EDCF in the Type 2 diabetic mesenteric artery remains unclear.

Otsuka Long-Evans Tokushima Fatty (OLETF) rats are characterized by an early increase in serum insulin and also by late-onset hyperglycemia, mild obesity, and mild Type 2 diabetes (24). There are several reports of abnormalities of vascular function in this diabetic model (22, 31–33, 45). Moreover, we recently demonstrated (32) that the EDHF-type relaxation in the isolated mesenteric artery is impaired in aged

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OLETF rats, and that the mechanisms underlying this impairment may be related to reductions both in cAMP/PKA signaling and in the activities of certain endothelial Ca^{2+} -activated K^+ (K_{Ca}) channels. However, no previous study has investigated whether the balance between EDRFs and EDCFs might be altered in mesenteric arteries in this model.

For the present study, we designed experiments to investigate the diabetes-related changes in ACh-induced relaxation and contraction in the OLETF rat mesenteric artery, and we tried to identify some of the molecular mechanisms involved.

MATERIALS AND METHODS

Reagents. AH-6809, AL-8810, 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34), SQ-29548, *tert*-butyl hydroperoxide (*t*-BOOH), apamin, phenylephrine (PE), indomethacin, *N*^o-nitro-L-arginine (L-NNA), phenylmethylsulfonyl fluoride (PMSF), and monoclonal β -actin antibody were all purchased from Sigma (St. Louis, MO). Resveratrol, NS-398, and antibodies against COX-1 and COX-2 were from Cayman Chemical (Ann Arbor, MI). Drugs were dissolved in saline, except for AH-6809, resveratrol, NS-398, SQ-29548, and TRAM-34 (dissolved in DMSO) and AL-8810 (dissolved in ethanol). Horseradish peroxidase (HRP)-linked secondary anti-mouse and anti-rabbit antibodies were purchased from Promega (Madison, WI), while the antibody against endothelial nitric oxide synthase (eNOS) was from BD Biosciences (San Jose, CA). Phospho-eNOS (Ser1177) antibody was purchased from Cell Signaling Technology (Danvers, MA), while extracellular superoxide dismutase (EC-SOD) antibody was from Upstate (Lake Placid, NY).

Animals and experimental design. Five-week-old male rats [OLETF and Long-Evans Tokushima Otsuka (LETO), a genetic control for OLETF] were supplied by the Tokushima Research Institute (Otsuka Pharmaceutical). Food and water were given *ad libitum* in a controlled environment (room temperature 21–22°C, room humidity 50 ± 5%) until the rats were 60–65 wk old. This study was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health 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).

Measurement of plasma glucose, cholesterol, triglyceride, insulin, adiponectin, and blood pressure. Plasma parameters and blood pressure were measured as described previously (28, 31–38). Briefly, plasma glucose, cholesterol, triglyceride, and high-density lipoprotein (HDL) cholesterol levels were each determined by the use of a commercially available enzyme kit (Wako Chemical, Osaka, Japan). Plasma insulin was measured by enzyme immunoassay (EIA) (Shibayagi, Gunma, Japan). Plasma adiponectin was determined by ELISA (Otsuka Pharmaceutical, Tokyo, 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 with a blood pressure analyzer (BP-98A; Softron, Tokyo, Japan).

Measurement of isometric force. Vascular isometric force was recorded as in our previous papers (29, 32, 34, 37, 38). At 60–65 wk of age, rats were anesthetized with diethyl ether and 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 (~0.1–3 μM) (i.e., so that the tension developed in response to PE was similar between LETO and OLETF groups). There was no significant difference in the response to PE between the LETO ($n = 29$) and OLETF ($n = 29$) groups (1.60 ± 0.05 and 1.68 ± 0.05 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 aliquots of the agonist sufficient 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 various factors that might constitute EDRF in the present preparations, we examined ACh-induced relaxation in the presence of various inhibitors, as follows: 1) 10 μM indomethacin (to investigate the influence of COX metabolites); 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) (to investigate NO-mediated relaxation); 3) 10 μM indomethacin plus 100 μM L-NNA (to investigate EDHF-type relaxation). Rings were incubated with the appropriate inhibitor(s) for 30 min before administration of PE.

For the contraction studies, mesenteric rings were first contracted with 80 mM K^+ , these responses being taken as 100%. The mean contractile responses induced by 80 mM K^+ in OLETF ($n = 46$) and LETO ($n = 44$) groups were 1.66 ± 0.03 and 1.65 ± 0.04 g, respectively (no significant difference). After being washed and equilibrated for 1 h, the mesenteric rings were treated with 100 μM L-NNA for 30 min. After this incubation period, ACh (10 nM–10 μM) was cumulatively applied. To investigate the effects of COX inhibitors or prostanoid receptor antagonists on ACh-mediated contraction, ACh-induced concentration-response curves were also generated by incubating rings for 30 min in the combined presence of L-NNA (100 μM) plus 10 μM indomethacin (nonselective COX inhibitor), 5 μM resveratrol (selective COX-1 inhibitor; Ref. 44), 1 μM NS-398 (selective COX-2 inhibitor; Ref. 14), 3 μM SQ-29548 (TxA₂ receptor antagonist; Refs. 26, 52), 10 μM AH-6809 [less selective PGD₂ receptor (DP)/PGE₂ receptor (EP₁, EP₂) antagonist; Refs. 19, 46], or 10 μM AL-8810 [PGF receptor (FP) antagonist; Refs. 16, 52]. In separate experiments, we investigated the effect on vascular tone induced by *t*-BOOH, a membrane-permeant oxidant that has been used extensively as a model of oxidative stress (12). In these experiments, *t*-BOOH (1 μM –1 mM) was cumulatively applied to mesenteric rings (with or without endothelium) isolated from LETO and OLETF rats. In some of these experiments, 100 μM L-NNA or 100 μM L-NNA plus 10 μM indomethacin was applied 30 min before the *t*-BOOH application and was present thereafter. Contractile responses were each expressed as a percentage of the contraction previously induced by 80 mM KCl. When required, the endothelium was removed from arterial segments by infusing a CHAPS solution (0.1%, for 60 s), which was subsequently flushed out with KHS; the inability of ACh to relax these segments confirmed the success of this procedure. The response to 80 mM KCl was unaltered by removal of the endothelium.

Release of prostaglandins. To allow us to measure the release of prostanoids, mesenteric arteries from LETO and OLETF 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 with a commercially available EIA kit (Cayman Chemical, Ann Arbor, MI). Two-time diluted 50- μl samples were used for measurement of PGE₂, PGF_{2 α} , and TxB₂ (a stable metabolite of TxA₂). The various assays were performed as described in the manufacturer's procedure booklet. The amounts of prostaglan-

dins released are expressed as picograms per milligram of wet weight of mesenteric artery.

