Mechanisms underlying the diabetes-related changes in the extracellular nucleotides-induced responses in superior mesenteric arteries from rats

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This dissertation is dedicated to my parents and brother.

Table of Contents

Chapter 3

Protein kinase C delta contributes to increase in EP3 agonist-induced contraction in mesenteric arteries from type 2 diabetic Goto-Kakizaki rats.

Chapter 4

Mechanisms underlying reduced $P2Y_1$-receptor-mediated relaxation in superior mesenteric arteries from long-term streptozotocin-induced diabetic rats.

List of abbreviations

2-MeSADP, 2-methylthio-ADP
6-keto-PGF1$_a$, 6-keto- prostaglandin $F_{1\alpha}$
AA, arachidonic acid
ACh, acetylcholine
ADP, adenosine diphosphate
ANG, angiotensin
ANOVA, analysis of varience
ARB, ANG II type 1 receptor blocker
ATP; adenosine triphosphate
BP, blocking peptide
CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate solution
COX, cyclooxygenase
cPLA$_2$, cytosolic Ca^{2+}-dependent phospholipase A_2
DMSO, dimethylsulfoxide
EC, endothelial cell
EDCF, endothelium-derived contracting
EDRF, endothelium-derived relaxing
eNOS, endothelial NOS
EP receptor, prostaglandin E_2 receptor
ERM, Ezrin/Radixin/Moesin
GK, Goto-Kakizaki
HDL, high-density lipoprotein
HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase
HRP, Horseradish peroxidase
KHS, Krebs-Henseleit solution
LETO, Long-Evans Tokushima Otsuka
L-NNA, N^G-nitro-L-arginine
NO, nitric oxide
NOS, NO synthase
NOx, nitric oxide metabolites
mPGES, microsomal prostaglandin E synthase
NBT, nitro-blue tetrazolium
NEFA, non-esterified fatty acid
OLETF, Otsuka Long-Evans Tokushima Fatty
P2 receptor, purinoceptor
PE, phenylephrine
PGE_2, prostaglandin E_2
$PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$
PKC, protein kinase C
PVDF, polyvinylidene difluoride
ROCK, Rho-kinase
SBP, systolic blood pressure
SE, standard error
SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMC, smooth muscle cell
STZ, streptozotocin
T2DM, type 2 diabetes mellitus
TP receptor, thromboxane receptor
TXA_2, thromboxane A_2
TXB_2, thromboxane B_2
UTP, uridine triphosphate

General introduction

Extracellular nucleotides contribute to the local regulation of vascular tone and play important roles in pathophysiological processes, including diabetes, hypertension, atherosclerosis, and vascular remodeling [1, 2, 3]. Nucleotides are released from aggregated platelets, from adventitial nerve and from activated endothelial cells and leucocytes in response to various pro-inflammatory stimuli, cell death and tissue damage [1, 4]. These nucleotide induce either vasoconstriction or vasodilation through cell surface purinergic (P2) receptors [5-7]. P2 receptors are located on cell surface, which belong to major two families of P2Y and P2X receptors [6, 8]. So far, characterizations of P2 receptors have been published of seven P2X receptor subtypes ($P2X_1$–$P2X_7$, ligand-gated ion channels) and eight P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$–$P2Y_{14}$, G protein-coupled receptors) [6, 8]. P2X receptors are exclusively activated by ATP, whereas P2Y receptors respond to both purine (ATP and ADP) and pyrimidine (UTP and UDP) nucleotides [1, 3, 6, 8]. Specifically, ATP is a ligand for $P2X_1$–$P2X_7$, $P2Y_2$, $P2Y_{11}$, and $P2Y_{13}$ receptors, whereas UTP is a ligand for $P2Y_2$ and $P2Y_4$ receptors [1, 3, 6, 8]. In the arterial system, extracellular nucleotides lead to vasoconstriction and increased blood pressure by the activation of both P2X and P2Y receptors on smooth muscle cells [1, 3, 6, 8, 9]. Some important effects of extracellular nucleotides are mediated by the activation of endothelial cells and the subsequent release of endothelium-derived relaxing factors (EDRFs; e.g., vasodilation and decreased blood pressure) [1, 3, 6, 8, 9]. Moreover, extracellular nucleotides release endothelium-derived contracting factors (EDCFs) in some pathophysiological states [10, 11].

Type 2 diabetes is associated with a markedly increased incidence of cardiovascular diseases [12]. A growing body of evidence indicates that endothelial dysfunction and smooth muscle dysfunction are present in various regions of the vasculature in type 2 diabetes in both humans and animal models [13-18]. Vascular tone is controlled by EDRFs and EDCFs [19-22]. Abnormality of this balance (viz. decreased production of EDRFs but also increased amounts of EDCF) results in diabetic vasculopathy [22-24].

Vasoconstrictor prostanoids such as PGE_2, $PGF_{2\alpha}$, PGD_2, and TXA_2 are released via the activation of arachidonic acid (AA) pathway. This pathway starts with the release of AA from cellular phospholipids thought phospholipase A_2 (PLA_2) activation, and AA can be metabolized by cyclooxygenases (COXs). COXs are generally considered that COX-1, in most tissues, is expressed constitutively while COX-2 is mainly induced by inflammatory stimuli. In the vascular system, COXs are expressed in both endothelial and vascular smooth muscle cells. These COXs pathways are a source of superoxide anions, which increase endothelium-dependent contraction [25]. COX-mediated EDCF diffuses to the underlying smooth muscle and activates prostanoid receptors. The most important prostanoid receptor is thromboxane-prostanoid (TP) receptor in endothelium-dependent contractions, which then leads to an influx of calcium ion into smooth muscle cells.

EDCF-mediated responses are observed not only in hypertension but also in diabetes, and they possibly reflect premature aging of vascular walls subjected to aggravated oxidative stress [20, 22]. 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 [22]. Identification of EDCF could therefore provide new insights into the mechanism responsible for endothelial dysfunction, and could potentially reveal new therapeutic targets for cardiovascular diseases.

Among the prostanoids, PGE_2 is an important and ubiquitously distributed vasoactive eicosanoid, and has been reported to act as a vasodilator and a vasoconstrictor. The prostanoid EP receptors are heterotrimetic G protein-coupled receptors (GPCRs) [26-28]. PGE_2 exerts a broad range of effects including inhibition of smooth muscle cells (mediated by the EP2 and EP4 receptor subtypes) and excitation of smooth muscle cells (mediated by the EP1 and EP3 receptor subtypes) [26-28].

On the other hand, EDRFs include nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF) [29]. NO is a soluble gas which mediates much of the endothelium's control of vascular relaxation. NO is formed by NO synthase

(NOS), the most important NOS isoform is endothelial NOS (eNOS) within the cardiovascular system. In the regulation of eNOS activity upon agonist stimulation, signal transduction depends not only on Ca^{2+}/calmodulin but also on eNOS phosphorylation by various kinases [30-32]. Regulation of eNOS is achieved by phosphorylation of multiple sites in the protein. Phosphorylation at Ser^{1177} stimulates eNOS activity [30-32], whereas phosphorylation at Thr^{495} inhibits its activity [34]. $P2Y_1$ and $P2Y_2$ are major nucleotide receptors on endothelial cells, and the activation of these receptors leads to vasodilation mainly via NO pathway. The oxidative stress contributes endothelial dysfunction via reduced NO bioavailability and increased COX activity.

Angiotensin II (ANG II) is a vasoactive peptide and also causes the increases of the oxidative stress in the vascular system. Therefore, ANG II plays an important role in the pathogenesis of cardiovascular diseases associated with type 2 diabetes, such as hypertension and atherosclerosis. Moreover, treatment with ANG II type 1 (AT_1) receptor blockers (ARBs) of patients with type 2 diabetes significantly improves both macro- and micro-vascular end points, including nephropathy, retinopathy, and neuropathy [35]. However, little information is available to indicate whether ARBs could normalize purinergic signalling once the progression of the disease process has begun.

For the present study, I designed experiments to investigate the mechanisms underlying the diabetes-related changes in the extracellular nucleotides-induced responses in superior mesenteric arteries from rats. Extracellular nucleotides mediate activation of endothelial cells and the subsequent release of EDRFs and EDCFs. I hypothesized that extracellular nucleotides-mediated relaxations and contractions would be dysfunction in chronic diabetes. Therefore, I also investigated the effect of diabetes on the response to extracellular nucleotides (ATP, UTP and ADP) in superior mesenteric artery isolated from rats.

Finally, I examined whether purinergic signalling can be used as potential therapeutic targets against diabetes-associated vascular diseases.

Chapter 1

Pravastatin normalizes endothelium-derived contraction factor-mediated response via suppression of Rho-kinase signalling in mesenteric artery from aged type 2 diabetic rat.

Introduction

Normal vascular endothelial function depends on a controlled balance between the production/release of endothelium-derived relaxing (EDRF) and contracting (EDCF) factors [1, 2]. EDCF-mediated responses contribute to the endothelial dysfunction seen in various animal models of ageing, diabetes and cardiovascular diseases [3, 4]. Appropriate modulation of EDCF-mediated signaling pathways might be expected to ameliorate endothelial dysfunction. Such modulations would therefore be of therapeutic interest in the treatment of cardiovascular diseases. However, the precise mechanisms underlying EDCF signalling in long-term type 2 diabetes mellitus (T2DM) remain unclear.

Type 2 diabetes mellitus, which is associated with a markedly increased incidence of cardiovascular diseases, is often part of an array of complex abnormalities referred to as 'metabolic syndrome', which is frequently accompanied by an elevated blood pressure [5]. Otsuka Long-Evans Tokushima Fatty (OLETF) rats are an established model of T2DM [6], and there are several existing reports of abnormalities of vascular function in this model [7-9]. Kamata's laboratory previously demonstrated (1) that endothelial dysfunction is present in the mesenteric arteries of aged OLETF rats, (2) that this may result from an imbalance between endothelium-derived factors (reduced EDRF signalling and increased EDCF signalling) and (3) that the mechanisms underlying this abnormality may involve increments in the activities of COX-1 and COX-2 [8, 9].

Pravastatin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, reduces adverse cardiovascular events and retards the development of diabetes in humans with hypercholesterolaemia [10]. Moreover, pravastatin has been reported to have pleiotropic vascular effects including inhibitions of the Rho-kinase activity and reactive oxygen species (ROS) production that occur via decreased formation of isoprenoid intermediates, such as farnesylpyrophosphate and geranylgeranylpyrophosphate [11, 12]. Beneficial effects on adverse cardiovascular events have been demonstrated in OLETF rats following continuous

administration of pravastatin from 5 to 6 weeks of age (pre-insulin resistance stage) [13, 14]. Moreover, Kajikuri et al. [15] suggested that pravastatin might prevent or retard the development of endothelial dysfunction in coronary arteries in OLETF rats by inhibiting endothelial superoxide production. However, it is unknown whether pravastatin would have beneficial effects on EDCF-mediated signalling in the superior mesenteric artery in OLETF rats if it is administered when hyperglycaemia has already developed.

For the present study, I designed experiments (1) to investigate the effect of 4 week treatment with pravastatin on EDCF-mediated responses in OLETF rats at the chronic stage of T2DM and (2) to identify some of the molecular mechanisms involved.

Experiment

Reagents

Phenylephrine (PE), N^G-nitro-L-arginine (L-NNA), nitro-blue tetrazolium (NBT) and an antibody against β-actin were purchased from Sigma Chemical (St. Louis, MO, USA), while acetylcholine chloride (ACh) was purchased from Daiichi-Sankyo Pharmaceuticals (Tokyo, Japan). Y27632 was from Calbiochem (La Jolla, CA, USA). All drugs were dissolved in saline. All concentrations are expressed as the final molar concentration of the base in the organ bath. Horseradish peroxidase (HRP)-linked secondary anti-mouse or anti-rabbit antibody was purchased from Promega (Madison, WI, USA). Antibodies against COX-1, COX-2, mPGES1 and mPGES2 were from Cayman Chemical (Ann Arbor, MI, USA), while antibodies against RhoA, ROCKI and ROCKII were obtained from BD Biosciences (San Jose, CA, USA). Antibodies against phospho-Ezrin (Thr^{567})/Radixin (Thr^{564})/Moesin (Thr^{558}) (PERM) and Ezrin/Radixin/Moesin (ERM) were obtained from Chemicon (Danvers, MA, USA).

Animals and experimental design

5 week-old male rats [OLETF rats and Long-Evans Tokushima Otsuka (LETO) rats, a genetic control for OLETF] were supplied by the Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan). All animals were allowed a standard laboratory diet (MF; Oriental Yeast Industry, Tokyo, Japan) and water ad libitum in a controlled environment (room temperature, 21–22 °C; room humidity, 50 ± 5%) until they were 56–60 weeks old. Some OLETF rats were given pravastatin (10 mg kg^{-1} per day, p.o.) [16, 17] for 4 weeks starting at 52–56 weeks old. Thus, I studied three groups: pravastatin-untreated LETO and OLETF groups and a pravastatin-treated OLETF group. This study was approved by the Hoshi University Animal Care and Use Committee, and all experiments were conducted in accordance both with 'Guide for the Care and Use of Laboratory Animals' published by the

US National Institutes of Health and with '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 blood glucose, cholesterol, triglyceride, insulin and non-esterified fatty acid, and blood pressure

Plasma parameters and systemic blood pressure were measured as described previously [8, 9, 16]. Briefly, the plasma glucose, cholesterol, triglyceride, high-density lipoprotein (HDL) cholesterol and 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 (Shibayagi, Gunma, Japan). After a given rat had been in a constant-temperature box at 37 °C for a few minutes, its systolic blood pressure (SBP) was measured by the tail-cuff method using a blood pressure analyser (BP-98A; Softron, Tokyo, Japan).

Measurement of isometric force

Vascular isometric force was recorded as in previous papers [8, 9]. At 56–60 weeks of age, rats were killed in the morning (at 09:00 hours). 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 glucose. 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, 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 pre-contracted with PE (10^{-6} M). When the PE-induced contraction had reached a plateau

level, ACh (10^{-9}–10^{-5} M) was added in a cumulative manner. 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 = 17), OLETF (n = 17) and pravastatin-treated OLETF (n = 7) groups (1.62 ± 0.03, 1.77 ± 0.06 and 1.81 ± 0.10 g respectively). To aid examination of EDCF-mediated responses, rings were treated with 10^{-4} M L-NNA for 30 min to increase the amplitude of endothelium-dependent contractions [18]. To investigate the effect of Rho-kinase on the EDCF-mediated response, mesenteric rings were incubated for 30 min in a medium containing 10^{-6} or 10^{-7} M Y27632 plus 10^{-4} M L-NNA before cumulative addition of ACh (10^{-8}–10^{-5} M). 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.

Release of prostaglandins

Prostanoid release was measured as in previous papers [8, 9]. To allow us to measure this release, mesenteric arteries from a given group were cut into transverse rings 4 mm in length. These were placed for 30 min in siliconized tubes containing 0.5 mL KHS in the presence of 10^{-4} M L-NNA at 37 °C, and then ACh (10^{-5} M) 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). Ten-timediluted samples were used for measurements of PGE_2. The various assays were performed as described in the manufacturer's procedure booklet. The amounts of prostaglandins released are expressed as pg mg^{-1} wet weight of mesenteric artery.

Western blotting

The protein levels of the COXs, mPGESs, RhoA, Rho-kinases, phosphorylated Ezrin/Radixin/Moesin (PERM) and ERM were quantified using immunoblotting procedures, essentially as described before [9].

Western immunoblots for PERM/ERM were obtained from mesenteric arteries after ACh stimulation (10^{-5} M for 15 min in the presence of L-NNA). 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, USA). The lysate was cleared by centrifugation at 16,000 g for 10 min at 4 °C. The supernatant was collected, and the proteins were solubilized in Laemmli's buffer containing mercaptoethanol. Protein concentrations were determined by means of a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL, USA). Samples (20 μg per lane) were resolved by electrophoresis on 10% SDSPAGE gels, then transferred onto polyvinylidene difluoride (PVDF) membranes. Briefly, after blocking the residual protein sites on the membrane with PVDF blocking reagent (Toyobo, Osaka, Japan), the membrane was incubated with rabbit anti-COX-1 (70 kDa; 1 : 1000), rabbit anti-COX-2 (72 kDa; 1 : 1000), rabbit anti-mPGES1 (16 kDa; 1 : 1000), rabbit anti-mPGES2 (33 kDa; 1 : 1000), mouse anti-RhoA (21 kDa; 1 : 1000), mouse anti-Rho-kinaseI (ROCKI) (160 kDa; 1 : 1000), mouse anti-ROCKII (160 kDa; 1 : 1000), rabbit anti-phospho-Ezrin (Thr^{567})/Radixin (Thr^{564})/Moesin (Thr^{558}) (PERM) (85 kDa; 1 : 1000) or rabbit anti-ERM (85 kDa; 1 : 1000) 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 using SuperSignal (Pierce). To normalize the data, I 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.

Measurement of superoxide by measurement of the amount of NBT reduced

Mesenteric artery rings were incubated with NBT to allow the superoxide generated by the tissue to reduce the NBT to blue formazan. The details of the assay have been published

previously [9]. Briefly, mesenteric arteries from the LETO and pravastatin-treated and untreated OLETF groups were cut into transverse rings 4 mm in length. Then, rings from a given group were placed for 120 min in KHS at 37 °C containing NBT (10^{-4} M) in the presence of ACh (10^{-5} M). The NBT reduction was stopped by the addition of 0.5 N HCl. After this incubation, the rings were minced and homogenized in a mixture of 0.1 N NaOH and 0.1% SDS in water containing 40 mg L^{-1} diethylenetriaminepentaacetic acid. After the mixture had been centrifuged at 16,000 g for 30 min, the resultant pellet was resuspended in pyridine at 80 °C for 60 min to extract formazan. The mixture was then subjected to a second centrifugation at 10,000 g for 10 min. The absorbance of the formazan was determined spectro-photometrically at 540 nm. The amount of NBT reduced (=quantity of formazan) was calculated as follows: amount of NBT reduced = $A \times V/(T \times Wt \times \varepsilon \times l)$, where A is the absorbance, V is the volume of pyridine, T is the time for which the rings were incubated with NBT, Wt is the blotted wet weight of the arterial rings, ε is the extinction coefficient (0.7 L $mmol^{-1}$ mm^{-1}) and l is the length of the light path. The results are reported in pmol min^{-1} mg Wt^{-1}.

Statistical analysis

Data are expressed as means ± SE. Relaxation responses are expressed as a percentage of the PE-induced contraction. Contractile responses are expressed as a percentage of the 80 mM KCl-induced contraction. Multiple comparisons between treatment groups were made using an analysis of variance (ANOVA) followed by Bonferroni's test ($P < 0.05$ being regarded as significant in each test).