Measurement of nitrite and nitrate. The concentration of nitrite (NO_2^-) plus nitrate (NO_3^-) in the plasma (plasma NO_x) was assayed by the method described by us previously (36). For the determination of plasma NO_x , 0.3 ml of 100% methanol was added to 0.3 ml of each plasma sample, and the sample was then centrifuged at 5,000 g for 10 min at 4°C. Briefly, NO_x in the plasma was separated by means of a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6×50 mm; Eicom, Kyoto, Japan), after which NO_3^- was reduced to NO_2^- in a reduction column packed with copper-plated cadmium filings (NO-RED; Eicom). The NO_2^- was mixed with a Griess reagent to form a purple azo dye in a reaction coil. The separation and reduction columns and the reaction coil were placed in a column oven set at 35°C. The absorbance of the product dye at 540 nm was measured with a flow-through spectrophotometer (NOD-10; Eicom). The mobile phase, which was delivered by a pump at a rate of 0.33 ml/min, was 10% methanol containing 0.15 M NaCl/ NH_4Cl and 0.5 g/l 4 Na-EDTA. The Griess reagent, which was 1.25% HCl containing 5 g/l sulfanilamide with 0.25 g/l *N*-naphthylethylenediamine, was delivered at a rate of 0.1 ml/min. The concentrations of NO_2^- and NO_3^- and the reliability of the reduction column were examined in each experiment.

Western blotting. Protein levels of COXs, eNOS, phospho-eNOS (Ser1177), and EC-SOD were quantified with immunoblotting procedures essentially as described previously (37). Mesenteric arterial tissues (2 pooled vessels per group) 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 1 mM PMSF. 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 buffer containing mercaptoethanol. The protein concentration was determined by means of a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL). Samples (20 $\mu\text{g}/\text{lane}$) were resolved by electrophoresis on 12% SDS-PAGE gels and then transferred onto polyvinylidene difluoride (PVDF) membranes. Briefly, after the residual protein sites on the membrane were blocked with ImmunoBlock (Dainippon-pharm, 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, 1:1,000), anti-phospho-eNOS (Ser1177) (140 kDa, 1:1,000), or anti-EC-SOD (45 kDa, 1:1,000) in blocking solution. HRP-conjugated anti-mouse or anti-rabbit antibody was used at a 1:10,000 dilution in Tween-PBS, followed by detection with SuperSignal (Pierce). 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, 1:5,000), with HRP-conjugated anti-mouse IgG as the secondary antibody. For analysis of phospho-eNOS expression, detection of phosphoprotein was followed by membrane stripping, detection of total eNOS expression, and then β -actin (see above). The optical densities of the bands on the film were quantified by densitometry, with correction for the optical density of the corresponding β -actin band.

Statistical analysis. Data are expressed as means \pm SE. 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 by a two-way ANOVA, with Bonferroni's correction for multiple comparisons being performed post hoc ($P < 0.05$ again being considered significant).

RESULTS

General parameters. As in previous reports (31–33), at the time of the experiment all OLETF rats (nonfasted) exhibited hyperglycemia, their blood glucose concentrations being significantly higher than those of the age-matched nondiabetic control LETO rats (also nonfasted) (Table 1). The body weight of the OLETF rats was greater than that of LETO rats (Table 1). The plasma total cholesterol, triglyceride, and HDL levels were all significantly higher in OLETF rats than in LETO rats, while the plasma insulin concentrations were similar between the two groups (Table 1). The plasma adiponectin concentrations were lower in OLETF rats than in LETO rats (Table 1). The systolic blood pressure of OLETF rats was higher than that of LETO rats (Table 1).

Relaxation responses to ACh. When the PE-induced contraction had reached a plateau, ACh (1 nM–10 μM) was added cumulatively (Fig. 1). In intact preparations of mesenteric artery rings from age-matched LETO and OLETF rats, ACh induced a concentration-dependent relaxation, with the maximum response at 100 nM and then progressively weaker responses up to 10 μM (Fig. 1A). Overall, this relaxation was significantly weaker in rings from OLETF rats (Fig. 1A). To investigate which endothelium-derived factors might be impaired in mesenteric arteries from OLETF rats, we examined ACh-induced relaxation in the presence of various inhibitors (Fig. 1, B–D). To assess the possible effects of COX metabolites, mesenteric rings were preincubated with 10 μM indomethacin for 30 min. This treatment led to a significant increment on the subsequently induced endothelium-dependent relaxation [vs. that in untreated rings (Fig. 1A)] in each group of rats, and, most notably, it eliminated the tendency for the relaxation to weaken as the concentration of ACh was increased (0.3–10 μM) in both the LETO and OLETF groups (Fig. 1B). This result indicates that products of COX metabolism make a prominent contribution to the decreased endothelium-dependent relaxation observed in these preparations at higher ACh concentrations. It should be noted that in the presence of indomethacin, ACh-induced relaxation was still significantly weaker in rings from OLETF rats than in those from LETO rats (Fig. 1B).

To investigate ACh-induced NO-mediated relaxation, we added ACh cumulatively to rings precontracted by PE in the combined presence of indomethacin, apamin, and TRAM-34. As shown in Fig. 1C, under these conditions the ACh-induced relaxation at higher ACh concentrations (i.e., 1–10 μM) was

Table 1. Parameter values in LETO and OLETF rats

Parameters	LETO	OLETF
Body weight, g	527.4 \pm 6.5	636.4 \pm 22.2‡
Plasma glucose, mg/dl	165.9 \pm 4.6	509.5 \pm 49.1‡
Plasma cholesterol, mg/dl	120.3 \pm 3.8	201.9 \pm 12.6‡
Plasma HDL, mg/dl	47.7 \pm 3.3	64.2 \pm 3.6†
Plasma triglyceride, mg/dl	93.2 \pm 3.8	388.9 \pm 43.9‡
Plasma insulin, ng/ml	1.8 \pm 0.2	1.8 \pm 0.3
Plasma adiponectin, $\mu\text{g}/\text{ml}$	3.8 \pm 0.1	2.9 \pm 0.3*
Systolic blood pressure, mmHg	114.2 \pm 2.6	140.4 \pm 3.7‡

Values are means \pm SE for 16 determinations per rat strain. LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka Long-Evans Tokushima Fatty; HDL, high-density lipoprotein. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ vs. LETO rats.

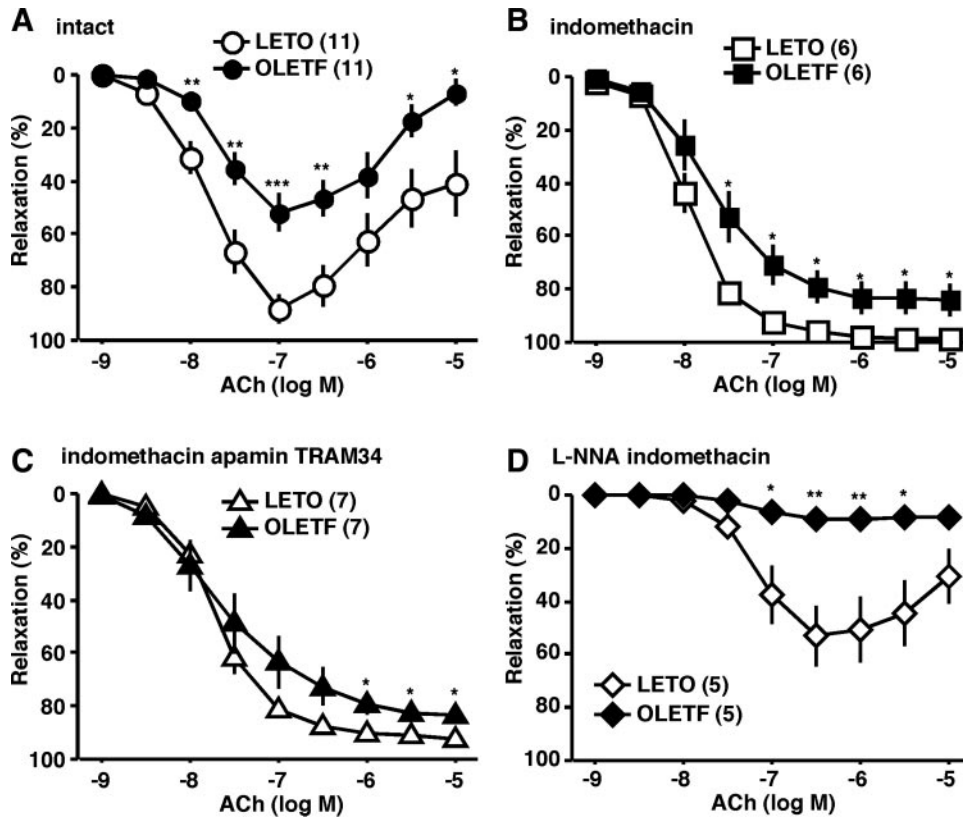


Fig. 1. Concentration-response curves for ACh-induced relaxation of isolated rings of mesenteric arteries obtained from Long-Evans Tokushima Otsuka (LETO) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats. Data were obtained in the absence (A) or presence (B–D) of the following drugs: 10 μ M indomethacin (B) or 10 μ M indomethacin in combination either with 100 nM apamin + 10 μ M 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) (C) or with 100 μ M N^G-nitro-L-arginine (L-NNA) (D). Details are given in MATERIALS AND METHODS. Data are means \pm SE; no. of determinations is shown within parentheses. * P < 0.05, ** P < 0.01, *** P < 0.001 vs. LETO.

significantly weaker in mesenteric arteries from OLETF rats than in those from LETO rats.

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 μ M L-NNA plus 10 μ M indomethacin. As in a previous report (32), this EDHF-type relaxation was significantly weaker in rings from OLETF rats than in those from LETO rats (Fig. 1D).

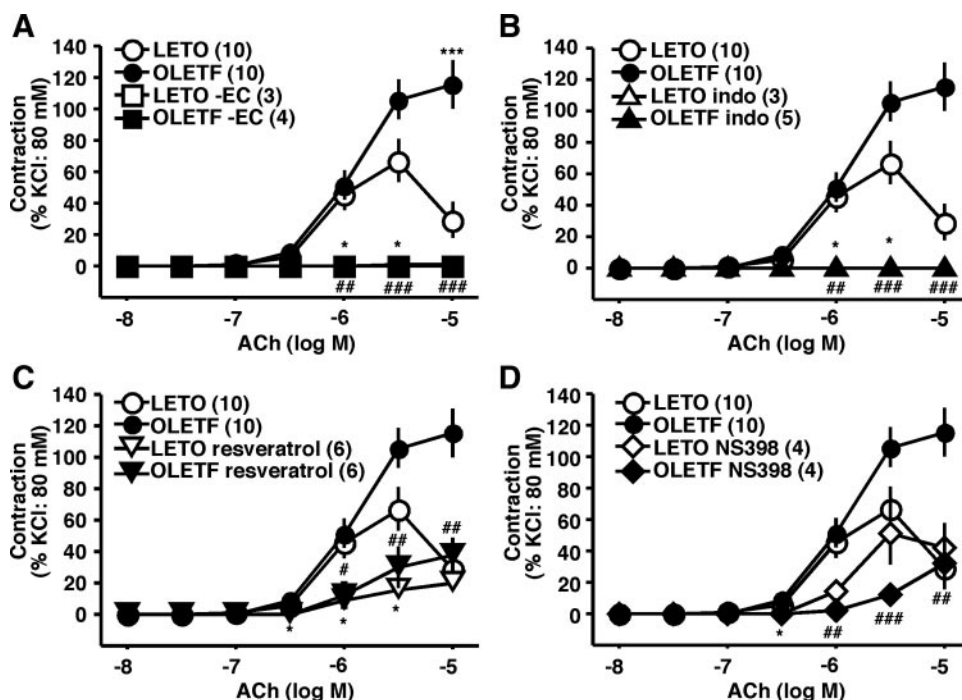
Endothelium-dependent contractions. As described above (Fig. 1), at higher concentrations of ACh (i.e., 1–10 μ M), a reduced ACh-induced relaxation was observed, with the relaxation being more nearly abolished in rings from OLETF rats than in those from LETO rats. To investigate the contractile component of these ACh-induced responses, we added ACh (10 nM–10 μ M) cumulatively to rings in the presence of L-NNA (100 μ M), because ACh-induced contraction was not observed in intact preparations (data not shown). As shown in Fig. 2A, under these conditions ACh-induced contraction was observed at higher ACh concentrations (i.e., 0.3–10 μ M) in rings from both groups. These responses were completely blocked by endothelial denudation, and the maximal response was significantly greater in mesenteric arteries from OLETF rats than in those from LETO rats (Fig. 2A). To assess the possible effects of COX metabolites, mesenteric rings were preincubated with 10 μ M indomethacin (nonselective COX inhibitor; Fig. 2B), 5 μ M resveratrol (COX-1-selective inhibitor; Fig. 2C), or 1 μ M NS-398 (COX-2-selective inhibitor; Fig. 2D) for 30 min. In LETO rats, the ACh-induced contractile response was significantly inhibited by indomethacin or

resveratrol treatment, but not by NS-398 treatment. However, this contraction was significantly inhibited by each of the three COX inhibitors in mesenteric rings from OLETF rats. Next, to assess the possible effects of prostanoid receptors, mesenteric rings were preincubated with 3 μ M SQ-29548 (TP antagonist; Fig. 3A), 10 μ M AH-6809 (DP/EP₁/EP₂ antagonist; Fig. 3B), or 10 μ M AL-8810 (FP antagonist; Fig. 3C). In LETO rats, the ACh-induced contractile response was significantly inhibited by SQ-29548 or AL-8810 treatment, but tended to be only slightly (nonsignificantly) inhibited by AH-6809 treatment. However, this contraction was significantly inhibited by all three prostanoid receptor antagonists in mesenteric rings from OLETF rats.