Abbreviations

ACh, acetylcholine; ANOVA, analysis of varience; COX, cyclooxygenase; DMSO, dimethylsulfoxide; EDCF, endothelium-derived contracting; EDRF, endothelium-derived relaxing; EP receptor, prostaglandin E_2 receptor; ERM, Ezrin/Radixin/Moesin; HDL, high-density lipoprotein; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; HRP, Horseradish peroxidase; KHS, Krebs-Henseleit solution; LETO, Long-Evans Tokushima Otsuka; L-NNA, N^G-nitro-L-arginine; NO, nitric oxide; NOS, NO Synthase; mPGES, microsomal prostaglandin E synthase; NBT, nitro-blue tetrazolium; NEFA, non-esterified fatty acid; OLETF, Otsuka Long-Evans Tokushima Fatty; PE, phenylephrine; PGE_2, prostaglandin E_2; PVDF, polyvinylidene difluoride; ROCK, Rho-kinase; SBP, systolic blood pressure; SE, standard error; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T2DM, type 2 diabetes mellitus

Results

General parameters

As shown in Table 1-1, at the time of the experiment, all OLETF rats (non-fasted) exhibited hyperglycaemia, their blood glucose concentrations being significantly higher than those of the age-matched non-diabetic control LETO rats (also non-fasted). Body weight was similar between the two groups. Plasma cholesterol, triglyceride, HDL and NEFA levels were all significantly higher in OLETF rats than in LETO rats. The SBP of OLETF rats was higher than that of LETO rats. Treatment of OLETF rats with pravastatin did not alter their plasma glucose, insulin, cholesterol, triglyceride or HDL levels or their SBP, but it did lower plasma NEFA.

Effect of pravastatin treatment on endothelium-dependent relaxation and contraction in OLETF rats

When the PE-induced contraction had reached a plateau, ACh was applied to induce relaxation (Fig. 1-1*A*). ACh induced a concentration-dependent relaxation (with the maximum response being at 10^{-7} to 3×10^{-7} M, and responses then being progressively weaker up to 10^{-5} M), and this response was weaker in rings from OLETF rats than in those from LETO rats (Fig. 1-1*A*). In rings from pravastatin-treated OLETF rats, the relaxation responses were similar to those seen in the LETO group (Fig. 1-1*A*).

To investigate the contractile component of the ACh-induced response, I added ACh (10^{-8}–10^{-5} M) cumulatively to rings in the presence of L-NNA (10^{-4} M). As shown in Figure 1-1B, in this condition, an ACh-induced contraction was observed at higher ACh concentrations (i.e. 10^{-6}–10^{-5} M) in rings from the three groups. However, this ACh-induced contraction was significantly greater in mesenteric arteries from OLETF rats than in those from LETO rats. Interestingly, it was reduced by 4 weeks treatment with pravastatin.

Table 1-1. Values of various parameters in LETO and pravastatin-treated and -untreated OLETF rats.

	LETO	OLETF	Pravastatin-treated OLETF
Body weight (g)	554.7 ± 5.9 (16)	544.4 ± 23.6 (16)	522.1 ± 9.9 (16)
Glucose (mg/dl)	134.9 ± 3.9 (16)	542.8 ± 20.1 (16)*	556.3 ± 44.0 (16)*
Insulin (ng/ml)	3.0 ± 0.2 (15)	2.3 ± 0.5 (15)	1.7 ± 0.2 (15)*
Cholesterol (mg/dl)	103.9 ± 2.1 (16)	281.3 ± 14.1 (16)*	262.5 ± 24.4 (16)*
Triglyceride (mg/dl)	86.5 ± 3.0 (16)	487.1 ± 46.5 (16)*	513.7 ± 82.2 (16)*
HDL (mg/dl)	62.6 ± 2.3 (16)	95.5 ± 8.0 (16)*	106.0 ± 9.2 (16)*
NEFA (mEq/l)	0.28 ± 0.01 (16)	0.53 ± 0.02 (16)*	0.42 ± 0.03 (16)*#
SBP (mmHg)	117.5 ± 2.2 (16)	147.7 ± 3.1 (16)*	148.3 ± 3.1 (16)*

Values are means ± S.E.M. Number of determinations is shown within parentheses. HDL, high-density lipoprotein; NEFA, non-esterified fatty acid; SBP, systolic blood pressure. *$P < 0.05$ vs. LETO. #$P < 0.05$ vs. OLETF.

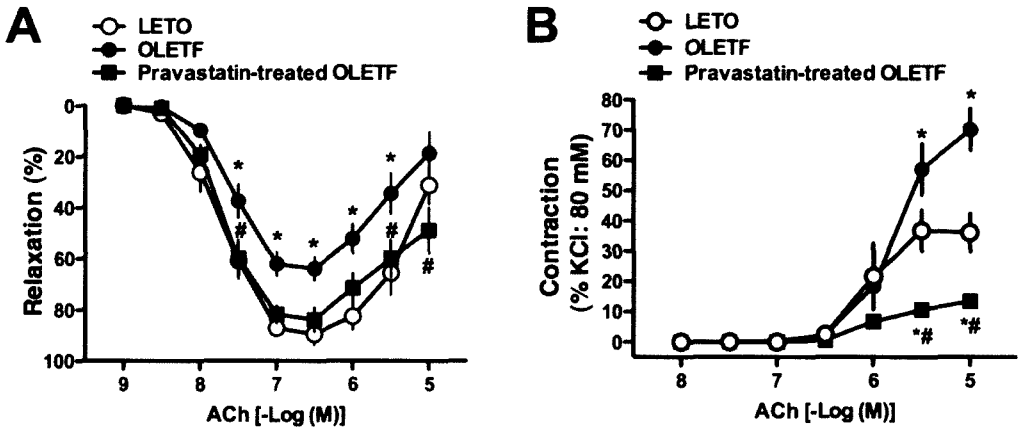


Fig. 1-1. Concentration-response curves for acetylcholine (ACh)-induced relaxation (*A*) and contraction (*B*) of isolated rings of mesenteric arteries isolated from Long-Evans Tokushima Otsuka (LETO), Otsuka Long-Evans Tokushima Fatty (OLETF) and pravastatin-treated OLETF rats. (*A*) Ordinate shows relaxation as a percentage of phenylephrine-induced contraction. (*B*) Responses were obtained in the presence of 10^{-4} M L-NNA. Data are means ± SE from eight (*A*) or seven (*B*) experiments. *P < 0.05 vs. LETO. $^{\#}$P < 0.05 vs. OLETF.

Effect of Rho-kinase inhibitor on EDCF-mediated contraction

Rho-kinase is involved in the pathogenetic modulation of vascular tone [19, 20]. Moreover, the Rho-kinase pathway contributes to EDCF-mediated arterial responses in pathophysiological states [20, 21]. I therefore assessed the effects of the Rho-kinase inhibitor Y27632 (10^{-7} or 10^{-6} M) on the ACh-induced contraction in the presence of L-NNA(10^{-4} M). Pre-treatment of rings from OLETF or LETO rats with Y27632 reduced the EDCF-mediated contraction in a concentration-related manner (Fig. 1-2). It should be noted that the quantitative difference in this contraction between the OLETF and LETO groups was abolished at each concentration of Y27632 (Fig. 1-2).

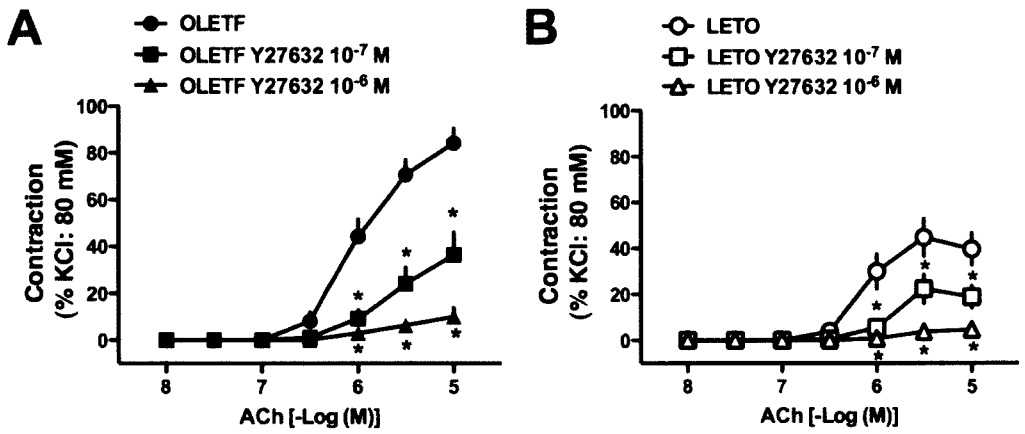


Fig. 1-2. Effect of Rho-kinase inhibitor on endothelium-dependent contraction in mesenteric arteries obtained from Long-Evans Tokushima Otsuka (LETO) and Otsuka Long-Evans Tokushima Fatty (OLETF) rats. (*A, B*) Effect of 10^{-6} or 10^{-7} M Y27632 on acetylcholine (ACh)-induced contraction in the presence of 10^{-4} M L-NNA in mesenteric arteries isolated from OLETF (*A*) and LETO (*B*) rats. Data are means ± SE from eight experiments. *P < 0.05 vs. without Y27632.

Effect of pravastatin treatment on COX pathway (assessed as release of PGE_2 and protein expressions of COXs and mPGESs)

Published evidence indicates that overproduction of prostanoids contributes to vascular dysfunction in diabetic arteries [9, 22, 23]. Moreover, I previously found that PGE_2 was the prostanoid responsible for increased EDCF signalling in mesenteric arteries from OLETF rats [8, 9, 24]. In view of these previous reports and the data we obtained using ACh (Fig. 1-1), I hypothesized that treatment of OLETF rats with pravastatin would reduce the ACh-stimulated release of PGE_2 from mesenteric artery rings isolated from such rats (Fig. 1-3*A*). ACh (10^{-5} M) evoked a release of PGE_2 that was significantly greater in rings from OLETF rats than in those from LETO rats. As I had hypothesized, this release of PGE_2 was significantly smaller in the pravastatin-treated OLETF group than in the untreated OLETF group.

Next, to investigate the possible mechanisms underlying the beneficial effects of pravastatin on the COX pathway in OLETF rats, I examined whether the protein expressions of COXs and mPGESs in mesenteric arteries might be altered by pravastatin treatment (Fig. 1-3). Western blotting analysis of mesenteric arteries from the three groups allowed detection of immunoreactive proteins. COX-1 expression was significantly greater in the OLETF group than in the LETO group (Fig. 1-3*B*), while the expressions of COX-2 and mPGES1 were not significantly increased in OLETF vs. LETO (Fig. 1-3*C, D*). The expression of mPGES2 was not different among the three groups of rats (Fig. 1-3*E*). Treatment of OLETF rats with pravastatin did not alter these protein levels (vs. OLETF).

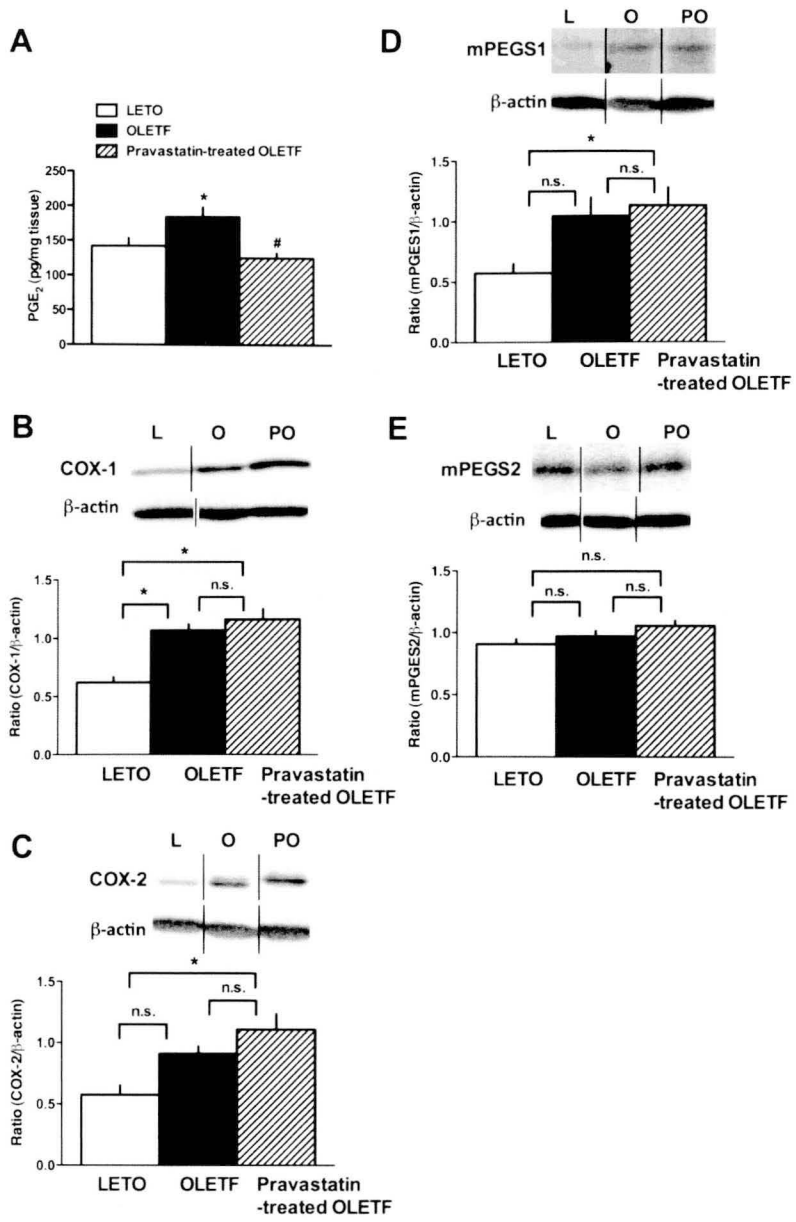


Fig. 1-3. Release of PGE_2 (*A*), under stimulation with 10^{-5} M acetylcholine (ACh), from mesenteric arteries isolated from Long-Evans Tokushima Otsuka (LETO), Otsuka Long-Evans Tokushima Fatty (OLETF) and pravastatin-treated OLETF rats. Data are means ± SE from eight experiments. *P < 0.05 vs. LETO. #P < 0.05 vs. OLETF. Analysis of COX-1 (*B*), COX-2 (*C*), mPGES1 (*D*) and mPGES2 (*E*) protein expressions in mesenteric arteries from LETO, OLETF and pravastatin-treated OLETF. The lanes, which were run on the same gel, were non-contiguous where indicated by the black lines. Data are means ± SE from five or six experiments. *P < 0.05 vs. LETO. n.s., not significant.

Effect of pravastatin on Rho-kinase activity

Statins modulate Rho-kinase signalling in the vasculature [25, 26]. To determine the effect of pravastatin on the protein expressions of RhoA, ROCKI and ROCKII [19], I performed immunoblotting for these proteins in mesenteric arteries and made comparisons among the three groups. Each of these protein levels was similar among the three groups (Fig. 1-4*A, B*). I next measured the level of phosphorylated ezrin, radixin and moesin (PERM). Those proteins are Rho-kinase targets and reflect Rho-kinase activity [27-29], so measuring PERM is a way of assessing Rho-kinase activity in ACh-stimulated rings. Western immunoblots were obtained from mesenteric arteries after ACh stimulation (10^{-5} M for 15 min in the presence of 10^{-4} M L-NNA) (Fig. 1-4*C*). Total ERM expression was similar among the three groups (Fig. 1-4*C*). However, the level of PERM was significantly greater in the OLETF group than in the LETO group and significantly lower in pravastatin-treated OLETF than in untreated OLETF (Fig. 1-4*C*).

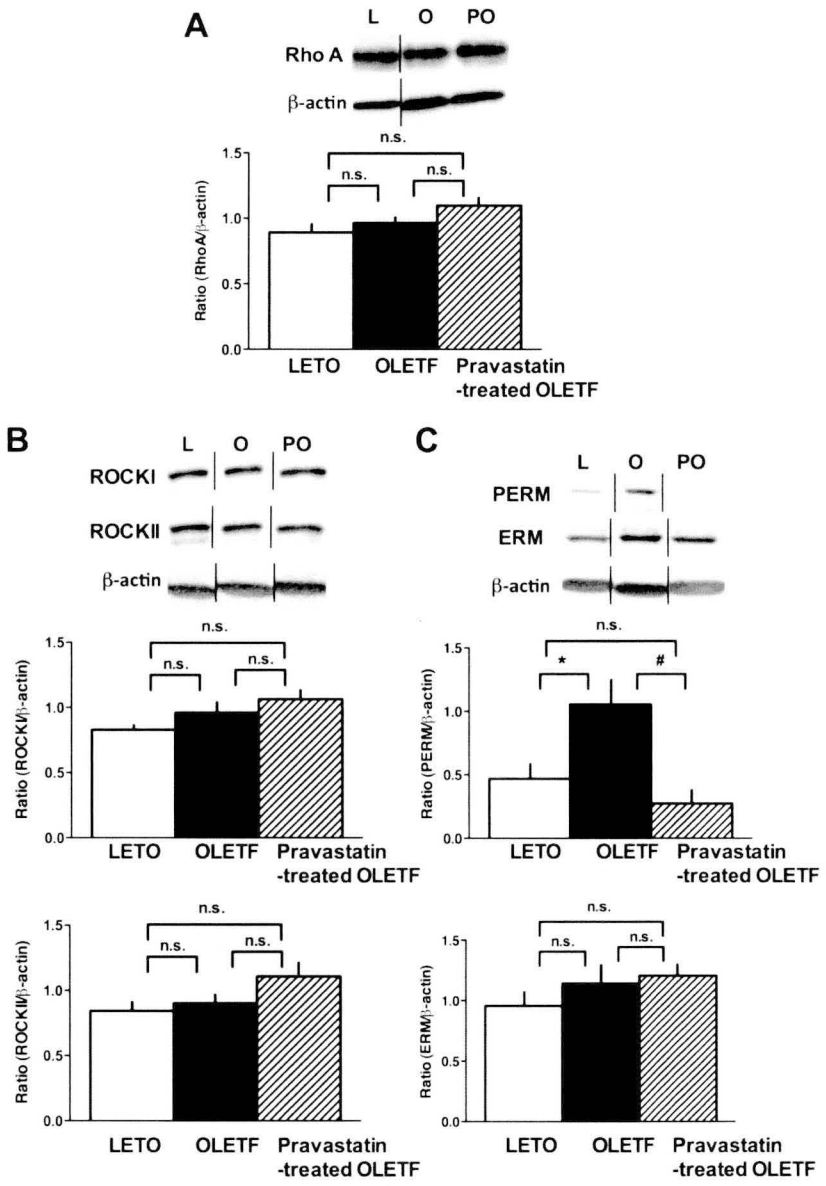


Fig. 1-4. Western blot results for RhoA (*A*), ROCKI (*B*), and ROCKII (*B*) protein expressions and for the acetylcholine (ACh) (10^{-5} M)-induced expressions of phosphorylated ERM [PERM (*C*)] and ERM (*C*) in mesenteric arteries obtained from Long-Evans Tokushima Otsuka (LETO), Otsuka Long-Evans Tokushima Fatty (OLETF) and pravastatin-treated OLETF rats. The lanes, which were run on the same gel, were non-contiguous where indicated by the black lines. Data are means ± SE from six experiments. *$P < 0.05$ vs. LETO. $^{\#}P < 0.05$ vs. OLETF. n.s., not significant.