ACh-induced release of prostaglandins. ACh (10 μ M) evoked the release of TxB₂ (stable metabolite of TxA₂; Fig. 4A), PGE₂ (Fig. 4B), and PGF_{2 α} (Fig. 4C) in mesenteric arteries from both LETO and OLETF rats. In the case of TxB₂ and PGE₂, the ACh-induced release was significantly greater in rings from OLETF rats than in those from LETO rats. However, there was no significant difference in the release of PGF_{2 α} between LETO and OLETF rats (Fig. 4C). In OLETF rats, indomethacin significantly reduced each ACh-induced release (TxB₂, PGE₂, and PGF_{2 α}).

Expressions of proteins for COXs. To investigate the possible mechanisms underlying the alterations in the ACh-induced relaxation and contraction responses seen in mesenteric arteries from OLETF rats, we examined whether the expressions of COX proteins in mesenteric arteries might differ between the two groups. Immunoblot analysis of mesenteric arteries from LETO and OLETF rats (using anti-COX antibodies) allowed detection of immunoreactive proteins (Fig. 5). For both COX-1

Fig. 2. Endothelium-dependent contractions in rat mesenteric arteries obtained from LETO and OLETF rats (in the presence of 100 μ M L-NNA). A: concentration-response curves for ACh-induced contractions of rings of isolated mesenteric arteries with or without (-EC) endothelial cells. B-D: effects of various cyclooxygenase (COX) inhibitors [10 μ M indomethacin (B), 5 μ M resveratrol (C), or 1 μ M NS-398 (D)] on ACh-induced contractions in the presence of 100 μ M L-NNA in mesenteric arteries isolated from LETO and OLETF rats. Details are given in MATERIALS AND METHODS. For comparison, the curves obtained for ACh-induced contractions in the presence of 100 μ M L-NNA (A) are shown again in B-D. Data are means \pm SE; no. of determinations is shown within parentheses. * P < 0.05, *** P < 0.001 vs. LETO. # P < 0.05, ### P < 0.01, #### P < 0.001 vs. OLETF.



and COX-2, protein expression was significantly greater in the OLETF group than in the LETO group.

***t*-BOOH-induced contraction.** Next, we analyzed the influence of oxidative stress, the endothelium, and diabetes. To this end, cumulative concentrations of *t*-BOOH (1 μ M–1 mM), a membrane-permeant oxidant (12), were added to rings (with or without endothelium) from LETO and OLETF rats (Fig. 6). *t*-BOOH induced concentration-dependent contractions in rings with or without endothelium from OLETF rats (Fig. 6B), but only very weak contractions in intact rings from LETO rats (Fig. 6A). We observed that 1) inhibiting NOS (with 100 μ M L-NNA) enhanced the contractile responses elicited by *t*-BOOH in intact rings from LETO rats (Fig. 6A) and 2) removal of the endothelium potentiated the contractile responses induced by *t*-BOOH in the LETO group (Fig. 6A). The two potentiating effects were not observed in rings from OLETF rats (Fig. 6B). Moreover, indomethacin (10 μ M) abolished the *t*-BOOH-induced contraction in the presence of L-NNA in rings from both groups (Fig. 6C). Thus, in mesenteric arteries from LETO and OLETF rats, *t*-BOOH-induced contraction is essentially mediated by COX metabolites, and whereas endothelium-derived factors (i.e., NO or other factors) negatively modulate the *t*-BOOH-induced contraction in mesenteric arteries from LETO rats, this modulation is defective in those from OLETF rats.

Evaluation of NO synthesis as plasma NO₂⁻ and expression of mesenteric eNOS protein. Since the plasma level of NO₂⁻ (an oxidation product of NO) correlates with the level of NO biosynthesis (25) and since the NO₃⁻-to-NO₂⁻ ratio is often used as an indirect marker of oxidative stress (40), we measured the levels of NO₂⁻ and NO₃⁻ in rat plasma as a measure of NO synthesis. Moreover, since recent reports have suggested that, in response to a variety of stimuli, efficient NO production requires eNOS phosphorylation through the phosphatidylinositol 3-kinase/Akt pathway (7, 27, 28), we used

Western blot analysis to examine whether the protein expressions of eNOS and phospho-eNOS (Ser1177) in the mesenteric artery might differ between our two groups of rats (Fig. 7). The plasma NO₂⁻ level was lower in OLETF rats than in LETO rats, while the NO₃⁻-to-NO₂⁻ ratio was significantly greater in OLETF rats than in LETO rats. As shown in Fig. 7, B and C, the densitometric ratio of eNOS to β -actin (Fig. 7B) was not different between arteries from the two groups of rats, but the densitometric ratio of phospho-eNOS to eNOS was significantly smaller in OLETF than in LETO rats (Fig. 7C).

Expressions of protein for EC-SOD. In our previous report (31), plasma SOD activity was found to be decreased in aged OLETF rats (vs. age-matched LETO rats). Since EC-SOD is a major superoxide scavenger in plasma and vascular tissues (49, 53), we examined whether EC-SOD protein expression in mesenteric arteries might differ between these two groups (Fig. 8). EC-SOD expression was significantly lower in the OLETF group than in the LETO group.

DISCUSSION

In the present study, we found that in rat mesenteric arteries 1) the ACh-induced relaxation was impaired in the OLETF group, and the relaxation became weaker at higher concentrations of ACh in both groups; 2) indomethacin enhanced the ACh-induced relaxation and eliminated the tendency for it to reverse, with the indomethacin-resistant relaxation being smaller in the OLETF group; 3) the ACh-induced NO-mediated relaxation was impaired in the OLETF group; and 4) the ACh-induced EDHF-type relaxation was impaired in the OLETF group [as previously reported (32)]. These results strongly suggest that the impairment of ACh-induced relaxation seen in intact preparations of OLETF rat mesenteric artery is due not only to decreases in NO and EDHF signaling but also to an increase in COX-mediated vasoconstriction.