Quantification of superoxide anion by measurement of the amount of NBT reduced

As superoxide leads to enhancements of EDCF-mediated contractions [30], I examined the effect of pravastatin on vascular superoxide generation by measuring the amount of NBT reduced by superoxide (Fig. 1-5). Mesenteric artery superoxide generation was significantly greater in OLETF than in LETO and significantly reduced in the pravastatin-treated OLETF group (vs. the untreated OLETF group).

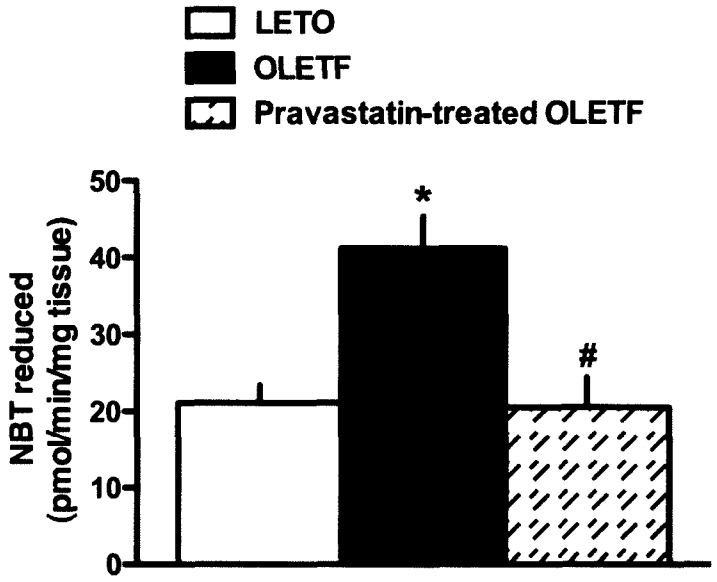


Fig. 1-5. Quantification of mesenteric artery superoxide production by measurement of amount of reduced nitro-blue tetrazolium (NBT). Tissues were obtained from Long-Evans Tokushima Otsuka (LETO), Otsuka Long-Evans Tokushima Fatty (OLETF) and pravastatin-treated OLETF rats. Data are means ± SE from six or ten experiments. *$P < 0.05$ vs. LETO. #$P < 0.05$ vs. OLETF.

Discussion

In this study, I have demonstrated that pravastatin improves EDCF signalling in mesenteric arteries in rats at the chronic stage of experimental T2DM.

Otsuka Long-Evans Tokushima Fatty rats manifest stable clinical and pathological features that resemble human T2DM [6]. Briefly, OLETF rats are characterized by *1*) increasing body weight just after weaning, *2*) a late onset of hyperglycaemia (after 18 weeks old) and diagnosable diabetes after 24 weeks old, *3*) a hyperinsulinaemia that is present at 24 weeks old and declines after 55 weeks old, and conversion to insulin-dependent diabetes after approx. 40 weeks old, and *4*) increasing plasma cholesterol and triglyceride concentrations after 21 weeks of age. In the present study, I used aged OLETF rats (i.e. 56–60 weeks old), which are then at the insulin-dependent stage of diabetes, to investigate EDCF-mediated signalling. I used such rats because long-term diabetic conditions entail severe diabetic complications associated with cardiovascular dysfunction and because previous studies have investigated the preventive, not the therapeutic, effect of pravastatin on vasoconstrictor-prostanoid signalling.

Pravastatin and other statins have clear beneficial effects on the atherosclerosis mediated by decreased LDL-cholesterol, and they improve endothelial function in part through their anti-inflammatory actions [31]. Recent experimental and clinical evidence indicates that some of the cholesterol-independent or 'pleiotropic' effects of statins involve improvements or restorations of endothelial functions and reductions in cardiovascular disease events [11, 32]. As regards such effects, it has been reported that statins inhibit the synthesis of isoprenoid intermediates in the cholesterol synthesis pathway, such as geranylgeranylpyrophosphate and farnesylpyrophosphate [11, 32]. These intermediates serve as important lipid attachments for the post-translational modification of a variety of proteins, including such small GTPases as RhoA and Rac1. RhoA/Rho-kinase plays a crucial role in endothelial dysfunction, including down-regulation of eNOS protein expression, whereas Rac1 induces assembly of the NADPH-oxidase complex, the most important source of the primordial oxygen radical in the

vessel wall [11]. Accordingly, statins reportedly ameliorate both hypertension and T2DM, with or without hypercholesterolaemia, in patients and in several animal models [34, 35], indicating that these diseases involve activation of RhoA/Rho-kinase and increased superoxide production.

In OLETF rats, SBP and plasma glucose, cholesterol, triglyceride, HDL and NEFA concentrations were all increased (vs. LETO). When I administered pravastatin for 4 weeks to such established OLETF rats, the treatment did not alter (vs. OLETF) SBP or the plasma glucose, insulin, cholesterol, triglyceride or HDL levels, although it did decrease the plasma NEFA level. Pravastatin has been reported to improve both hypercholesterolaemia and insulin sensitivity in diabetes, both in patients and in animal models [35]. Moreover, several experiments have demonstrated that it reduces blood pressure in hypertensive patients [36], although in another report pravastatin did not affect blood pressure in angiotensin-II-induced hypertensive mice [26]. This discrepancy may be related to the model, to a species difference or to the pravastatin treatment regimen [16, 17]. Possibly, if OLETF rats were treated with pravastatin for a long time, or if treatment was started at an earlier stage, this drug might reduce the levels of hypercholesterolaemia and/or hypertension and significantly improve insulin sensitivity. In this study, I found that in OLETF mesenteric arteries (vs. LETO) *1*) ACh-induced relaxation was reduced, *2*) both the EDCF-mediated contraction and PGE_2 production induced by ACh were increased, as was the expression of COX-1, while the expressions of COX-2 and mPGES1 were not significantly increased, and *3*) the ACh-stimulated PERM level was increased. Using our regimen (viz. 4 week treatment of OLETF rats already at the chronic stage), I found that pravastatin treatment normalized ACh-induced relaxation, EDCF-mediated contraction and PGE_2 production, and also the PERM level, without changing the expressions of two COXs, two mPGESs, RhoA and two Rho-kinases. To judge from these results, pravastatin exerted vasculoprotective actions in the present OLETF mesenteric arteries that can be largely attributed to a suppression of EDCF signalling by a cholesterol-independent pleiotropic effect, such as a suppression of

Rho-kinase.

Endothelial dysfunction results not only from decreased EDRF signalling but also from increased EDCF signalling. In aortas isolated from hypertensive rats *1*) the ACh-induced EDCF-mediated contraction was suppressed by pre-treatment with a superoxide scavenger and *2*) the H_2O_2-induced contraction was increased, and these responses could be inhibited by Y27632, by a COX inhibitor or by a TP-receptor antagonist [21]. Indeed, in the hypertensive rat aorta, the ACh-induced release of endothelial COX-dependent ROS is augmented [30]. From such evidence, ideas have been proposed for the identity of EDCF, including release of PGE_2, as have suggestions that endothelium- dependent contraction may involve the production of ROS, activation of endothelial COX and a subsequent stimulation of TP receptors located on smooth muscle cells. Recent experiments have suggested that Rho-kinase may act as a molecular switch, transducing signals from endothelium-derived prostaglandins and ROS [20, 21]. Rho-kinase activation upregulates NADPH-oxidase expression [37], and Rho-kinase plays a role in vascular smooth muscle contraction (via inhibition of myosin phosphatase [38]. In the present study, I found that the EDCF-mediated contraction was suppressed by Y27632 in both the LETO and OLETF groups and that the difference between those groups in the magnitude of that contraction was abolished by Y27632. I also found that in OLETF mesenteric arteries *1*) the superoxide generation was suppressed by pravastatin treatment, *2*) the PERM level was reduced by pravastatin treatment, and *3*) the RhoA, ROCKI and ROCKII protein expressions were not altered by pravastatin treatment. These results suggest that in OLETF mesenteric arteries, pravastatin can suppress EDCF-mediated signalling, an effect that may be attributable to a suppression of the activities of Rhokinase and that may be associated with a lowering of superoxide production.

There are limitations of the present study that should be admitted. As mentioned above, the circulating NEFA level was increased in OLETF rats, and this elevated level was reduced by the treatment with pravastatin. A possible interpretation of the present data is that the endothelial dysfunction present in OLETF rats is attributed to an increase in blood NEFA, and

that endothelial dysfunction is improved by pravastatin treatment at least in part through a lowering of this parameter. Indeed, evidence of endothelial dysfunction has been observed in various vessels in states in which there is an increment in the circulating level of free fatty acids [39-41]. Moreover, in the present study, I used one dose of pravastatin as reported previously [16, 17]. Several reports suggest that pravastatin has beneficial effects in various disease states by using different dose regimens [10, 13-15, 33, 35, 36, 42]. However, to establish a causal relationship and appropriate dose of pravastatin would require research focusing, for example, *1*) on time-course alterations both in circulating substances and in endothelial dysfunction in this model because this OLETF rat manifests symptoms of metabolic syndrome [42,43], and *2*) on comparison of various doses of pravastatin with regard to the impact on endothelial dysfunction.

I conclude that pravastatin may have beneficial effects against the enhancement of EDCF-mediated signalling seen at the chronic stage of T2DM. The mechanism responsible for the pravastatin-induced suppression of EDCF-mediated responses may involve an inhibition of Rho-kinase activity and a reduction in superoxide generation. These findings not only support the pleiotropic effects of pravastatin previously demonstrated in large intervention studies in T2DM patients but also offer a credible explanation for the beneficial effects that this drug has on the vascular system in T2DM.

Chapter 2

Mechanisms underlying altered extracellular nucleotide-induced contractions in mesenteric arteries from rats in later-stage type 2 diabetes: effect of ANG II type 1 receptor antagonism.

Introduction

Extracellular nucleotides play important roles in physiological and pathological processes, including the regulation of vascular tone, atherosclerosis, and remodeling [1, 2]. These nucleotides are released from endothelial cells in response to mechanical stimuli, such as shear stress, and also in response to hypoxia, hyperoxia, or agonist stimulation [1, 2]. Extracellular nucleotides act through cell surface receptors, which can be divided into P2Y and P2X receptor families [3, 4]. So far, characterizations have been published of seven P2X receptor subtypes ($P2X_1$–$P2X_7$, ligand-gated ion channels) and eight P2Y receptor subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$–$P2Y_{14}$, G protein-coupled receptors) [3, 4]. P2X receptors are exclusively activated by ATP, whereas P2Y receptors respond to both purine (ATP and ADP) and pyrimidine (UTP and UDP) nucleotides [1-4]. Specifically, ATP is a ligand for $P2X_1$–$P2X_7$, $P2Y_2$, $P2Y_{11}$, and $P2Y_{13}$ receptors, whereas UTP is a ligand for $P2Y_2$ and $P2Y_4$ receptors [1-4]. In the arterial system, extracellular nucleotides cause vasoconstriction and increased blood pressure by the activation of both P2X and P2Y receptors on smooth muscle cells [1-5]. Some important effects of extracellular nucleotides are mediated by the activation of endothelial cells and the subsequent release of endothelium-derived relaxing factors (EDRFs; e.g., vasodilation and decreased blood pressure) [1-5]. Moreover, extracellular nucleotides release endothelium-derived contracting factors (EDCFs) in some pathophysiological states [6, 7].

Type 2 diabetes is associated with a markedly increased incidence of cardiovascular diseases [8]. Various animal models have been used to gain insights into the pathogenesis of the vasculopathy associated with type 2 diabetes [8], and an accumulating body of evidence indicates that endothelial dysfunction is seen in several regions of the vasculature in animals and humans with type 2 diabetes [9-14]. For example, Kamata et al. [9-11, 15] have previously reported that abnormal endothelium-derived signalings [i.e., impaired EDRF signaling and enhanced EDCF (vasoconstrictor prostanoids) signaling] exist in mesenteric arteries from type 2 diabetic rats. However, many of the animal models exhibit features of metabolic

syndrome other than diabetes itself, such as hyperlipidemia, obesity, or hypertension. This makes it difficult to assess the pathogenetic relevance of each of these confounding factors in the development of diabetic vasculopathy in these models. The Goto-Kakizaki (GK) rat offers a convenient model for the study of type 2 diabetes without the confounding effects of obesity or hypertension [16]. GK rats are a highly inbred strain of Wistar rats that spontaneously develop type 2 diabetes [16]. This genetic rat model is particularly useful as a model of human type 2 diabetes because a defect in glucose-stimulated insulin secretion, peripheral insulin resistance, hyperinsulinemia, and hyperglycemia are seen as early as 4 weeks after birth [16]. Although Kamata et al. [15, 17-19] and others [13] have reported that abnormalities of vascular function exist in GK rats, no study on arterial reactivity to ATP, or the associated molecular mechanisms, has been conducted using GK rats at the chronic stage of diabetes.

Treatment with ANG II type 1 (AT1) receptor blockers (ARBs) in patients with type 2 diabetes significantly improves both macrovascular and microvascular end points, including nephropathy, retinopathy, and neuropathy [20]. Although various studies using animal models of cardiovascular diseases have demonstrated prevention of disease when treatment is started before the onset of complications, treatment of diabetic patients does not begin until after the symptoms have been diagnosed [21]. Moreover, although several studies have demonstrated that treatment with ARBs has beneficial effects on diabetic vasculopathy [20], little information is available to indicate whether ARBs might normalize ATP/UTP-mediated signaling once the progression of the disease process has begun.

For the present study, I hypothesized that nucleotide-induced arterial contractions are augmented in diabetic GK rats and, further, that treatment of such rats with an ARB (losartan) would normalize these contractions. I designed experiments to investigate *1*) the changes in ATP/UTP-induced contractions of mesenteric arteries that might occur as a result of long-term diabetes, focusing especially on the relationship between nucleotide-induced contractions and vasoconstrictor prostanoids signaling, and *2*) the effects on these altered nucleotide-induced contractions that might be seen if rats had received short-term losartan

treatment.

Experiment

Reagents

Phenylephrine, indomethacin, N^G-nitro-L-arginine (L-NNA), ATP (disodium salt), 8,8' -carbonylbis(imino-3,1-phenylene carbonylimino)- bis(1,3,5-naphthalenetrisulfonic acid) (NF-023), suramin, α,β-methylene-ATP, nitroblue tetrazolium (NBT), and antibodies against $P2Y_2$, $P2Y_4$, and β -actin were purchased from Sigma Chemical (St. Louis, MO). UTP (trisodium salt) and arachidonyltrifluoromethyl ketone ($AACOCF_3$) were from Wako (Osaka, Japan). U-46619, valeroyl salicylate, NS-398, and antibodies against cyclooxygenase (COX)-1 and COX-2 were from Cayman Chemical (Ann Arbor, MI). 5-Iodouridine-5'-*O*-diphosphate trisodium salt (MRS-2693) was obtained from Tocris Bioscience (Ellisville, MO). Antibodies against cytosolic phospholipase A_2 ($cPLA_2$) and phospho-$cPLA_2$ were from Cell Signaling Technology (Danvers, MA), whereas the antibody against $P2Y_6$ was from Alomone Labs (Jerusalem, Israel). Drugs were dissolved in saline except for $AACOCF_3$, U-46619, valeroyl salicylate, and NS-398 (which were dissolved in DMSO) and indomethacin (which was dissolved first in a small amount of 0.1 M Na_2CO_3 solution and then made up to the final volume with distilled water). All concentrations are expressed as the final molar concentration of the base in the organ bath.

Animals and experimental design

Male Wistar control rats and GK rats were obtained at the age of 4 weeks (Clea, Tokyo, Japan). All animals were allowed a standard laboratory diet (MF, Oriental Yeast Industry, Tokyo, Japan) and water ad libitum in a controlled environment (room temperature: 21–22°C, room humidity: 50 ± 5%) until they were 37–42 weeks old. Starting at 35–40 week old, some GK and Wistar rats were given losartan for 2 weeks (25 $mg \cdot kg^{-1} \cdot day^{-1}$ p.o. at 17:00 hours, "Nulotan," Banyu, Ibaraki, Japan). Thus, I studied three groups: losartan-untreated Wistar and GK groups and a losartan-treated GK group. This study was approved by the Hoshi University Animal Care and Use Committee, and all experiments were conducted in

accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and with 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 of Japan).

Measurement of blood glucose and insulin levels and blood pressure

Plasma glucose and insulin levels were measured as previously described [9, 10, 15, 17-19]. Briefly, the plasma glucose level was determined using a commercially available enzyme kit (Wako). The plasma insulin level was measured by enzyme immunoassay (Shibayagi, Gunma, Japan). After a given rat had been in a constant-temperature box at 37°C for a few minutes, its systolic 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 previous studies [9-12, 15, 18, 19]. At 37–42 weeks of age, some rats were euthanized each morning (at 09:00 hours). 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 glucose. The artery was carefully cleaned of all fat and connective tissue, and ring segments of 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 KHS at 37°C. 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). In all experiments, tissues were equilibrated for 30 min in the presence of 10^{-4} M L-NNA before the administration of any experimental agents. This was done because without this nitric oxide (NO) synthase (NOS) inhibitor, nucleotide-induced contractions were too small for use in elucidating the

mechanisms underlying the difference in responses to nucleotides between GK and Wistar rats. For the contraction experiments, ATP (10^{-6}–10^{-3} M), UTP (10^{-7}–10^{-4} M), the selective P2X agonist α,β-methylene-ATP [10^{-8}–10^{-5} M [22, 23)], or the selective $P2Y_6$ agonist MRS-2693 [10^{-8}–3×10^{-5} M [24)] was added cumulatively to the bath until a maximal response was achieved. To investigate the effects on the ATP- or UTP-induced contractile response induced by drugs, a given ring was incubated for 30 min in the appropriate drug-containing medium {viz. 10^{-6} M NF-023 [a $P2X_1$ receptor antagonist [25, 26)], 10^{-4} M suramin [a nonselective P2 receptor antagonist [27, 28)], 10^{-5} M indomethacin [a nonselective COX inhibitor [10)], 3×10^{-6} M SQ-29548 [a TP receptor antagonist [29)], 10^{-4} M valeroyl salicylate [a selective COX-1 inhibitor [30)], 10^{-6} M NS-398 [a selective COX-2 inhibitor [31)], and 10^{-5} M $AACOCF_3$ [a $cPLA_2$ inhibitor [32)]} before the cumulative addition of an agonist. When required, removal of the endothelium from arterial segments was achieved by infusing CHAPS (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. Finally, the wet weight of the mesenteric ring was measured.