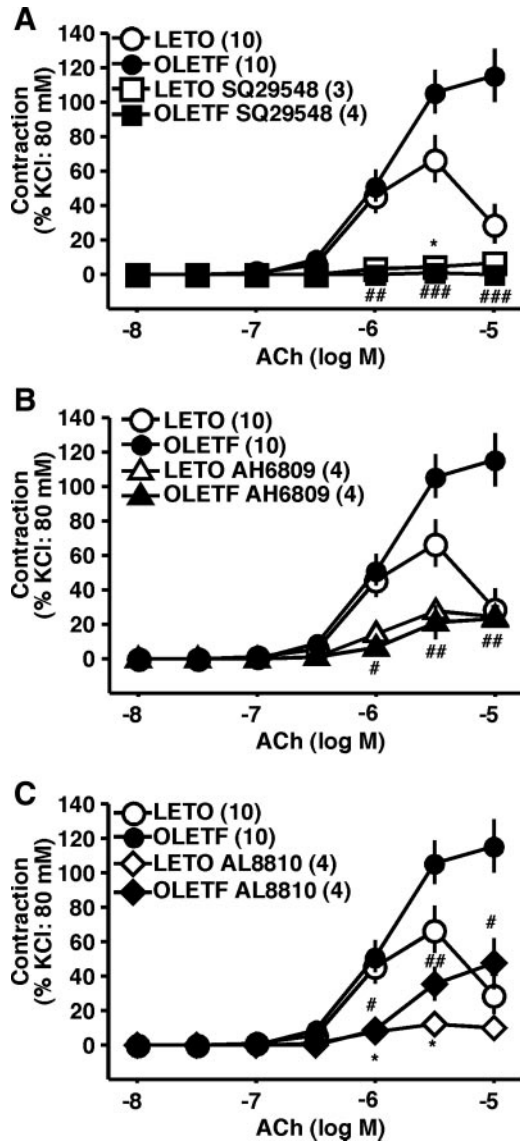


Fig. 3. Effects of various prostanoid receptor antagonists [3 μ M SQ-29548 (TP antagonist; A), 10 μ M AH-6809 (DP/EP₁/EP₂ antagonist; B), or 10 μ M AL-8810 (FP antagonist; C)] on ACh-induced contraction in the presence of 100 μ M L-NNA in mesenteric arteries isolated from LETO and OLETF rats. Details are given in MATERIALS AND METHODS. For comparison, the curves obtained for ACh-induced contractions in the presence of 100 μ M L-NNA (see Fig. 2A) are shown again in each panel. Data are means \pm SE; no. of determinations is shown within parentheses. **P* < 0.05 vs. LETO. #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001 vs. OLETF.

EDCF-mediated responses are observed not only in hypertension but also in diabetes (14, 15, 46, 50), and they probably reflect premature aging of blood vessel walls when they are subjected to exaggerated oxidative stress (see below). In the present experiments, the endothelium-dependent contraction induced by ACh was abolished by the selective COX-1 inhibitor resveratrol (44) and by the selective COX-2 inhibitor NS-398 (14) in the OLETF group. In contrast, in the LETO group NS-398 had no inhibitory effect on this contraction. Furthermore, Western blot analysis revealed that COX-1 and COX-2 levels were significantly augmented in mesenteric arteries from the OLETF group (vs. the LETO group). These results suggest that in a disease such as Type 2 diabetes, the

observed reduction in the relaxation to endothelium-dependent dilators can be attributed, at least in part, to an enhanced production of EDCF (via COX-1 and COX-2), with this EDCF then activating prostanoid receptors.

Prostanoids, which are metabolites of arachidonic acid produced via COX activity, may stimulate different receptor

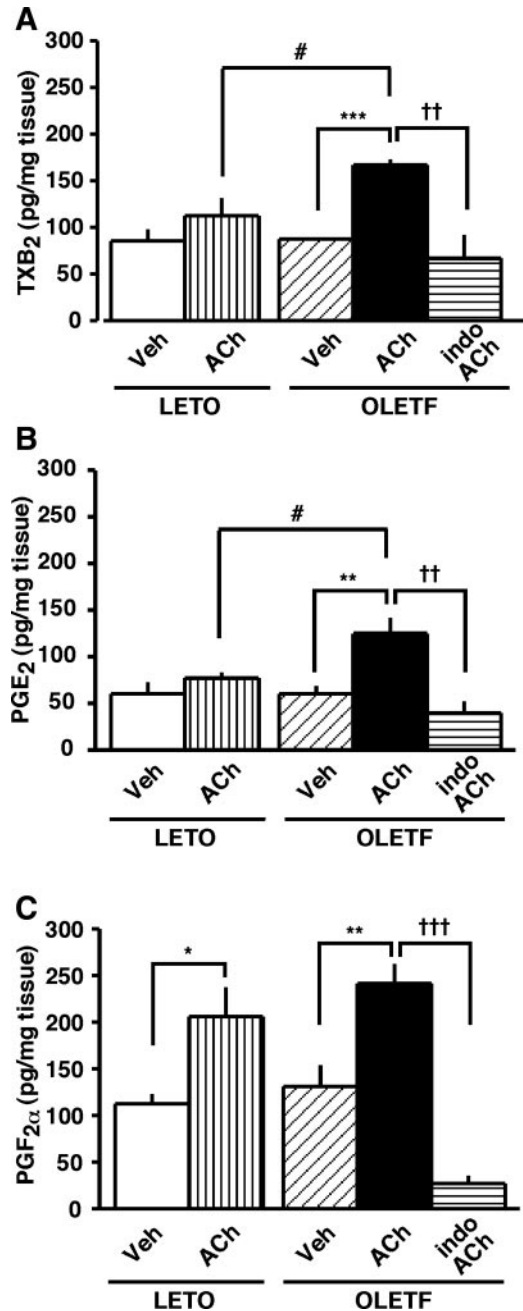


Fig. 4. Release of prostanoids [thromboxane (Tx)B₂ (stable metabolite of TxA₂; A), prostaglandin (PG)E₂ (B), and PGF_{2 α} (C)] evoked by 10 μ M ACh in mesenteric artery rings isolated from LETO (*n* = 6) and OLETF (*n* = 6) rats, together with the effect of indomethacin (indo; 10 μ M) on the ACh (10 μ M)-induced release of prostanoids in mesenteric arteries from OLETF rats [*n* = 3 (TxB₂) or 4 (PGE₂, PGF_{2 α})]. Details are given in MATERIALS AND METHODS. Data are means \pm SE from 3–6 experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. corresponding vehicle (Veh)-treated group. #*P* < 0.05 vs. ACh-treated LETO group. ††*P* < 0.01, †††*P* < 0.001 vs. ACh-treated OLETF group.