Release of prostaglandins

Prostanoid release was measured as in previous studies [10-12)]. To allow us to measure this release, mesenteric arteries from a given group were cut into transverse rings of 4 mm in length. These were placed for 30 min in siliconized tubes containing 0.5 ml KHS in the presence of 10^{-4} M L-NNA at 37°C and then further incubated with or without a given nucleotide (3×10^{-4} M ATP or 10^{-4} M UTP) for 15 min. In some experiments, such mesenteric rings (with or without endothelium) from GK and Wistar rats were incubated for 30 min at 37°C in KHS containing 10^{-4} M L-NNA plus 10^{-5} M indomethacin, 10^{-4} M valeroyl salicylate, or 10^{-6} M NS-398. Next, after the mesenteric rings had been removed, the tubes were freeze clamped in liquid nitrogen and stored at -80°C for later analysis. Prostaglandins were measured using a commercially available enzyme immunoassay kit

(Cayman Chemical). Ten time-diluted samples were used for measurements of PGE_2, thromboxane B_2 [TXB_2; a stable metabolite of thromboxane A_2 (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 PGI_2. The various assays were performed as described in the manufacturer's procedure booklet. The amounts of prostaglandins released are expressed as picograms or nanograms per milligram of wet weight of the mesenteric artery.

Western blot analysis

Protein levels of COX-1, COX-2, $cPLA_2$, phospho-$cPLA_2$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ were quantified using immunoblot analysis procedures, essentially as previously described [9-12, 18]. Mesenteric arterial tissues were homogenized in ice-cold lysis buffer containing 50 mM Tris·HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS containing protease and phosphatase inhibitor cocktails (Complete Protease Inhibitor Cocktail and PhosSTOP, Roche Diagnostics, Indianapolis, IN). The lysate was cleared by centrifugation at 16,000 *g* for 10 min at 4°C. The supernatant was collected, and proteins were solubilized in Laemmli's buffer containing mercaptoethanol. Protein concentrations were determined using a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL) and kept the same in all samples. Samples (20 μg/lane) were resolved by electrophoresis on 10% SDS-PAGE gels and then transferred onto polyvinylidene difluoride (PVDF) membranes. Briefly, after blockade of residual protein sites on the membrane with ImmunoBlock (Dainipponpharm, Osaka, Japan) or PVDF blocking reagent (Toyobo, Osaka, Japan), the membrane was incubated with anti-COX-1 (70 kDa, 1:1,000), anti-COX-2 (72 kDa, 1:1,000), anti-$cPLA_2$ (95 kDa, 1:1,000), anti-phospho-$cPLA_2$ (95 kDa, 1:1,000), anti-$P2Y_2$ receptor (65 kDa, 1:200), anti-$P2Y_4$ receptor (78–79 kDa, 1:300), or anti-$P2Y_6$ receptor (42 kDa, 1:200) in blocking solution. Horseradish peroxidase-conjugated anti-rabbit antibody was used at a 1:10,000 dilution in Tween-PBS followed by detection using SuperSignal (Pierce). To normalize the data, we used β-actin as a housekeeping protein. β-Actin protein levels were

determined after the membrane was stripped and probed with β-actin monoclonal primary antibody (42 kDa, 1:5,000) with horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody. Optical densities of the bands on the film were quantified using densitometry with correction for the optical density of the corresponding β-actin band. In some experiments for COX protein detection, the same samples were loaded on the same gel for COX-1, COX-2, and β-actin. Therefore, the β-acting loading control blot in figure appeared to be the same (Fig. 2-6, *A* and *B*).

Measurement of superoxide by measurement of the amount of NBT reduced

Mesenteric artery rings were incubated with NBT to allow the superoxide generated by the tissue to reduce the NBT to blue formazan. Details of the assay have been published previously [12]. Briefly, mesenteric arteries from the Wistar and losartan-treated and -untreated GK groups were cut into transverse rings of 4 mm in length. Rings from a given group were then placed for 120 min in 1 ml KHS at 37°C containing NBT (10^{-4} M). The NBT reduction was stopped by the addition of 0.5 N HCl (1 ml). After this incubation, rings were minced and homogenized in a mixture of 0.1 N NaOH and 0.1% SDS in water containing 40 mg/l diethylentriaminepentaacetic acid. The mixture was centrifuged at 16,000 *g* for 30 min, and the resultant pellet was resuspended in 250 μl pyridine at 80°C for 60 min to extract formazan. The mixture was then subjected to a second centrifugation at 10,000 *g* for 10 min. The absorbance of the formazan was determined spectrophotometrically at 540 nm. The amount of NBT reduced (equal to the quantity of formazan) was calculated as follows: amount of NBT reduced= $A \times V/(T \times Wt \times \varepsilon \times l)$, where *A* is the absorbance, V is the volume of pyridine, *T* is the time for which the rings were incubated with NBT, Wt is the blotted wet weight of the arterial rings, ε is the extinction coefficient ($0.7\ l \cdot mmol^{-1} \cdot mm^{-1}$), and *l* is the length of the light path. Results are reported as picomoles per minute per milligram wet weight.

Statistical analysis

Data are expressed as means ± SE. The contractile force developed by mesenteric artery rings is expressed in milligrams of tension per milligram of tissue. Concentration-response curves were analyzed by two-way ANOVA for repeated measures, and multiple comparisons between treatment groups were made using ANOVA followed by Bonferroni's test (with $P < 0.05$ being regarded as significant in each test).

Abbreviations

6-keto-PGF1$_a$, 6-keto- prostaglandin $F_{1\alpha}$; AA, arachidonic acid; ACh, acetylcholine; ANG, angiotensin ; ANOVA, analysis of varience; ARB, ANG II type 1 receptor blocker; ATP; adenosine triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate solution; COX, cyclooxygenase; cPLA$_2$, cytosolic Ca^{2+}-dependent phospholipase A_2; DMSO, dimethylsulfoxide; EC, endothelial cell; EDCF, endothelium-derived contracting; EDRF, endothelium-derived relaxing; GK, Goto-Kakizaki, HRP, Horseradish peroxidase; KHS, Krebs-Henseleit solution; L-NNA, N^G-nitro-L-arginine; NO, nitric oxide; NOS, NO Synthase; NBT, nitro-blue tetrazolium; P2 receptor, purinoceptor; PE, phenylephrine; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PVDF, polyvinylidene difluoride; SBP, systolic blood pressure; SE, standard error; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SMC, smooth muscle cell, T2DM, type 2 diabetes mellitus; TP receptor, thromboxane receptor; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; UTP, uridine triphosphate

Results

General parameters

As shown in Table 2-1, body weight was significantly lower in GK rats than in Wistar rats, whereas non-fasted blood glucose and insulin levels were significantly higher in GK rats than in Wistar (also non-fasted). Treatment with losartan did not alter the above parameters in GK rats. Systolic blood pressure was significantly lower in GK rats than in Wistar rats. Short-term treatment with losartan lowered systolic blood pressure (vs. the untreated GK group).

Table 2-1. Values of various parameters in Wistar and losartan-treated and –untreated GK rats.

	Wistar	GK	Losartan-treated GK
Body weight (g)	629.2 ± 11.4 (16)	415.7 ± 4.8 (16)*	394.7 ± 4.6 (16)*
Glucose (mg/dl)	177.7 ± 6.3 (16)	501.5 ± 12.9 (16)*	461.1 ± 15.8 (16)*
Insulin (ng/ml)	2.5 ± 0.2 (16)	4.7 ± 0.3 (16)*	4.0 ± 0.3 (16)*
SBP (mmHg)	119.1 ± 2.6 (16)	108.5 ± 1.9 (16)*	98.1 ± 2.3 (16)*#

Values are means ± S.E.M. Number of determinations is shown within parentheses. SBP, systolic blood pressure. *$P < 0.05$ vs. Wistar. #$P < 0.05$ vs. GK.

Contractile responses to ATP

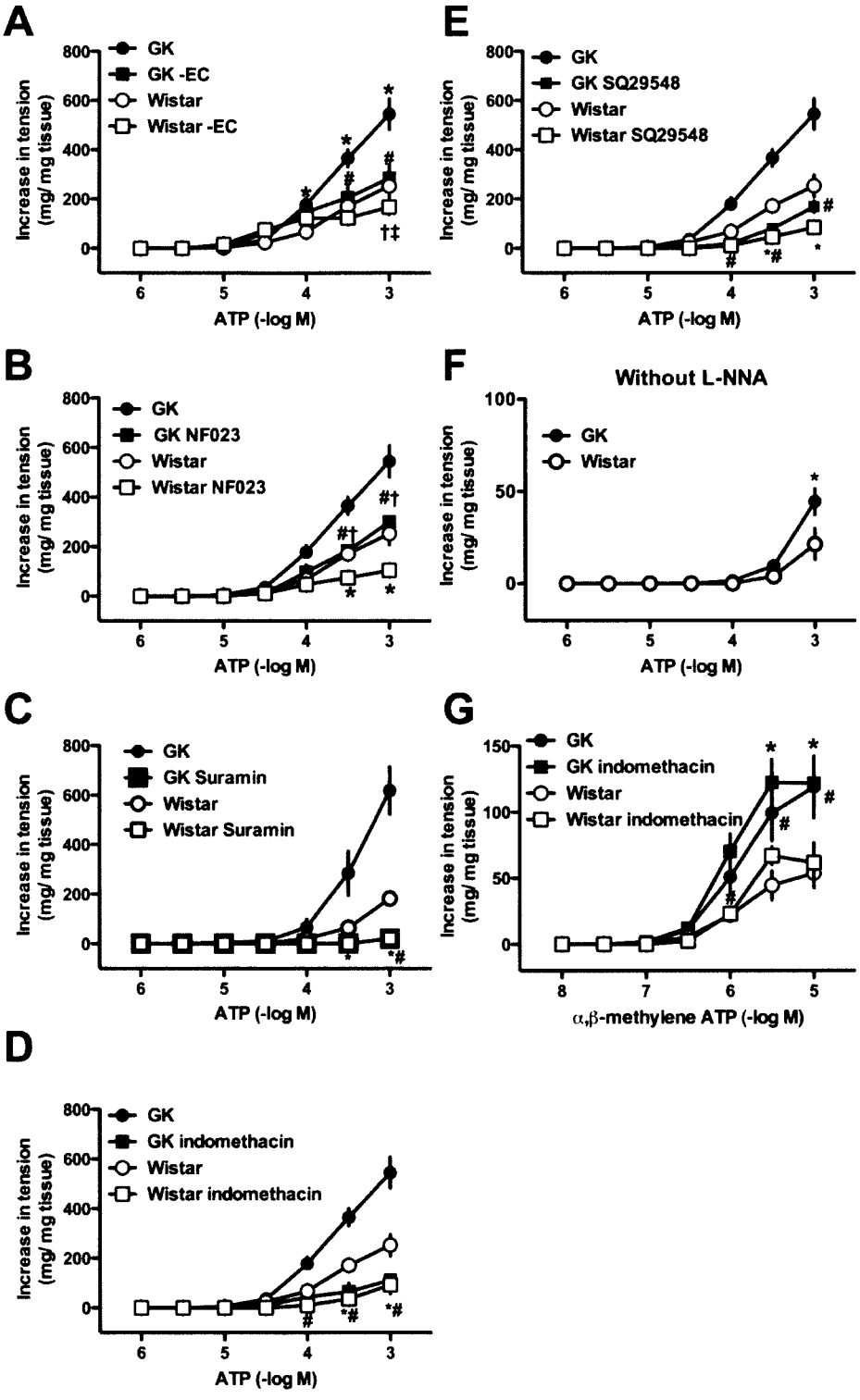
Since ATP can stimulate the release of NO, which plays an important role in the regulation of vascular tone and negatively modulates contraction, I investigated such contractions in the presence of a representative NOS inhibitor (10^{-4} M L-NNA), which inhibits both basal and agonist-induced NOS activity. As shown in Fig. 2-1*F*, I confirmed that in the absence of L-NNA, cumulative administration of ATP (10^{-6}–10^{-3} M) led to a small concentration-dependent increase in tension in mesenteric artery rings from both Wistar and GK rats (note the expanded ordinate scale in Fig. 2-1*F*). At the highest ATP concentration used (i.e., 10^{-3} M), this contraction was significantly greater in GK rats than in Wistar rats. Although L-NNA enhanced the ATP-induced contraction in both groups (Fig. 2-1, *A* vs. *F*; note the difference in ordinate scales), the ATP-induced contraction was significantly greater in rings from GK rats than in those from Wistar in the presence of L-NNA (Fig. 2-1*A*). The ATP-induced contraction was reduced by endothelial denudation, and this suppressive effect of endothelial denudation was larger in GK rats than in Wistar rats (Fig. 2-1*A*). Higher concentrations of ATP induced contraction even in endothelium-denuded rings, and this response, too, was greater in GK rats than in Wistar rats (Fig. 2-1*A*). The existence of such endothelium-independent vasoconstriction at higher concentrations of ATP is consistent with results obtained in a previous study [7] using a different vessel, the rat aorta.

To investigate the possible involvement of $P2X_1$ or P2Y receptors in the ATP-induced contraction, I examined the effects of antagonists of those receptors on the ATP-induced contraction. Incubation with NF-023, a $P2X_1$ receptor antagonist (10^{-6} M), reduced the ATP-induced contraction in both Wistar and GK groups (Fig. 2-1*B*). Likewise, incubation with suramin, a nonselective P2 receptor antagonist (10^{-4} M), reduced such contraction in both Wistar and GK groups (Fig. 2-1*C*). It should be noted that in the presence of NF-023, the ATP-induced contraction was still significantly greater in rings from GK rats than in those from Wistar rats (Fig. 2-1*B*), whereas in the presence of suramin, this contraction was similar between the two groups (Fig. 2-1*C*). Suramin treatment did not affect the contractile

responses induced by 10–80 mM KCl or 10^{-9}–10^{-5} M phenylephrine in mesenteric arteries (data not shown).

Since ATP induces the release not only of NO but also of EDCF in some vessels [7], and since previous reports [9-12] suggested that EDCF (i.e., vasoconstrictor prostanoids) signaling is altered in diabetic mesenteric arteries, we next examined whether the ATP-induced contraction might be altered after the acute inhibition of COX or antagonism of the TP receptor. Treatment with either 10^{-5} M indomethacin, a nonselective COX inhibitor, or 3×10^{-6} M SQ-29548, a TP receptor antagonist, reduced the ATP-induced contraction in both Wistar and GK groups (Fig. 2-1*D* and *E*). Furthermore, incubation with indomethacin abolished the difference between the two groups (Fig. 2-1*D*), as did treatment with SQ-29548 (Fig. 2-1*E*). To investigate the difference between Wistar and GK rats in the contraction induced by P2X receptor activation, we cumulatively applied α,β-methylene ATP, a selective $P2X_1$ agonist, to rings from GK and Wistar rats (Fig. 2-1*G*). The contraction induced by α,β -methylene ATP was greater in the GK group than in the Wistar group. Moreover, indomethacin did not affect such contractions in either group (Fig. 2-1*G*).

Fig. 2-1. Concentration-response curves for ATP-induced contractions in rings of mesenteric arteries obtained from Wistar and Goto-Kakizaki (GK) rats. (*A–E*) effects of endothelial denudation (-EC; *A*), the $P2X_1$ receptor antagonist NF-023 (10^{-6} M; *B*), the nonselective P2 receptor antagonist suramin (10^{-4} M; *C*), the nonselective cyclooxygenase (COX) inhibitor indomethacin (Indo; 10^{-5} M; *D*), and the TP receptor antagonist SQ-29548 (3×10^{-6} M; *E*) on ATP-induced contractions in the presence of 10^{-4} M *N*G-nitro-L-arginine (L-NNA). (*F*) ATP-induced contractions in the absence of 10^{-4} M L-NNA. (*G*) concentration-response curves for $P2X_1$ agonist (α,β-methylene ATP)-induced contractions in rings of mesenteric arteries obtained from Wistar and GK rats. Indo (10^{-5} M) did not affect the α,β-methylene ATP-induced contraction in either the Wistar or GK group. The ordinate shows the increase in tension (expressed in mg tension/mg tissue) measured at the peak of the response. Note the expanded ordinate scales in (*F*) and (*G*). Data are means ± SE from 4–8 experiments. The curves shown in (*A*) for the GK and Wistar groups are shown again in (*B*), (*D*), and (*E*). $^{*}P < 0.05$ vs. the Wistar group in (*A–F*). $^{\#}P < 0.05$ vs. the GK group in (*A–F*). $^{\dagger}P < 0.05$, the Wistar group vs. the Wistar group with endothelium denudation in *A* or the GK group with NF-023 vs. the Wistar group with NF-023 in (*B*). $^{\ddagger}P < 0.05$, the GK group with endothelium denudation vs. the Wistar group with endothelium denudation in (*A*). In (*G*), $^{*}P < 0.05$ vs. the Wistar group and $^{\#}P < 0.05$, the GK group with Indo vs. the Wistar group with Indo.


A
GK
GK -EC
Wistar
Wistar -EC
Increase in tension (mg/ mg tissue)
ATP (-log M)
B
GK
GK NF023
Wistar
Wistar NF023
C
GK
GK Suramin
Wistar
Wistar Suramin
D
GK
GK indomethacin
Wistar
Wistar indomethacin
E
GK
GK SQ29548
Wistar
Wistar SQ29548
F
Without L-NNA
GK
Wistar
G
GK
GK indomethacin
Wistar
Wistar indomethacin
α,β-methylene ATP (-log M)

Contractile responses induced by the P2Y receptor agonist

I next looked for differences between Wistar and GK rats in the contractions induced by a $P2Y_2$ and $P2Y_4$ receptor agonist. Cumulative administration of UTP (10^{-7}–10^{-4} M), a $P2Y_2/P2Y_4$ receptor agonist, induced contraction that was significantly greater in rings from GK rats than in those from Wistar in the presence of 10^{-4} M L-NNA (Fig. 2-2*A*). The UTP-induced contraction was largely blocked by endothelial denudation, and this maneuver abolished the difference between the two groups (Fig. 2-2*A*). The enhanced UTP-induced contraction seen in the GK group was largely suppressed by suramin (Fig. 2-2*B*), indomethacin (Fig. 2-2*C*), or SQ-29548 (Fig. 2-2*D*). Furthermore, each of these treatments abolished the difference between the two groups. Like the ATP response, the UTP-induced contraction was small in the absence of L-NNA treatment (Fig. 2-2*E*; note the expanded ordinate scale). The contraction induced by another $P2Y_2/P2Y_4$ agonist (UTPγS) in the presence of L-NNA was greater in the GK group than in the Wistar group (data not shown). A selective $P2Y_6$ receptor agonist (MRS-2693) induced a very small contraction in each group (Fig. 2-2*F*; note the expanded ordinate scale).

To assess the possible involvement of the $cPLA_2$/COX pathway in the enhanced nucleotide-induced contractions in GK rats, mesenteric rings were preincubated for 30 min with 10^{-4} M valeroyl salicylate (a COX-1-selective inhibitor; Fig. 3, *A* and *D*), 10^{-6} M NS-398 (a COX-2-selective inhibitor; Fig. 2-3, *B* and *E*), or 10^{-5} M $AACOCF_3$ (a $cPLA_2$ inhibitor; Fig. 2-3, *C* and *F*). Interestingly, the contractions induced by ATP (Fig. 2-3, *A–C*) and UTP (Fig. 2-3, *D–F*) were significantly inhibited by each of these inhibitors. It should be noted that in the presence of each inhibitor, however, the two nucleotide-induced contractions were still significantly greater in rings from GK rats than in those from Wistar rats (Fig. 2-3).

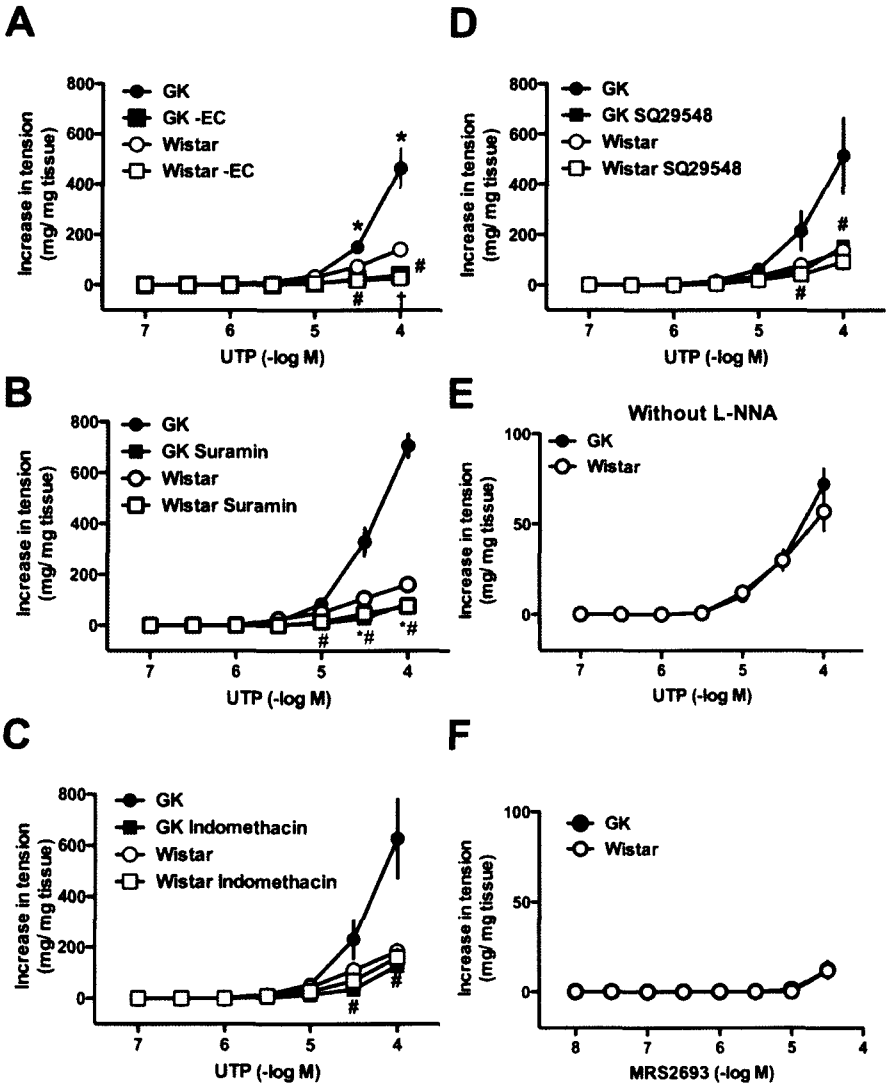


Fig. 2-2. Concentration-response curves for UTP-induced contractions in rings of mesenteric arteries obtained from Wistar and GK rats. (*A–D*) effects of endothelial denudation (*A*), the nonselective P2Y receptor antagonist suramin (10^{-4} M; *B*), the nonselective COX inhibitor Indo (10^{-5} M; *C*), and the TP receptor antagonist SQ-29548 (3×10^{-6} M; *D*) on UTP-induced contractions in the presence of 10^{-4} M L-NNA. (*E*) UTP-induced contractions in the absence of 10^{-4} M L-NNA. (*F*) MRS-2693 (selective $P2Y_6$ agonist)-induced contractions in the presence of 10^{-4} M L-NNA. The ordinate shows the increase in tension (expressed in mg tension/mg tissue) measured at the peak of the response. Note the expanded ordinate scales in (*E*) and (*F*). Data are means ± SE from 4–8 experiments. $^{*}P < 0.05$ vs. the Wistar group in (*A*) and (*B*). $^{\#}P < 0.05$ vs. the GK group in (*A–D*). $^{\dagger}P < 0.05$, the Wistar group vs. the Wistar group with endothelium denudation in (*A*).

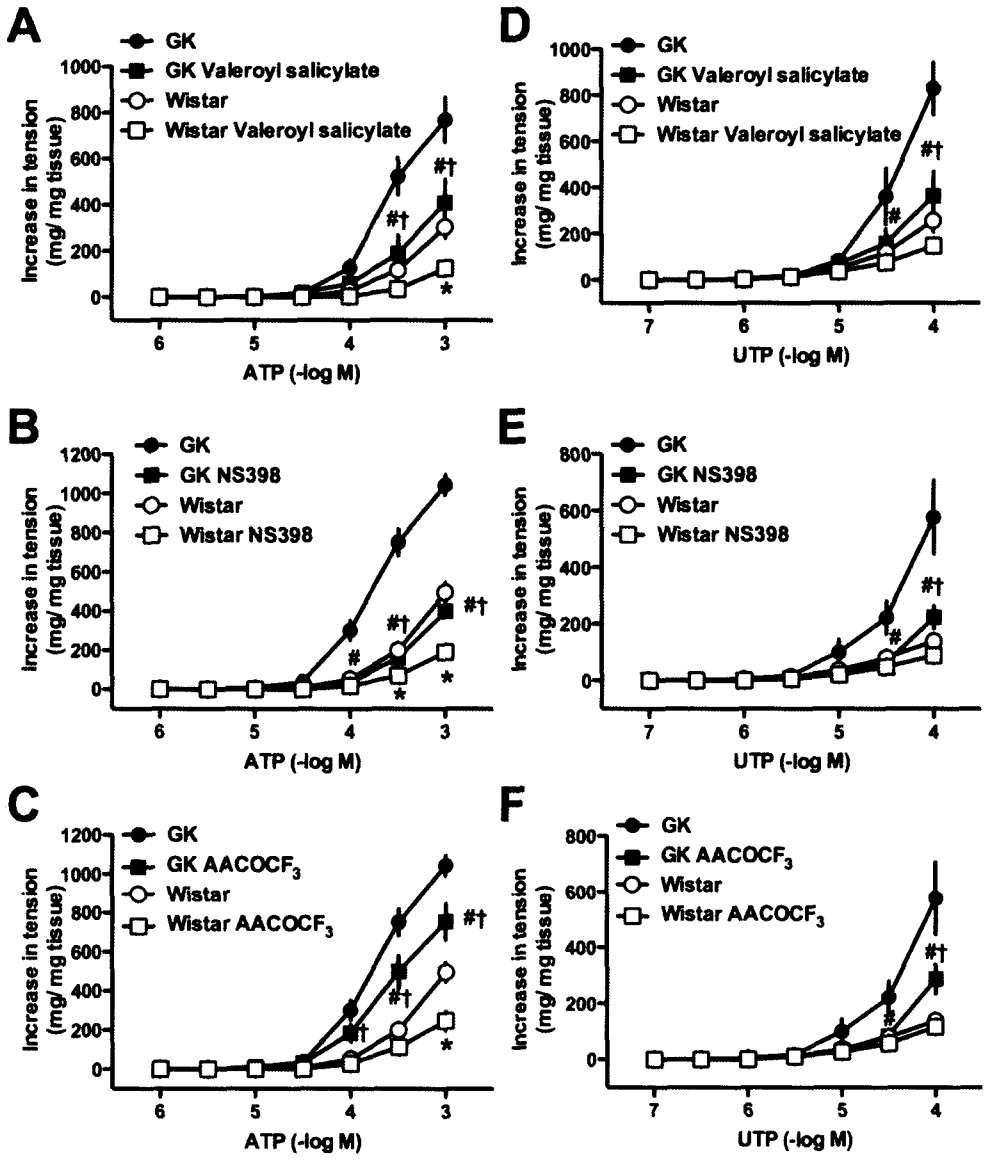


Fig. 2-3. Effects of a specific COX-1 inhibitor [10^{-4} M valeroyl salicylate (VAS); (*A*) and (*D*)], a specific COX-2 inhibitor (10^{-6} M NS-398; *B* and *E*), or a specific cystolic phospholipase A_2 (cPLA$_2$) inhibitor [10^{-5} M arachidonyltrifluoromethyl ketone (AACOCF$_3$); *C* and *F*] on ATP-induced (*A–C*) or UTP-induced (*D–F*) contractions in rings of mesenteric arteries isolated from Wistar and GK rats. Data are means ± SE from 6 experiments. The curves shown in (*A*) for GK and Wistar rats are shown again in (*B*) and (*C*). The curves shown in (*D*) for GK and Wistar rats are shown again in (*E*) and (*F*). $^*P < 0.05$ vs. the Wistar group. $^{\#}P < 0.05$ vs. the GK group. $^{\dagger}P < 0.05$, the inhibitor-treated GK group vs. the inhibitor-treated Wistar group.

Effect of short-term treatment with losartan on ATP- or UTP-induced contraction

Kamata et al. [9, 15, 19] and others [33] have demonstrated that ARBs have beneficial effects on vascular function in diabetic models. Moreover, I have previously reported that the mesenteric artery ANG II content was increased in GK rats [19] and further that short-term losartan treatment of GK rats at the chronic stage of diabetes normalized both the endothelin-1-induced contraction [19] and EDRF-mediated relaxation [15]. To investigate the effect on nucleotide-induced contractions caused by blockade of the AT_1 receptor, I investigated the effect of a 2-wk treatment with losartan on contractions (Fig. 2-4). Surprisingly, the increased ATP-induced (Fig. 2-4*A*) and UTP-induced (Fig. 2-4*B*) contractions were each strongly suppressed by such treatment in GK rats.

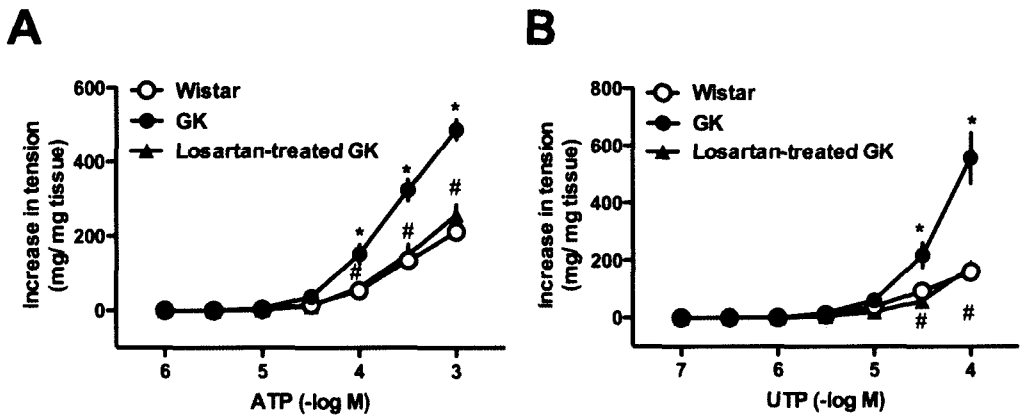


Fig. 2-4. Short-term (2 weeks) treatment with losartan suppresses nucleotide-induced contractions in GK rats. (*A*) and (*B*) concentration-response curves for ATP-induced (*A*) or UTP-induced (*B*) contractions in rings of mesenteric arteries obtained from Wistar, GK, and losartan-treated GK rats. Data are means ± SE from 8–12 experiments. $^{*}P < 0.05$, the GK group vs. the Wistar group. $^{\#}P < 0.05$, the losartan-treated GK group vs. the GK group.

ATP- or UTP-stimulated release of prostanoids

The results described above (see Figs. 2-1–2-3) could be consistent with the enhanced nucleotide-induced contractions in GK rats being attributable to activation of the $cPLA_2$/COX pathway. I therefore investigated the release of prostanoids from mesenteric arteries isolated from Wistar, GK, and losartan-treated GK rats (Fig. 2-5). In nonstimulated mesenteric rings, the production of PGE_2 (Fig. 2-5*A*), $PGF_{2\alpha}$ (Fig. 2-5*B*), 6-keto-$PGF_{1\alpha}$ (a stable metabolite of PGI_2; Fig. 2-5*C*), and TXB_2 (a stable metabolite of TXA_2; Fig. 2-5*D*) did not differ among the three groups. ATP (3×10^{-4} M) and UTP (10^{-4} M) each induced the release of PGE_2 (Fig. 2-5*E*) and $PGF_{2\alpha}$ (Fig. 2-5*G*), and each response was significantly greater in GK rats than in Wistar rats. Surprisingly, the UTP-induced release of each of those two prostanoids was significantly reduced in the losartan-treated GK group. Neither the ATP-induced nor UTP-induced production of 6-keto-$PGF_{1\alpha}$ differed among the Wistar, GK, and losartan-treated GK groups (Fig. 2-5*F*). The ATP-induced release of TXB_2 was greater in GK rats than in Wistar rats, but this enhancement was not affected by losartan treatment (Fig. 2-5*H*).

To examine the mechanism responsible for the nucleotide-induced release of PGE_2 from GK mesenteric artery rings, I investigate the effects of endothelial denudation and COX inhibitors on ATP-induced (Fig. 2-5*I*) or UTP-induced (Fig. 2-5*J*) PGE_2 production. The increased ATP stimulated PGE_2 production seen in the GK group tended (but not significantly) to be reduced by endothelial denudation, and it was significantly reduced by indomethacin, valeroyl salicylate, and NS-398 (Fig. 2-5*I*). The increased UTP-stimulated PGE_2 production seen in the GK group was significantly reduced by each of the four maneuvers (endothelial denudation, indomethacin, valeroyl salicylate, and NS-398; Fig. 2-5*J*).

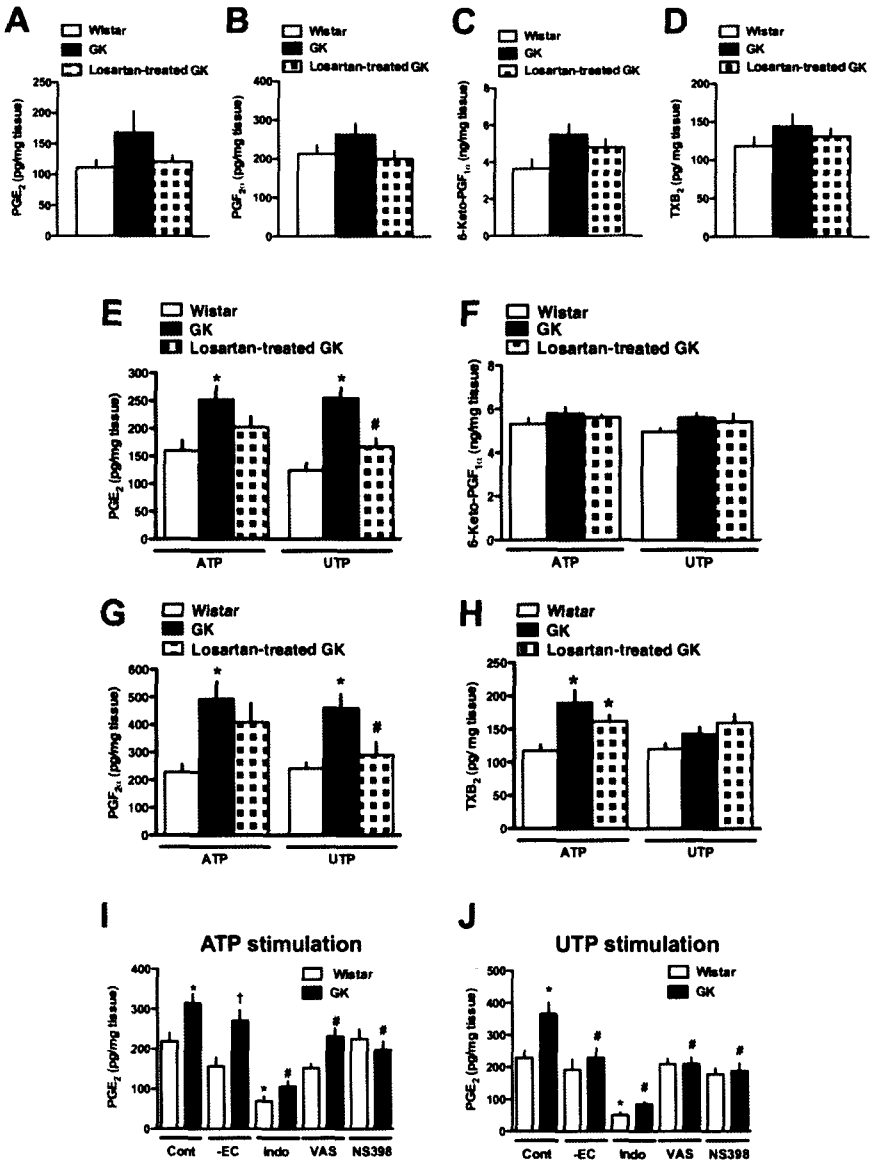


Fig. 2-5. Release of prostanoids [PGE_2 (*A* and *E*), $PGF_{2\alpha}$ (*B* and *G*), 6-keto-$PGF_{1\alpha}$ (a stable metabolite of prostacyclin; *C* and *F*), and thromboxane (TX)B_2 (a stable metabolite of TXA_2; *D* and *H*)] from rings of mesenteric arteries isolated from Wistar, GK, and losartan-treated GK rats either without (*A–D*) or with (*E–H*) stimulation by 3×10^{-4} M ATP or 10^{-4} M UTP. *I* and *J*: effects of endothelial denudation or COX inhibitors (10^{-5} M Indo, 10^{-4} M VAS, or 10^{-6} M NS-398) on PGE_2 release from rings of mesenteric arteries isolated from Wistar and GK rats upon stimulation with either 3×10^{-4} M ATP (*I*) or 10^{-4} M UTP (*J*). Data are means ± SE from 6–12 experiments. $^*P < 0.05$ vs. the Wistar group in (*E*), (*G*), and (*H*). $^{\#}P < 0.05$, the losartan-treated GK group with UTP vs. the GK group with UTP in (*E*) and (*G*). In (*I*) and (*J*), $^*P < 0.05$ vs. the Wistar control group. $^{\#}P < 0.05$ vs. the GK control group. $^{\dagger}P < 0.05$ vs. the Wistar group with endothelium denudation.