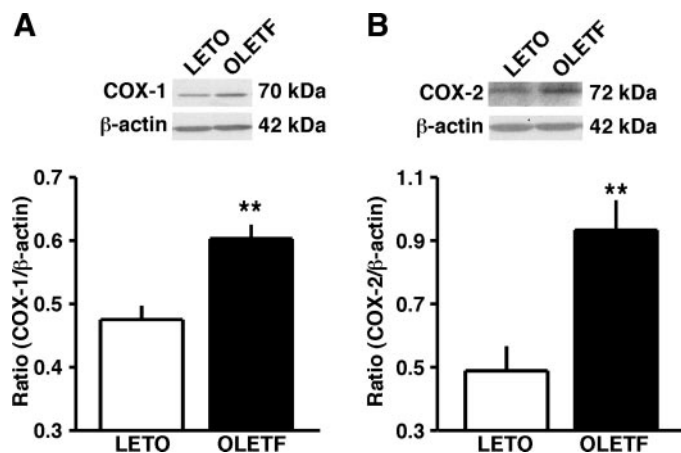


Fig. 5. Analysis of COX-1 (A) and COX-2 (B) protein expression in mesenteric arteries obtained from LETO and OLETF rats. *Top*: representative Western blots for COX-1, COX-2, and β -actin. Proteins (20 μ g) were subjected to 12% SDS-PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. These were then incubated with a primary antibody specific for either COX-1 (70 kDa) or COX-2 (72 kDa) or for β -actin (42 kDa), and also with a secondary anti-rabbit or anti-mouse antibody. *Bottom*: bands were quantified as described in MATERIALS AND METHODS. Ratios were calculated for the optical density of each COX over that of β -actin. Values are means \pm SE of 7 determinations. ** P < 0.01 vs. LETO group.

subtypes with various potencies (4). These subtypes include DP, EP, FP, IP, and TP receptors, which respond preferentially to PGD_2 , PGE_2 , $\text{PGF}_{2\alpha}$, PGI_2 , and TxA_2 , respectively. This classification does not exclude an effect, for example, of TxA_2 on the EP receptor (4), of PGE_2 on the TP receptor (4, 14, 50), or of $\text{PGF}_{2\alpha}$ on the TP receptor (14, 50, 52). In the present study, to investigate whether the ACh-induced contraction was mediated by prostanoid receptors, we used three prostanoid receptor antagonists, namely, SQ-29548 [TP antagonist (26, 52)], AH-6809 [less selective DP/EP₁/EP₂ antagonist (19, 46)], and AL-8810 [FP antagonist (16, 52)]. In the arteries used in the present study, we found that 1) the ACh-mediated contraction was inhibited by all three of these antagonists in both groups of rats; 2) production of these prostanoids [i.e., TxB_2 (a stable metabolite of TxA_2), PGE_2 , and $\text{PGF}_{2\alpha}$] was increased by ACh stimulation; and 3) the ACh-induced productions of TxB_2 and PGE_2 were significantly greater in mesenteric arteries from the OLETF group (vs. the LETO group). These results suggest that increased production of TxA_2 and/or PGE_2 may be responsible for the increased ACh-induced contraction in the mesenteric artery obtained in the OLETF group. This is supported, for instance, by evidence that among the COX derivatives that may be produced in mesenteric arteries, TxA_2 , $\text{PGF}_{2\alpha}$, and PGE_2 are the main vasoconstrictor products (1, 2) and that accumulation of endoperoxides favors the metabolism of PGE_2 , which is involved in the COX-dependent contraction of rat resistance arteries (2). However, the ligand-receptor interaction (activation) involved in prostanoid signaling is generally complex. For example, the TP receptor can be activated by several prostanoids other than TxA_2 in vascular smooth muscle cells (2, 14, 50, 52). Moreover, Vysniauskienė et al. (52) demonstrated that in isolated porcine ciliary arteries both AL-8810 (FP receptor antagonist) and SQ-29548 (TP receptor antagonist) inhibited the contraction induced by the FP receptor agonist $\text{PGF}_{2\alpha}$ as well as that induced by the TP receptor agonist U-46619. We obtained similar results using

isolated mesenteric arteries from LETO rats (data not shown). Although some agents may activate one receptor preferentially, they may also possess a modest affinity for other prostanoid receptor subtypes. In any event, it should be stressed that since the specificities of the TP, EP, and FP receptor antagonists

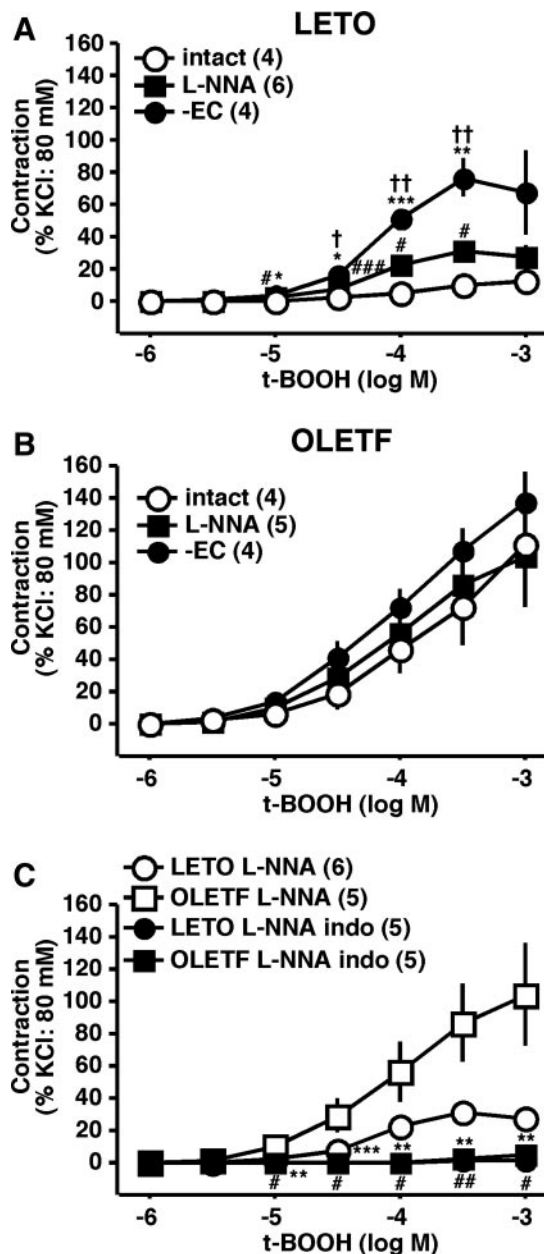


Fig. 6. Concentration-response curves for *tert*-butyl hydroperoxide (*t*-BOOH)-induced contractions of rings of mesenteric arteries isolated from LETO (A) and OLETF (B) rats. C: effects of 10 μ M indomethacin on *t*-BOOH-induced contractions in the presence of 100 μ M L-NNA in mesenteric arteries isolated from LETO and OLETF rats. Data were obtained from intact or endothelium-denuded (-EC) preparations and from intact preparations in the presence of 100 μ M L-NNA. Details are given in MATERIALS AND METHODS. For comparison, the curves obtained for *t*-BOOH-induced contractions in the presence of 100 μ M L-NNA (A and B) are shown again in C. Data are means \pm SE; no. of determinations is shown within parentheses. A and B: * P < 0.05, ** P < 0.01, *** P < 0.001, -EC vs. intact. # P < 0.05, ## P < 0.01, ### P < 0.001, L-NNA vs. intact. † P < 0.05, †† P < 0.01, -EC vs. L-NNA. C: ** P < 0.01, *** P < 0.001, L-NNA-treated LETO vs. L-NNA + indo-treated LETO. # P < 0.05, ## P < 0.01, L-NNA-treated OLETF vs. L-NNA + indo-treated OLETF.