Expression of COX-1, COX-2, phospho-cPLA$_2$, and cPLA$_2$

To investigate the possible mechanisms underlying the above beneficial effects of losartan on ATP/UTP-mediated responses in mesenteric arteries from GK rats, I first examined the protein expression of COX-1, COX-2, and cPLA$_2$ (Fig. 2-6). The protein expressions of COX-1 (Fig. 2-6*A*) and COX-2 (Fig. 2-6*B*) were each significantly greater in GK rats than in Wistar rats, but losartan treatment reduced only the elevated COX-2 expression.

cPLA$_2$ is activated by a rise in cytosolic Ca^{2+} induced by receptor stimulation and by its own phosphorylation (Ser^{505}) [34]. I examined the expression of phospho-cPLA$_2$ after P2Y stimulation. In the GK group, ATP-stimulated and UTP-stimulated cPLA$_2$ phosphorylation levels were each significantly increased (vs. those in Wistar rats), and the UTP-induced increase was completely normalized in the losartan-treated GK group (vs. the UTP-stimulated GK group; Fig. 2-6*C*). Expression of total cPLA$_2$ was not different among the Wistar, GK, and losartan-treated GK groups (data not shown).

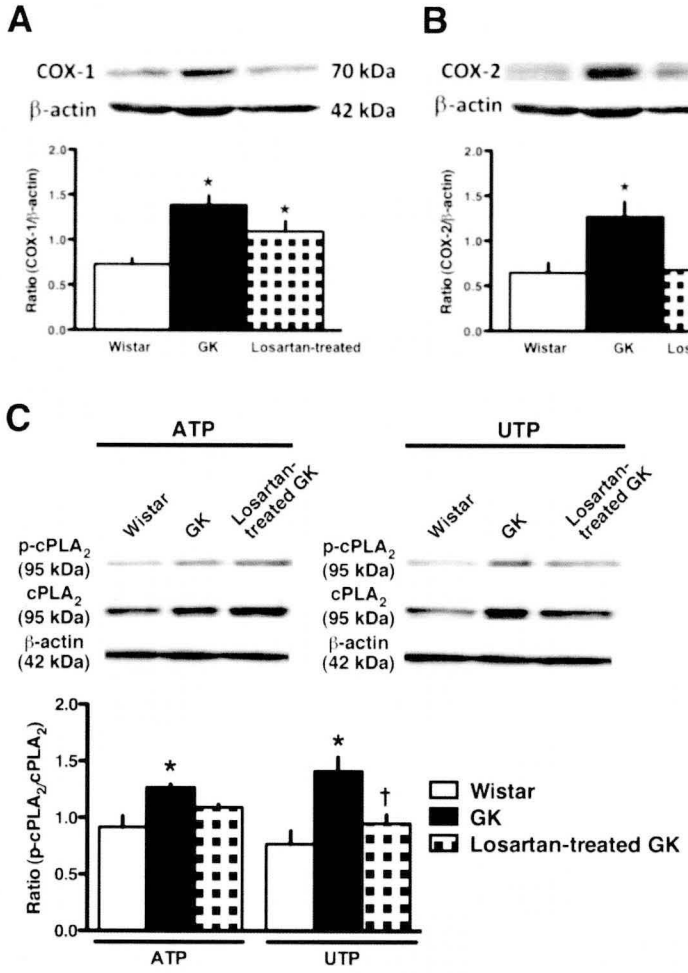


Fig. 2-6. *A* and *B*: analysis of COX-1 (*A*) and COX-2 (*B*) protein expressions in mesenteric arteries from Wistar, GK, and losartan-treated GK rats. Data are means ± SE from 6–8 experiments. $^{*}P < 0.05$ vs. the Wistar group. $^{\#}P < 0.05$, the losartan-treated GK group vs. the GK group. *Top*: representative Western blot is shown (The same samples were loaded on the same gel for COX-1; COX-2, and β-actin. Therefore, the β-acting loading control blot in figure appeared to be the same.) (*C*) Western blots for ATP (3×10^{-4} M)-induced or UTP (10^{-4} M)-induced $cPLA_2$ phosphorylation in mesenteric arteries obtained from Wistar, GK, and losartan-treated GK rats. Ratios were calculated for the optical density of phosphorylated (p-)$cPLA_2$ over that of $cPLA_2$. Data are means ± SE from 6 experiments. $^{*}P < 0.05$ vs. the corresponding Wistar group. $^{\#}P < 0.05$, the losartan-treated GK group vs. the GK group. $^{\dagger}P < 0.05$, the losartan-treated GK group with UTP vs. the GK group with UTP.

Expression of $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors

I measured $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptor expressions in mesenteric arteries by immunoblot analysis (Fig. 2-7). The expressions of $P2Y_2$ (Fig. 2-7*A*) and $P2Y_6$ (Fig. 2-7*C*) were each similar between GK and Wistar rats, whereas $P2Y_4$ (Fig. 2-7*B*) expression was lower in GK rats than in Wistar rats. Losartan treatment of GK rats did not affect $P2Y_2$ and $P2Y_6$ expressions, but it increased $P2Y_4$ expression.

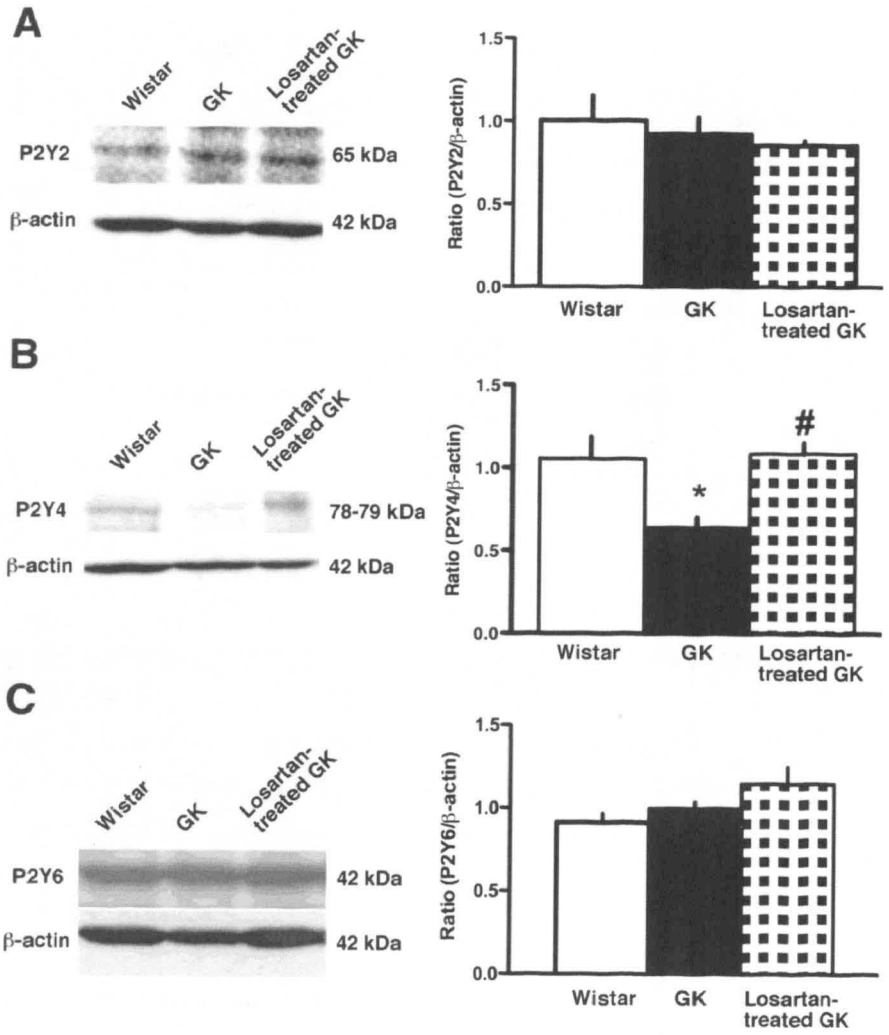


Fig. 2-7. (*A–C*) Western blots for $P2Y_2$ (*A*), $P2Y_4$ (*B*), and $P2Y_6$ (*C*) receptor expressions in mesenteric arteries from Wistar, GK, and losartan-treated GK rats. Data are means ± SE from 5–8 experiments. $^{*}P < 0.05$, the GK group vs. the Wistar group. $^{\#}P < 0.05$, the losartan-treated GK group vs. the GK group.

Effects of losartan on superoxide production in GK rats

Superoxide leads to an enhancement of EDCF signaling [35-38]. Here, I investigated the effect of losartan treatment on superoxide generation from the nonstimulated mesenteric artery by measuring the amount of NBT reduced by superoxide (Fig. 2-8) [12, 39]. Mesenteric artery superoxide generation was *1*) significantly greater in rings from GK rats than in those from Wistar rats and *2*) significantly weaker in the losartan-treated GK group than in the losartan-untreated GK group.

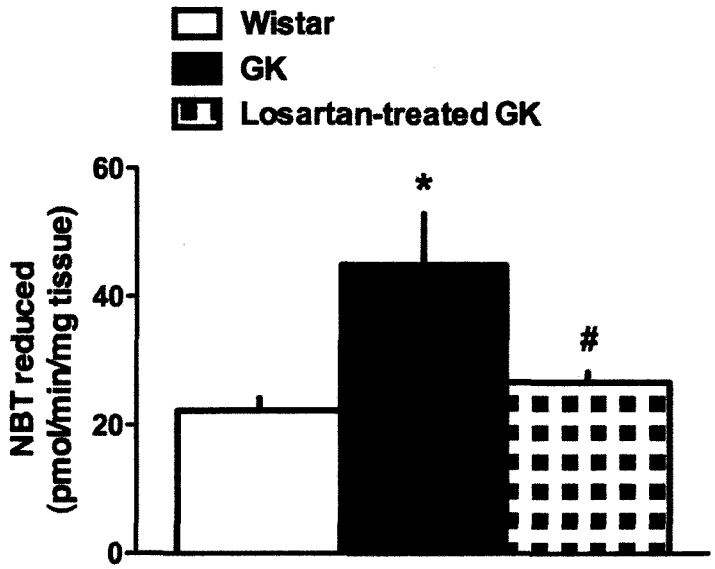


Fig. 2-8. Quantification of mesenteric artery superoxide production by measurement of the amount of reduced nitroblue tetrazolium (NBT). Tissues were obtained from Wistar, GK, and losartan-treated GK rats. Data are means ± SE from 5 or 6 experiments. $^{*}P < 0.05$, the GK group vs. the Wistar group. $^{\#}P < 0.05$, the losartan-treated GK group vs. the GK group.

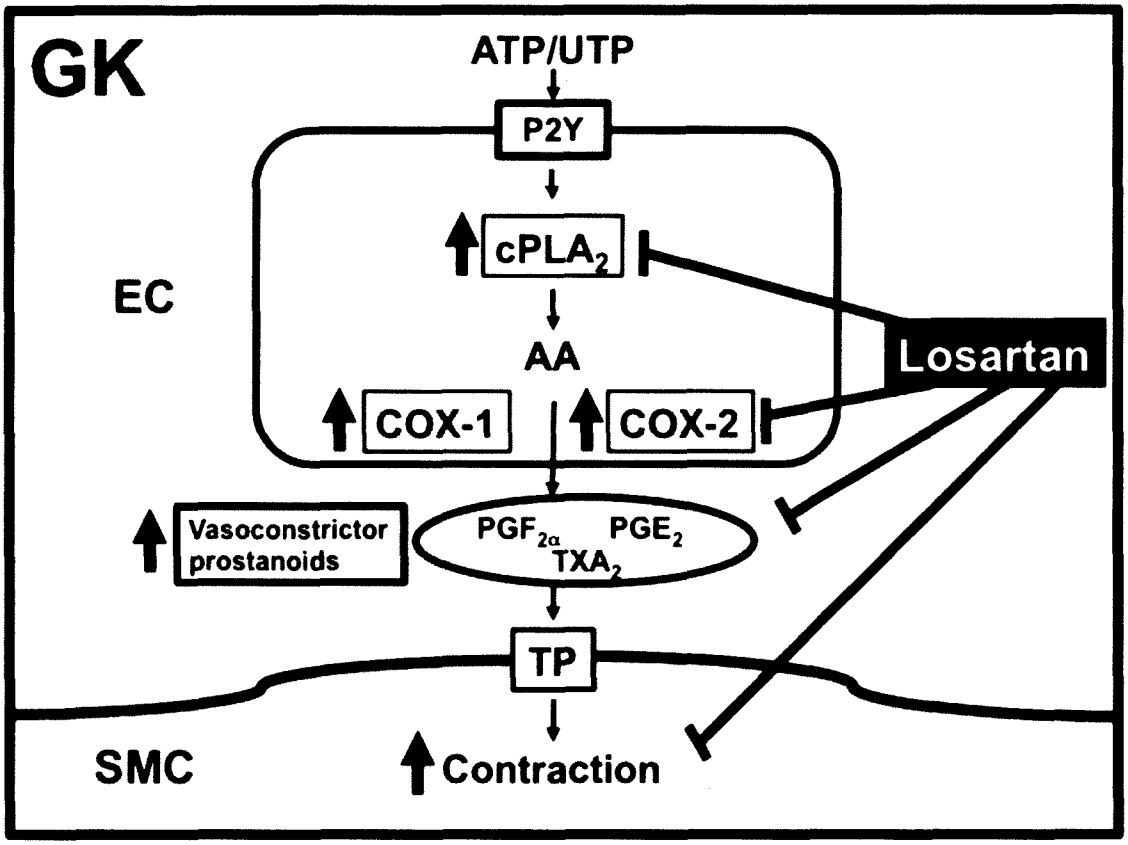


Fig. 2-9. Schematic diagram showing the possible pathways mediating the enhanced nucleotide-induced contractions observed in superior mesenteric artery rings obtained from type 2 diabetic GK rats. I suggest that this enhancement is due to *1*) abnormal release of prostanoids from the endothelium after P2Y receptor stimulation and *2*) increased expressions of p-cPLA$_2$, COX-1, and COX-2. In the present study, short-term losartan treatment of such diabetic rats normalized *1*) the increased nucleotide-induced contractions and the levels of PGE_2 and $PGF_{2\alpha}$ release and *2*) the expressions of COX-2 and p-cPLA$_2$. AA, arachidonic acid; EC, endothelial cell; SMC, smooth muscle cell.

Discussion

The main inference to be drawn from the present study is that in superior mesenteric arteries, nucleotide-induced contractions are enhanced in type 2 diabetic GK rats (vs. their nondiabetic controls). This enhancement may be due to *1*) abnormal release of prostanoids from the endothelium and *2*) increases in the expressions of phospho-cPLA$_2$, COX-1, and COX-2. Furthermore, short-term losartan treatment of such diabetic rats normalized *1*) the increased nucleotide-induced contractions and the release of PGE_2 and $PGF_{2\alpha}$ and *2*) the expressions of COX-2 and phospho-cPLA$_2$. To help the reader follow the discussion of the present results and the putative underlying mechanisms, I have included a schematic summary of the pathways and interactions that may be involved (Fig. 2-9).

The GK rat, a model of type 2 diabetes without the confounding effects of obesity or hypertension [16], was used here at the chronic stage of diabetes (37–42 weeks old) because long-term diabetic conditions are associated with severe diabetic complications, including cardiovascular dysfunction. Indeed, numerous reports [13, 15, 17-19, 40-42] have indicated that abnormal vascular reactivity is present in vessels from GK rats at this chronic stage. However, no previous study has investigated nucleotide-induced contractile responses and how the relevant downstream pathways or receptor expressions might be altered in a long-term diabetic state.

In the present study, the ATP-induced contraction was enhanced in mesenteric arteries isolated from such GK rats (vs. those from the controls) under conditions in which NOS activity was inhibited. To investigate the mechanisms underlying this enhanced contraction, I first focused on the relationship between ATP-induced contraction and EDCFs (viz. vasoconstrictor prostanoids) because *1*) ATP can release such factors [7]; *2*) Kamata et al. [9-12] previously found that signaling mediated by these factors was abnormal in mesenteric arteries from Otsuka Long-Evans Tokushima Fatty rats, another type 2 diabetic model; and *3*) ATP-induced (Fig. 2-1*F*) and UTP-induced (Fig. 2-2*E*) contractions were smaller in the absence than in the presence of NOS inhibitor treatment. Indeed, some previous studies [7, 29]

have used an NOS inhibitor (10^{-4} M L-NNA) in investigations of EDCF signaling. Here, I found that *1*) the ATP-induced contraction was suppressed by endothelial denudation and by COX inhibitors in both GK and Wistar rats, *2*) ATP-stimulated prostanoid release (viz. PGE_2, $PGF_{2\alpha}$, and TXA_2) was increased in GK rats, and *3*) COX-1 and COX-2 expressions were increased in GK rats. Interestingly, indomethacin and the TP receptor antagonist SQ-29548 each abolished the difference in the ATP-induced contraction between the GK and Wistar groups. These results suggest that the enhancement of ATP-induced contraction seen in type 2 diabetic mesenteric arteries may be attributable to increases in the COX-mediated release of endothelium-derived prostanoids.

It has previously been demonstrated that nucleotide-induced vasoconstriction is mediated by P2X and P2Y receptors in various arteries [1, 2]. To investigate which receptors might be responsible for the enhancement of ATP-induced contraction in GK mesenteric arteries, I used several agonists/antagonists of these receptors. In vascular smooth muscle cells, $P2X_1$ is the dominant P2X subtype [43], although $P2X_2$, $P2X_3$, $P2X_4$, and $P2X_5$ have also been found [44, 45]. NF-023 inhibits $P2X_1$ receptors more effectively than $P2X_2$, $P2X_3$, or $P2X_4$ receptors [46], whereas α,β-methylene ATP is a relatively selective agonist for $P2X_1$ and $P2X_3$ receptors [46]. I found that *1*) the contraction induced by ATP was suppressed by both NF-023 and suramin [a nonselective P2 antagonist [27, 28, 47, 48]]; *2*) suramin, but not NF-023, abolished the difference between the GK and Wistar groups; *3*) the α,β -methylene ATP-induced contraction was increased in the GK group versus the Wistar group, but this response was not changed by indomethacin treatment in either group; and *4*) the enhancement of the UTP ($P2Y_2/P2Y_4$ agonist)-induced contraction seen in GK rats (vs. Wistar rats) was greatly suppressed by endothelial denudation, suramin, indomethacin, and SQ-29548, and each of these four maneuvers abolished the difference between the two groups. Taken together, these results suggest that the enhancement of ATP-induced contraction seen in the GK mesenteric artery may be attributable attributable to increased endothelium-derived vasoconstrictor prostanoid signaling after activation of the P2Y receptor, rather than of the P2X receptor. Actually,

several reports [49-51] have suggested that activation of the P2Y receptor stimulates the production of prostanoids.