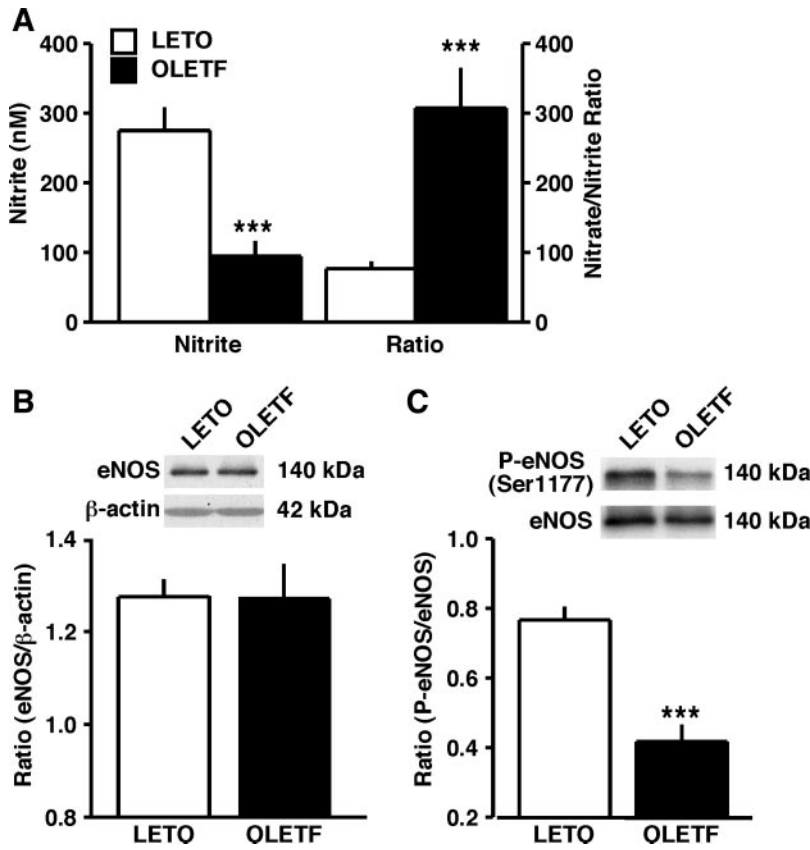


Fig. 7. Analysis of the plasma level of nitrite and nitrate-to-nitrite ratio and of endothelial nitric oxide synthase (eNOS) protein and phospho-eNOS (Ser1177; P-eNOS) protein expression in mesenteric arteries obtained from LETO and OLETF rats. Details are given in MATERIALS AND METHODS. A: plasma level of nitrite (left) and nitrate-to-nitrite ratio (right) in LETO and OLETF rats. Each column represents the mean ± SE of 10 determinations. ****P* < 0.001 vs. corresponding LETO group. B, top: representative Western blots for eNOS and β-actin. Proteins (20 μg) were subjected to 7.5% SDS-PAGE and then transferred to PVDF membranes. These were then incubated with a primary antibody specific either for eNOS (140 kDa) or for β-actin (42 kDa) and also with a secondary anti-mouse antibody. Bottom: bands were quantified as described in MATERIALS AND METHODS. Ratios were calculated for the optical density of eNOS over that of β-actin. Values are each the mean ± SE of 7 determinations. C, top: representative Western blots for P-eNOS and eNOS. Bottom: bands were quantified as described in MATERIALS AND METHODS. Ratios were calculated for the optical density of P-eNOS over that of eNOS. Values are each the mean ± SE of 7 determinations. ****P* < 0.001 vs. LETO group.

tested can be questioned, no definite conclusion can be drawn regarding the possible stimulation of different receptors by prostanoids induced by ACh in the present study. Although the above published evidence and our results, taken together,

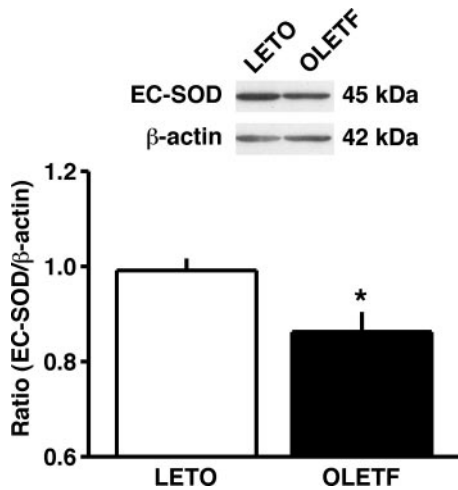


Fig. 8. Analysis of extracellular superoxide dismutase (EC-SOD) protein expression in mesenteric arteries obtained from LETO and OLETF rats. Top: representative Western blots for EC-SOD and β-actin. Proteins (20 μg) were subjected to 12% SDS-PAGE and then transferred to PVDF membranes. These were then incubated with a primary antibody specific either for EC-SOD (45 kDa) or for β-actin (42 kDa) and also with a secondary anti-rabbit or anti-mouse antibody. Bottom: bands were quantified as described in MATERIALS AND METHODS. Ratios were calculated for the optical density of EC-SOD over that of β-actin. Values are each the mean ± SE of 7 determinations. **P* < 0.05 vs. LETO group.

suggest that activation of TP, EP, and FP receptors may be responsible for ACh-induced contraction in mesenteric arteries from OLETF rats, further studies will be required to determine whether other prostanoids (and how and to what extent activations of prostanoid receptors) are involved in the augmented EDCF signaling observed in this Type 2 diabetic model.