Vascular endothelial cells reportedly express several subtypes of P2Y receptors [43, 52]. In the present study, I found that *1*) the contractions induced by $P2Y_2/P2Y_4$ agonists (UTP or UTPγS) were increased in the GK group; *2*) a $P2Y_6$ agonist induced only a very small contraction that was similar between the two groups; and *3*) expressions of $P2Y_2$ and $P2Y_6$ were each similar between the two groups, whereas $P2Y_4$ expression was reduced in the GK mesenteric artery. Currently, it is not possible to make a proper study of single P2 receptor subtypes due to the lack of selective antagonists. From my experiments, I cannot completely exclude the possibility that alterations in the P2 receptor might play a role in the enhancement of nucleotide-induced vasoconstrictions in the GK mesenteric artery. However, it seems more likely that the pathway downstream of P2 receptors is altered in diabetic mesenteric arteries.

Prostanoids are released via the arachidonic acid (AA) pathway, which starts with PLA_2 activation and leads to the release of AA from cellular phospholipids. Here, I found that nucleotide-induced contractions were reduced by $AACOCF_3$, a potent and selective inhibitor of $cPLA_2$. Moreover, both ATP-and UTP-stimulated $cPLA_2$ phosphorylation (activation) were increased in the GK group. COX- and endothelium-dependent contractions have been reported in the arteries of several species in response to various agonists and substances [6, 36, 38, 53]. In the present study, expressions of COX-1 and COX-2 were increased in mesenteric arteries isolated from GK rats. Moreover, the increased nucleotide-induced contractions in GK arteries were reduced by selective inhibitors of each of the COXs. The increased nucleotide-stimulated PGE_2 release seen in mesenteric arteries from GK rats was suppressed by indomethacin (ATP or UTP stimulation), valeroyl salicylate (ATP or UTP stimulation), and NS-398 (ATP or UTP stimulation). These results suggest that increased $cPLA_2$/COX pathway activity contributes to the enhancement of nucleotide-induced contractions seen in mesenteric arteries from GK rats. It should be noted that neither of the selective inhibitors of COX-1 or COX-2 abolished the differences in nucleotide-induced contractions between GK

and Wistar rats, but that indomethacin did abolish these differences. I speculate that activations of COX-1 and COX-2 contribute synergistically to the enhancement of nucleotide-induced vasoconstrictions in mesenteric arteries in diabetic rats. This idea is supported by published evidence showing that in several arteries from individuals with type 2 diabetes or other disease conditions, EDCF signalings are mediated not only by COX-1 activation but also by COX-2 activation [6, 10, 36, 38, 53].

Although I am uncertain as to the precise mechanism underlying the above differences between Wistar and GK rats in P2Y receptor expression and in the downstream components, various changes related to the long-term diabetic state might be involved. For instance, Thaning et al. [54] noted attenuated purinergic receptor function in type 2 diabetic patients, whereas hypoglycemia induced $P2Y_4$ upregulation [55]. Furthermore, activation of $cPLA_2$ can be induced by high glucose [56], or insulin [57], or high ANG II [58] [which is increased in mesenteric arteries from GK rats [19]]. Thus, it is possible that the abnormalities observed in the P2Y receptor-dependent $cPLA_2$/COX pathway in GK arteries may have been secondary to one or more of the above variables changing as a result of the long-term disease state present in chronically diabetic GK rats. However, to establish a relationship between the levels of extracellular nucleotides and P2Y receptor expressions and/or functions will require research focusing, for example, on time course changes in the above factors in the diabetic state, because extracellular nucleotide levels are altered in diabetic states [1].

ARBs are very effective, safe antihypertensive drugs with pleiotropic effects (such as antiatherogenic, and antidiabetic, antiplatelet-aggregating effects) [59]. A novel, intriguing, and potentially important finding of the present study is that 2 weeks losartan treatment normalized the enhanced contractile responses to nucleotides seen in GK arteries. In fact, losartan improved *1*) ATP- and UTP-induced contractions, *2*) UTP-stimulated $cPLA_2$ phosphorylation and the UTP-induced release of PGE_2 and $PGF_{2\alpha}$, and *3*) COX-2 expression, all in arteries from GK rats at the established stage of diabetes. The above finding is supported by evidence from some others models. For example, ANG II increases $cPLA_2$

activity in vascular smooth muscle cells [58] as well as both COX-2 expression and PGE_2 production in aortic fibroblasts from normotensive and hypertensive rats through AT_1 receptors [60]. Moreover, we [9] have previously reported that losartan treatment of type 2 diabetic rats suppressed both EDCF-mediated contraction and prostanoid release in their isolated mesenteric arteries. Together, the previous evidence and the present data suggest that losartan normalizes nucleotide-induced contractions via suppression of the $cPLA_2$/COX pathway.

One possibility for the mechanism underlying the losartan-induced improvement of nucleotide-mediated responses is that there may be a reduction in oxidative stress. There is a number of reports [6, 35, 37, 61] suggesting that oxidative stress augments EDCF-mediated responses. Indeed, I have preliminary evidence showing that nucleotide-induced vasoconstrictions in mesenteric arteries from GK rats can be reduced by tempol (a superoxide dismutase mimetic) or by apocynin [an inhibitor of NAD(P)H oxidase] (unpublished observations). Since previous reports [9, 62, 63] and the present data (Fig. 2-8) suggest that losartan decreases oxidative stress, the normalization of nucleotide-induced signaling by losartan may be mediated by its antioxidative actions. As yet, it remains unclear whether there is a direct relationship between the effects of losartan on responses mediated by nucleotides and its effects on these cellular pathways. Further investigations will be required on this point.

Several limitations of the present study should be mentioned. In the mesenteric arteries of our GK rats, there were differences between ATP and UTP in terms of their prostanoid release profiles and in the effects of losartan on the release of prostanoids. As mentioned above, it is not yet possible to study single P2 receptor subtypes due to the lack of selective antagonists. The ligand-receptor interactions (activation) involved in nucleotide signaling are generally complex. Most of these receptors are capable of mediating responses to several nucleotides, resulting in multiple receptors having overlapping ligand preferences [2, 3, 4]. Moreover, there is a possibility that release of prostanoids may be induced by nucleotides in cells other than endothelial cells. Indeed, there are reports [64, 65] of prostanoid release in

vascular smooth muscle cells. Without further studies, I cannot distinguish which P2 receptor activated signaling (endothelial cells vs. smooth muscle cells) might contribute to the enhanced nucleotide-induced vasoconstrictions present in GK diabetic mesenteric arteries.

In conclusion, my study demonstrate that the enhanced ATP-induced contraction seen in mesenteric arteries taken from GK rats at the chronic stage of diabetes is due to increased $cPLA_2$/COX pathway activity and that AT_1 receptor antagonism normalizes the P2Y-induced contraction in such arteries via a suppression of that pathway's activity. I believe that my findings should stimulate further interest in the manipulation of ATP and/or ANG II signaling as potential therapeutic targets in the battle against diabetes-associated vascular diseases.

Chapter 3

Protein kinase C delta contributes to increase in EP3 agonist-induced contraction in mesenteric arteries from type 2 diabetic Goto-Kakizaki rats.

Introduction

That prostaglandins (PGs) play important roles in the regulation/maintenance of vascular function is well recognized [1-7]. Among the PGs, prostaglandin E_2 (PGE_2), an important and ubiquitously distributed vasoactive eicosanoid, has been reported to be a potent vasodilator in various vessels in both humans and experimental animals [1, 3, 5]. However, PGE_2 may be a vasoconstrictor in some vessels such as human pulmonary artery [8], guinea pig aorta [9], porcine large cerebral arteries [10], rat aorta [11] and rat mesenteric artery [12]. The actual PGE_2-mediated vascular response appears to depend on species and vessel type as well as on conditions such as physiological and pathophysiological states. For those reasons, the precise mechanisms mediating PGE_2-induced vasocontraction have not been thoroughly explored.

The prostanoid EP receptors are heterotrimetic G proteincoupled receptors (GPCRs) [1, 3, 5]. PGE_2 exerts a broad range of effects including inhibition of smooth muscle cells (mediated by the EP2 and EP4 receptor subtypes) and excitation of smooth muscle cells (mediated by the EP1 and EP3 receptor subtypes) [1, 3, 5]. Concerning the signaling pathway mediating PGE_2-mediated vasocontractions, Shum et al. [13] reported that the EP3 receptor agonist sulprostone induces a contraction in guinea pig aorta that can be inhibited by a Rho kinase (ROCK) inhibitor. More recently, Kobayashi et al. [12] found that the vasocontraction induced by EP3 receptor activation is mediated by Ca^{2+}-independent pathways involving protein kinase C (PKC) δ and ε and ROCK in a main branch of rat mesenteric artery. However, the details of the mechanisms underlying the PGE_2-mediated vasocontraction in diabetic states remain unclear.

Type 2 diabetes is associated with a markedly increased incidence of cardiovascular diseases [14-18]. A growing body of evidence indicates that endothelial dysfunction and smooth muscle dysfunction are present in various regions of the vasculature in type 2 diabetes in both humans and animal models [14-16, 19-21]. For instance, Kamata et al. previously found in vessels isolated from type 2 diabetic Goto-Kakizaki (GK) rats at the chronic stage of diabetes: *1*) that endothelium-dependent relaxation and endothelial nitric oxide synthase (eNOS) activity were

impaired in the aorta [22, 23], *2*) that endothelium-dependent relaxation was impaired in superior mesenteric arteries [24, 25] and *3*) that although endothelin-1 (ET-1)-induced contraction was increased in superior mesenteric arteries, angiotensin II- and arginine vasopressin-induced contractions were similar between the GK and Wistar arteries [21]. Kamata's laboratory proposed that upregulation of the ET_A receptor, a defect in ET_B receptor-mediated NO signaling, and activation of the MEK/ERK pathway together represent a likely mechanism underlying the hyperreactivity to ET-1 that exists at the chronic stage of type 2 diabetes in GK rats [21]. In addition, Kamata's laboratory recently reported that the release of vasoconstrictor prostanoids including PGE_2 that occurs upon agonist stimulation was increased in superior mesenteric arteries from type 2 diabetic rats [26, 27].

There is a growing body of evidence concerning the roles played by PGs in diabetic cardiovascular complications [2, 28]. Cellular and animal models indicate that PGs are induced under diabetic conditions, have proatherogenic effects and also mediate the actions of growth factors and cytokines [28]. Further, it is known that many PGs have specific vasoactive properties, thereby contributing to the local regulation of vascular tone [2, 28]. Indeed, earlier reports had (*a*) proposed that alterations in the vascular metabolism of PGs might play a crucial role in diabetes-associated changes in local vasoregulatory mechanisms [30] and (*b*) found that in mesenteric arteries isolated from type 1 diabetic dogs, exogenous arachidonic acid led to thromboxane A_2-mediated contraction, whereas in those from control animals, it caused prostacyclin-dependent dilatation [31]. In aortas from type 2 diabetic mice, phenylephrine-induced contraction has been found to be reduced and acetylcholine (ACh)-induced relaxation to be enhanced by the nonselective cyclooxygenase (COX) inhibitor indomethacin, suggesting agonist-induced release of vasoconstrictor PGs [32]. Moreover, in type 2 diabetic mice, the basal tone of skeletal muscle arterioles is increased due to an enhanced COX-2-dependent production of vasoconstrictor PGs [33].

The above background indicates not only that vasoconstrictor prostanoids are important for the development of diabetic vasculopathy but also that a proper understanding of PGE_2-

mediated responses and signaling in diabetic arteries is indispensable if effective preventive and therapeutic approaches are to be devised against the development of diabetic vascular complications.

The main aim of the present study was to test my basic hypothesis that the vasoconstrictor effects of PGE_2 are enhanced in the type 2 diabetic GK rat. This is a highly inbred strain of Wistar rats that spontaneously develops type 2 diabetes [34] and offers a convenient model for the study of that disease without the confounding effects of obesity or hypertension [34] at the chronic stage. I also hypothesized that EP3 receptor-mediated contraction might be enhanced in such rats via increased activation of the PKCδ pathway. I used superior mesenteric arteries because my previously found that prostanoid release in response to agonists is increased in such arteries isolated from GK rats, indicating that PGE_2 signaling is likely to be important in setting vascular tone [26]. To test my hypotheses, I performed functional studies as well as experiments to identify some of the molecular mechanisms involved.

Experiment

Reagents

N^G-nitro-L-arginine (L-NNA) and an antibody against β-actin were purchased from Sigma Chemical Co. (St. Louis, MO, USA), while PGE_2, sulprostone, sc19220, an antibody against EP3 receptor and the relevant blocking peptide were from Cayman (Ann Arbor, MI, USA). Acetylcholine (ACh) chloride was purchased from Daiichi Sankyo Pharmaceuticals (Tokyo, Japan), while rottlerin and Gö6976 were from Calbiochem-Novabiochem (La Jolla, CA, USA). $_L$-NNA was dissolved in HPLC grade water, while PGE_2, sulprostone, sc19220, rottlerin and Gö6976 were first dissolved in DMSO. All concentrations are expressed as the final molar concentration of the base in the organ bath. Horseradish peroxidase (HRP)-linked secondary antimouse or antirabbit antibody was purchased from Promega (Madison, WI, USA), antibodies against PKCδ and phospho-PKCδ (Thr^{505}) were from Cell Signaling Technology (Danvers, MA, USA) and antibodies against phospho-caldesmon (Ser^{534} for rat; the corresponding human residue being Ser^{789}) and caldesmon were from Abcam (Cambridge, MA, USA).

Animals and experimental design

Male Wistar control rats and GK rats were obtained at 4 weeks old (Clea, Tokyo, Japan). The experimental design 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 US National Institutes of Health, and with 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 (accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan). All animals were allowed a standard laboratory diet (MF; Oriental Yeast Industry, Tokyo, Japan) and water ad libitum. The experiments described here were performed when the rats were 37–44 weeks old.

Measurement of blood glucose

Plasma glucose was measured by using a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan).

Vascular functional study

Vascular isometric force was measured as in previous papers [21, 24-27]. After euthanasia, the superior mesenteric artery was rapidly removed and immersed in oxygenated, modified Krebs-Henseleit solution (KHS). This solution consisted of 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$ and 11.0 mM glucose. 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 the optimal resting tension (1.0 g) was reached, which value was derived from preliminary length-active tension curves [21, 24-27]. They were then allowed to equilibrate for at least 1 h. Force generation was monitored by means of an isometric transducer (model TB-611 T; Nihon Kohden, Tokyo, Japan).

For vasocontraction studies, PGE_2 (10^{-9}–10^{-5} M) or the EP1/EP3 agonist sulprostone (10^{-9}–10^{-5} M) [11, 13, 35, 36] was added cumulatively to the organ bath until a maximal response was obtained. To avoid the influence of NO on the contractile response, I obtained all data for the concentration–response curves in the presence of the NOS inhibitor L-NNA (10^{-4} M) [21, 26], except when endothelium-denuded preparations were used. To study the role of the EP1 receptor in the PGE_2- and sulprostone-mediated responses obtained under NOS inhibition, we generated concentration–response curves for PGE_2 and sulprostone in the presence of 10^{-5} M sc19220 (selective EP1 antagonist [11, 37]). To study the roles played by PKCα and PKCβ in PGE_2-induced contractions, such contractions were obtained in the presence of 10^{-6} M Gö6976 (PKCα/β inhibitor [12, 38]). To study the role of PKCδ in the PGE_2- and sulprostone-induced contractions, these were obtained in the presence of 10^{-6} M rottlerin

(PKCδ inhibitor [12]). After the addition of sufficient aliquots of a given agonist to produce the chosen concentration, a stable response was allowed to develop before the addition of the next dose of the same agonist. When required, removal of the endothelium from arterial segments was achieved by infusing a 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate solution (CHAPS, 0.1%, for 60 s), followed by flushing out with KHS. The inability of ACh to relax these segments confirmed the success of this procedure, as reported previously [21, 26]. Finally, the wet weight of the mesenteric ring was measured. This weight was slightly but significantly lower in the GK (1.02 ± 0.03 mg; n = 65) group than in the Wistar (1.19 ± 0.03 mg; n = 67, $P < 0.001$) group.

Western blotting

The protein levels of EP3 receptor, phospho-PKCδ (Thr^{505}), total PKCδ, phospho-caldesmon (Ser^{534}), total caldesmon and β-actin were quantified using an immunoblotting procedure, essentially as described before [21, 26]. Superior mesenteric arteries from control and diabetic rats were isolated, cleaned of fat, dissected and frozen in liquid nitrogen. To investigate the expression of phospho-PKCδ or that of phospho-caldesmon in such mesenteric arteries upon sulprostone stimulation, mesenteric arterial rings were incubated with KHS containing 10^{-4} M L-NNA at 37°C for 30 min and then exposed to 10^{-5} Msulprostone or vehicle (water) for 10 min (phospho-PKCδ) or 30 min (phospho-caldesmon). Mesenteric arterial protein extracts (20 μg) were applied to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were incubated with anti-EP3 (1 : 500), anti-phospho-PKCδ (Thr^{505}) (1 : 1,000), anti-PKCδ (1 : 1,000), anti-phospho-caldesmon (Ser^{534} for rat; the corresponding human residue being Ser^{789}) (1 : 1,000) or anti-caldesmon (1 : 1,000) antibody and anti-β-actin (1 : 5,000) antibody. Detection was achieved using a HRP-conjugated IgG followed by enhanced chemiluminescence. Band intensity was quantified by densitometry. Results were normalized to β-actin and are shown relative to control (vehicle-treated Wistar). The specificity of the EP3 antibody was assessed using

blocking peptide according to the manufacturer's instructions.

Statistical analysis

The contractile force developed by rings of superior mesenteric artery is shown here in grams of tension per milligram of tissue. Concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism5.0; Graph-Pad Software, Inc., San Diego, CA, USA). Data are shown as means ± SE. Statistical analysis of the concentration–response curves was performed using two-way analysis of variance (ANOVA) for repeated measures. For comparisons between groups, data were analyzed using ANOVA with a post hoc Bonferroni test or Student's t test. Western blot data were analyzed by one-sample t test or one-way ANOVAwith a post hoc Bonferroni test. Values of $P<0.05$ were considered statistically significant.