The idea that oxidative stress plays an important role in the regulation of vascular tone in pathophysiological conditions has received a great deal of attention (17, 18, 20, 33, 35, 36, 40, 42, 43). Although the importance of oxidative stress in endothelial dysfunction has been recognized, a complete assessment of the possible relationship between oxidative stress and an imbalance between endothelium-derived relaxing and contracting factors had not been made, hence our decision to investigate this issue using mesenteric arteries from aged OLETF rats. In this context, we previously found (31) that in aged OLETF rats plasma SOD activity (an antioxidant system) is decreased while the plasma malondialdehyde level (lipid peroxides) is increased. In the present study, we found that EC-SOD protein expression was reduced in mesenteric arteries from OLETF rats (vs. those from LETO rats). Since EC-SOD is a major superoxide scavenger in the plasma and in vascular tissues (49, 53), an increase in oxidative stress in our OLETF group might be at least in part attributable to this decrease in EC-SOD expression. Moreover, in the present OLETF mesenteric arteries the protein level of phospho-eNOS (Ser1177) was reduced (vs. LETO). Phosphorylation of eNOS results in increased NO production (7, 27, 28), and so NO production in vascular tissue may be reduced in OLETF rats. Indeed, in OLETF rats the plasma NO₂⁻ level [oxidation product of NO and correlated

with NO biosynthesis (25)] was decreased (vs. the LETO group), despite eNOS protein expression in the mesenteric artery showing no change, and the plasma NO_3^- -to- NO_2^- ratio [often used as an indirect marker of oxidative stress (36, 40)] was increased. Moreover, in our diabetic OLETF rats the plasma adiponectin level was significantly reduced (vs. the LETO group). Since adiponectin has been suggested to have not only anti-inflammatory and antiatherogenic properties (21) but also a suppressive effect on oxidative stress (41), this hypoadiponectinemia may be causally related to the endothelial dysfunction observed in our OLETF mesenteric arteries. Furthermore, we found here that the contraction induced by the membrane-permeant oxidant (12) *t*-BOOH was more powerful in mesenteric arteries from OLETF rats than in those from LETO rats, and also that this contraction was increased by endothelial denudation and/or L-NNA treatment in LETO but not in OLETF. These results suggest that while endothelium-derived factors (i.e., NO or other factors) negatively modulate *t*-BOOH-induced contraction in the mesenteric artery, this modulation is defective in our diabetic model. The contraction induced by this agent in the presence of L-NNA was completely blocked by indomethacin in both the LETO and OLETF groups, and the oxidative stress-induced modulation of the activity of COXs tended to be increased in mesenteric arteries from OLETF rats. These results suggest that oxidative stress is increased and is associated with endothelial dysfunction in our diabetic model. These oxidative stress-induced abnormalities could augment or exacerbate various factors in the circuit leading to endothelial dysfunction in the diabetic mesenteric artery. This idea is supported by published evidence that in several arteries from individuals with Type 2 diabetes or other disease conditions, 1) reactive oxygen species have a detrimental effect on NO-mediated responses (6, 18, 20, 33, 36, 43, 48), 2) EDHF-signaling is impaired by oxidative stress (17, 35), 3) oxidative stress can increase COX activity (8, 10, 42, 51), 4) TP receptor stimulation leads to reductions in arterial hyperpolarization and EDHF-mediated relaxation (5), and 5) the defects in NO and/or EDHF signaling may make the vessels more sensitive to factors tending to increase vascular tone (6, 12, 35, 42, 50). Taken together, all this suggests that the increased oxidative stress might be responsible for the imbalance between EDRFs and EDCFs observed in the mesenteric arteries from our diabetic rats.

In experimental studies using streptozotocin-induced diabetic rats (a Type 1 diabetes model), an impaired EDHF-mediated response and an increased EDCF-mediated response have been reported both by us (29, 30, 34) and by others (11, 46). However, there is little experimental data concerning such responses in Type 2 diabetes because a suitable model of Type 2 diabetes has not previously been available. OLETF rats manifest stable clinical and pathological features that resemble human Type 2 diabetes (24). Briefly, OLETF rats are characterized by 1) increasing body weight just after weaning, in contrast to streptozotocin-induced diabetic rats, 2) a late onset of hyperglycemia (after 18 wk of age) and diagnosable diabetes after 24 wk of age, 3) a hyperinsulinemia that is present at 24 wk of age and declines after 55 wk of age and conversion to insulin-dependent diabetes after ~40 wk of age, and 4) increasing plasma cholesterol and triglyceride concentrations after 21 wk of age (24). In the present study, aged OLETF rats (i.e., 60–65 wk of age), which are in the stages of insulin-

dependent diabetes, were used to investigate endothelial dysfunction-related vascular changes. We used such rats because long-term diabetic conditions entail severe diabetic complications associated with cardiovascular dysfunction and because no previous study has investigated the balance between EDRF and EDCF signaling in this model in the established phase of diabetes. Although hypertension is one of the major risk factors for cardiovascular diseases and is closely related to Type 2 diabetes (47), the mechanism underlying the hypertension seen in OLETF rats remains to be elucidated. In the present study, the systolic blood pressure of the OLETF rats at 60–65 wk of age was higher than that of the age-matched LETO rats. Since the contribution made by EDHF-mediated responses appears significantly greater in small than in large arteries (9), such responses may be important for blood pressure homeostasis. Indeed, impaired EDHF-mediated responses have been demonstrated in several hypertensive models (9). Moreover, an increase in the prostanoids derived from COXs can contribute to increases in vascular tone and blood pressure both under physiological conditions (2) and in such pathophysiological states as hypertension (10, 50) and Type 2 diabetes (1, 8).

Together, the above observations suggest to us that the increased blood pressure seen in OLETF rats may be attributable not only to impairments of EDHF-mediated responses but also to increments in EDCF-mediated responses. These abnormalities could conceivably be secondary to the long-term presence of disease states such as hyperglycemia, hyperlipidemia, increased abdominal fat accumulation, and/or insulin resistance in individuals with Type 2 diabetes. Be that as it may, there are several limitations of the present study that should be mentioned. Although we used OLETF rats in the chronic phase of diabetes, this model exhibits not only hypertension but also hypercholesterolemia and high plasma levels of triglyceride. All of these risk factors can cause endothelial dysfunction (9, 10, 13, 35, 48). Hence, the contributions made by risk factors other than the diabetes itself are difficult to determine in studies of this model's mesenteric artery. Further investigation will therefore be required, for example, to determine whether the endothelial dysfunction due to the above factors might be improved by the therapeutic administration of agents such as statins, angiotensin-converting enzyme inhibitors, and antagonists of the angiotensin receptor.

Collectively, our data suggest 1) that endothelial dysfunction is present in the mesenteric arteries of aged OLETF rats, 2) that this may result from an imbalance of endothelium-derived factors (reduced EDRF signaling and increased EDCF signaling), and 3) that the mechanisms underlying this abnormality may involve increments in both COX-1 and COX-2 activities. We propose an important role for COXs-derived constrictor prostaglandins in the altered regulation of mesenteric arterial responsiveness seen in OLETF rats. It still remains questionable, however, whether and to what extent changes in mesenteric prostaglandin synthesis might contribute to the alterations in EDRF signaling and blood pressure in Type 2 diabetes. Nevertheless, on the basis of the present data, alterations in COX-dependent modulation of vasomotor function should be taken into consideration in future investigations of Type 2 diabetes.

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