Abbreviations

ACh, acetylcholine; ANOVA, analysis of varience; BP, blocking peptide; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate solution; COX, cyclooxygenase; DMSO, dimethylsulfoxide; EC, endothelial cell; EDCF, endothelium-derived contracting; EDRF, endothelium-derived relaxing; GK, Goto-Kakizaki, HRP, Horseradish peroxidase; KHS, Krebs-Henseleit solution; L-NNA, N^G-nitro-L-arginine; NO, nitric oxide; NOS, NO Synthase; PGs, prostaglandins; PGE_2, prostaglandin E_2; PKC, protein kinase C; PVDF, polyvinylidene difluoride; SE, standard error; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis;

Results

Blood glucose and body weight

At the time of the experiment (when the rats were 37–44 weeks old), GK rats displayed a higher blood glucose (non-fasted values) than control Wistar rats (442.0 ± 20.3 mg dL^{-1}; n = 20 vs. 166.5 ± 6.2 mg dL^{-1}; n =2 0, $P < 0.001$). The body weight of the GK rats was lower than that of the Wistar rats (386.2 ± 6.9 g; n = 20 vs. 615.0 ± 15.5 g; n = 20, $P<0.001$).

PGE_2- and sulprostone-induced responses in superior mesenteric artery

NO plays an important role in the regulation of vascular tone and negatively modulates contraction, leading to complexities in analyzing the signaling pathways in the vasculature in some situations [21, 26, 39-41]. To mask any putative NO component in the present vasoconstrictor responses, I performed functional experiments in the presence of a NOS inhibitor (10^{-4} M L-NNA). This inhibits both basal and agonist-induced NOS activities. In that condition, cumulative administration of PGE_2 (10^{-9}–10^{-5} M) (Fig. 3-1*A*) or sulprostone (10^{-9}–10^{-5} M) (Fig. 3-1*B*) induced concentration-dependent contraction in superior mesenteric arterial rings, whether they were from GK or Wistar rats. In the presence of L-NNA, the PGE_2-induced and sulprostone-induced contractions were each greater in rings from GK rats than in those from Wistar rats (Fig. 3-1*A* and *B*). Likewise, the PGE_2-induced contraction was greater in endothelium-denuded rings from GK rats than in those from Wistar rats (Fig. 3-1*C*). To determine which receptor (EP1 or EP3) mediates the above contractile responses, we investigated the effect of a selective EP1 receptor antagonist (Fig. 3-1*A* and *B*). That antagonist, sc19220 (10^{-5} M), did not affect the PGE_2-induced vasocontraction in either group (Fig. 3-1*A*) and had a significant suppressive effect on the sulprostone-induced vasocontraction only at 10^{-6} M in the GK group and only at 10^{-5} M in the Wistar group (Fig. 3-1*B*). It should be noted that in the presence of that EP1 antagonist, the PGE_2- and sulprostone-induced vasocontractions were still greater in rings from GK rats than in those from Wistar rats. These results suggest that the augmentations of the PGE_2-induced and

sulprostone-induced contractile responses seen in superior mesenteric arteries from GK rats were attributable to activation of the EP3 receptor rather than of the EP1 receptor.

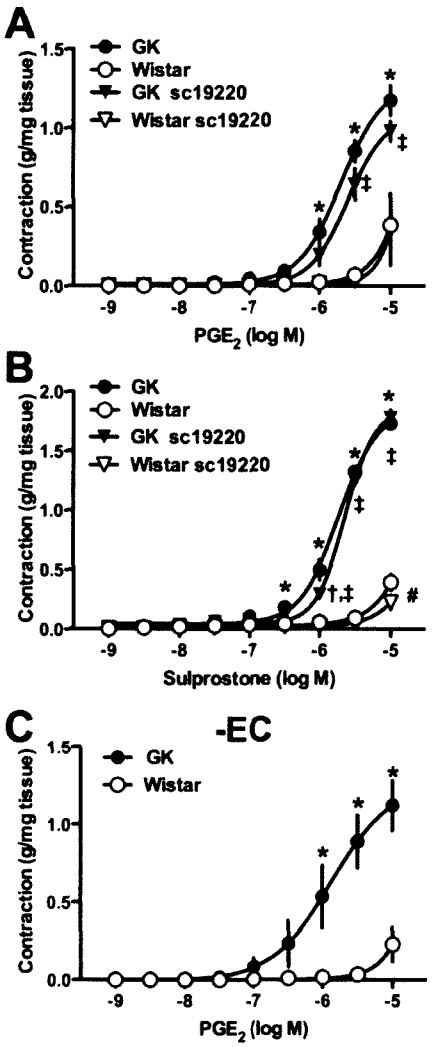


Fig. 3-1. Effects of EP1 antagonist on PGE_2-induced vasocontraction in superior mesenteric artery rings from GK rats. Concentration–response curves for PGE_2 (*A*) and sulprostone (*B*) in the presence of 10^{-4} M LNNA or 10^{-4} M L-NNA plus 10^{-5} M sc19220, and for PGE_2 following endothelial denudation (*C*). Data, which are shown for superior mesenteric arteries from diabetic GK and control Wistar rats, are means ± SE (n = 5 or 6). *P < 0.05, GK vs. Wistar. #P < 0.05, Wistar vs. Wistar sc19220. †P<0.05, GK vs. GK sc19220. ‡P < 0.05, GK sc19220 vs. Wistar sc19220.

Effect of PKCδ inhibition on EP3 receptor-inducedvasocontraction

The published evidence suggests that PKCδ plays an important role in vasocontraction in various arteries [12, 38, 42]. Therefore, we investigated the relative contribution made by PKCδ to EP3 receptor-mediated responses in diabetic superior mesenteric arteries under NOS inhibition. The PGE_2-induced contraction was significantly reduced by the selective PKCδ inhibitor rottlerin (10^{-6} M) (Fig. 3-2*A*) but not by the PKCα/β inhibitor Gö6976 (10^{-6} M) (Fig. 3-2*B*). Similarly, the sulprostone-induced contraction in GK mesenteric arteries was significantly reduced by rottlerin (Fig. 3-2*C*).

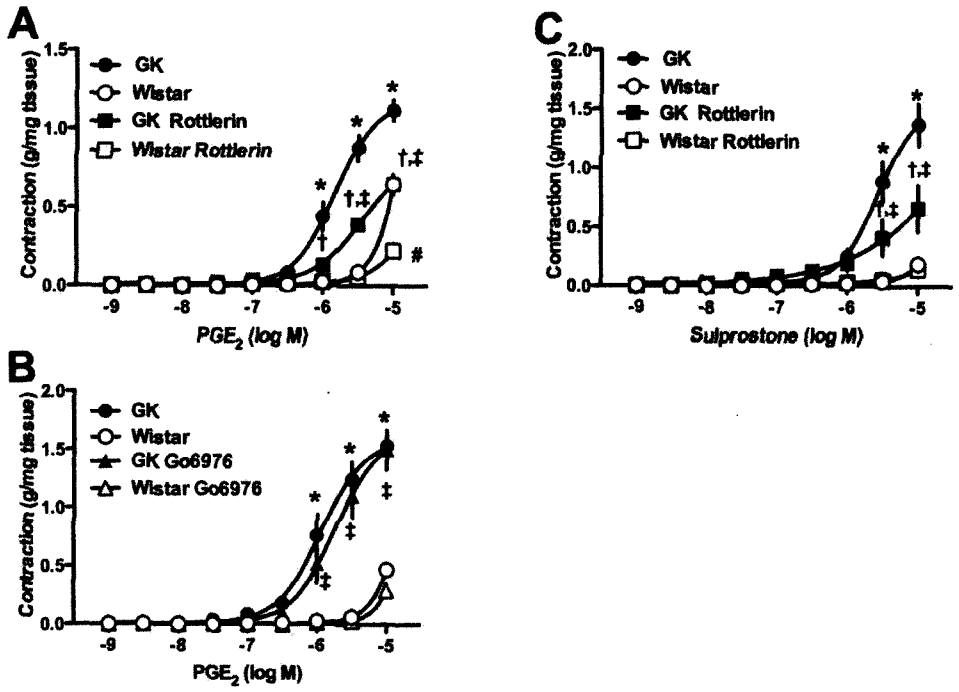


Fig. 3-2. Effects of PKCδ inhibitor or PKCα/β inhibitor on PGE_2-induced vasocontraction in superior mesenteric artery rings from GK rats. Concentration–response curves for PGE_2 (*A*, *B*) or sulprostone (*C*). All experiments were performed in the presence of 10^{-4} M L-NNA with or without either 10^{-6} M rottlerin (*A*, *C*) or 10^{-6} M Gö6976 (*B*). Data, which are shown for superior mesenteric arteries from diabetic GK and control Wistar rats, are means ± SE (n = 5 or 6). *P < 0.05, GK vs. Wistar. #P < 0.05, Wistar vs. Wistar rottlerin. †P < 0.05, GK vs. GK rottlerin. ‡P < 0.05, GK rottlerin vs. Wistar rottlerin.

Sulprostone-induced phosphorylation of PKCδ is increased in GK mesenteric artery

Since PKCs are phosphorylated in the process of activation, the level of phosphorylation indicates their activation level [43]. To assess PKCδ activity, I examined the levels of phosphorylated PKCδ (Thr^{505}) (p-PKCδ). Western immunoblots were obtained from superior mesenteric arteriesafter sulprostone (10^{-5} M for 10 min) or vehicle treatment (Fig. 3-3). Although total PKCδ expression in those arteries was similar between GK and Wistar rats (Fig. 3-3*C*), the p-PKCδ level induced by sulprostone stimulation was significantly greater in the GK than in the Wistar group (Fig. 3-3*B*). These data suggest that an increase in PKCδ activity due to increased phosphorylation at Thr^{505} may contribute to the augmentation of EP3-mediated contraction seen in superior mesenteric arteries from GK rats.

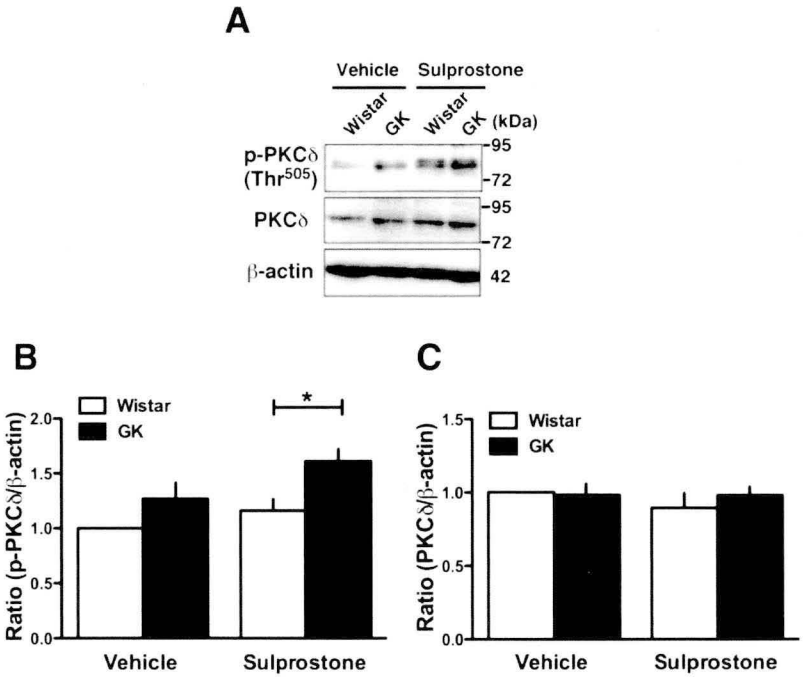


Fig. 3-3. Phosphorylation of PKCδ by EP3 agonist in superior mesenteric arteries from diabetic GK rats. Phospho-PKCδ (Thr^{505}) and total PKCδ protein contents were assessed by Western blotting in superior mesenteric artery rings stimulated with sulprostone (10^{-5} M), or treated with vehicle, for 10 min. (*A*) Representative blots are shown for phosphorylation of PKCδ at Thr^{505} (p-PKCδ) and for PKCδ in Wistar and GK arterial rings stimulated with sulprostone or without stimulation (vehicle), in each case in the presence of 10^{-4} M L-NNA. Bands for phospho-PKCδ (*B*) or total PKCδ (*C*), or for β-actin, were quantified as described in "Methods", ratios being calculated for the optical density of phospho- or total PKCδ over that of β-actin. Data are means ± SE from eight experiments. *$P < 0.05$, GK vs. corresponding Wistar.

Sulprostone-induced phosphorylation of caldesmon is increased in superior mesenteric arteries from GK rats

Caldesmon has been extensively studied as a modulator of smooth muscle contraction and phosphorylation of caldesmon in intact smooth muscle increases in parallel with isometric force [44, 45]. Moreover, previous studies have clearly demonstrated that PKCs phosphorylate caldesmon in various tissues and cells [46, 47]. Therefore, I examined whether the sulprostone-stimulated expression of phosphorylated caldesmon (Ser^{789}) protein in superior mesenteric arteries might be increased in the GK group. Immunoblot analysis of sulprostone (10^{-5} M for 30 min)-stimulated and vehicle-treated arteries from Wistar and GK groups (using anti-phospho-caldesmon and anti-caldesmon antibodies) allowed detection of immunoreactive proteins (Fig. 3-4). For both *h*-caldesmon and *l*-caldesmon, the phosphorylated levels induced by sulprostone were significantly greater in the GK than in the Wistar group (Fig. 3-4*B* and *D*), whereas total caldesmon levels did not differ between those groups (Fig. 3-4*C* and *E*).

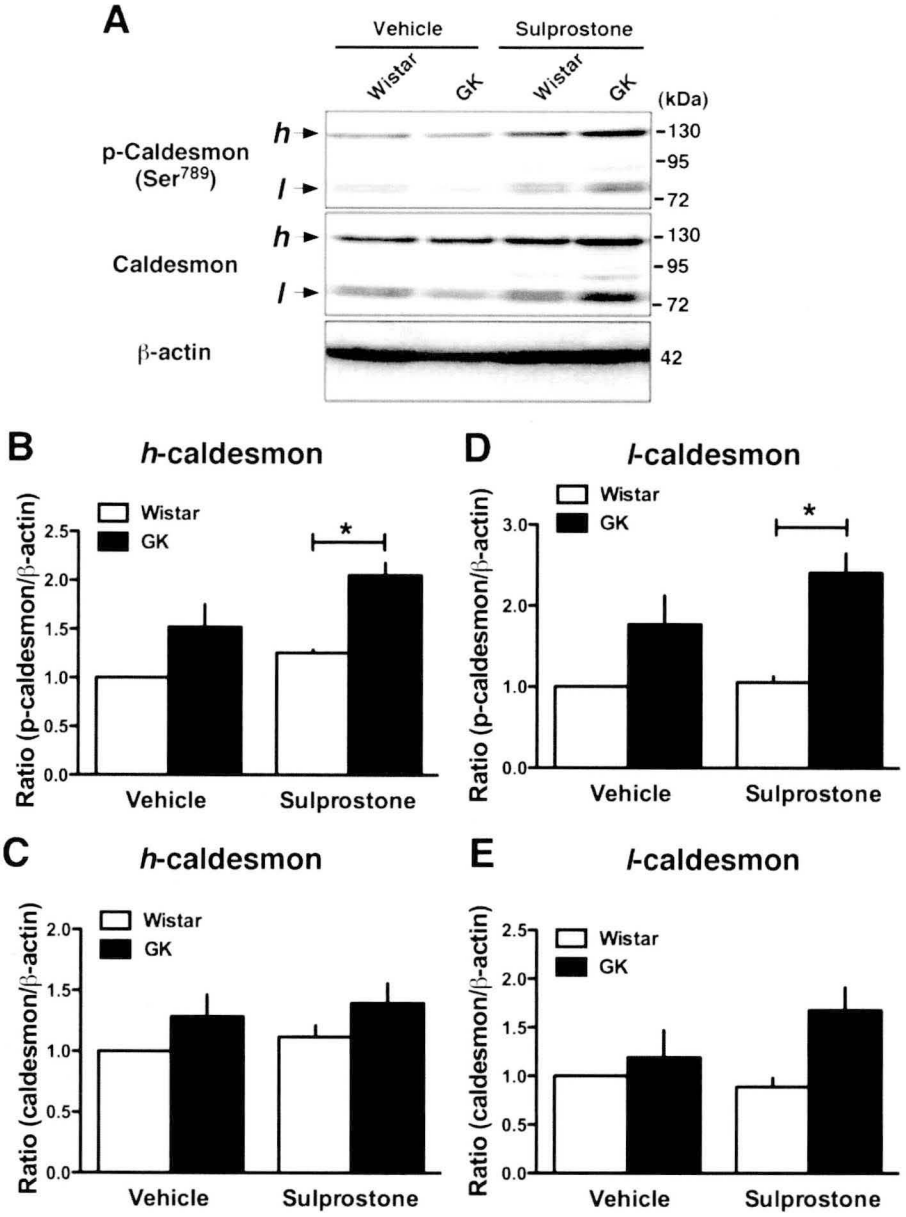


Fig. 3-4. Phosphorylation of caldesmon by EP3 agonist in superior mesenteric arteries from diabetic GK rats. Phospho-caldesmon (Ser^{789}) and total caldesmon protein contents were assessed by Western blotting in superior mesenteric artery rings stimulated with sulprostone (10^{-5} M), or treated with vehicle, for 30 min. (*A*) Representative blots are shown for phosphorylation of caldesmon at Ser^{789} (p-caldesmon) and for caldesmon in Wistar and GK arterial rings either stimulated with sulprostone or without stimulation (vehicle), in each case in the presence of 10^{-4} M L-NNA. Bands for phospho-*h*-caldesmon (*B*), total *h*-caldesmon (*C*), phospho-*l*-caldesmon (*D*) or total *l*-caldesmon (*E*), and for β-actin, were quantified as described in "Methods", ratios being calculated for the optical density of phospho- or total caldesmon over that of β-actin. Data are means ± SE from four or five experiments. *P < 0.05, GK vs. corresponding Wistar.

Expression of EP3 receptor in superior mesenteric arteries

Conceivably, an alteration in EP3 receptor expression could underlie the enhanced EP3 receptor-mediated vasocontraction seen in the GK artery. Accordingly, I investigated EP3 receptor protein expression in superior mesenteric arteries by Western blotting (Fig. 3-5). The EP3 polyclonal antibody detected multiple bands (Fig. 3-5*A*). Importantly, these multiple bands disappeared completely when the primary antibody was pre-incubated with the appropriate antigenic peptide (Fig. 3-5*A*). The protein expression of EP3 (Fig. 3-5*A*; analyzed sum of all multiple bands) in superior mesenteric arteries was similar between Wistar and GK rats (Fig. 3-5*B*).

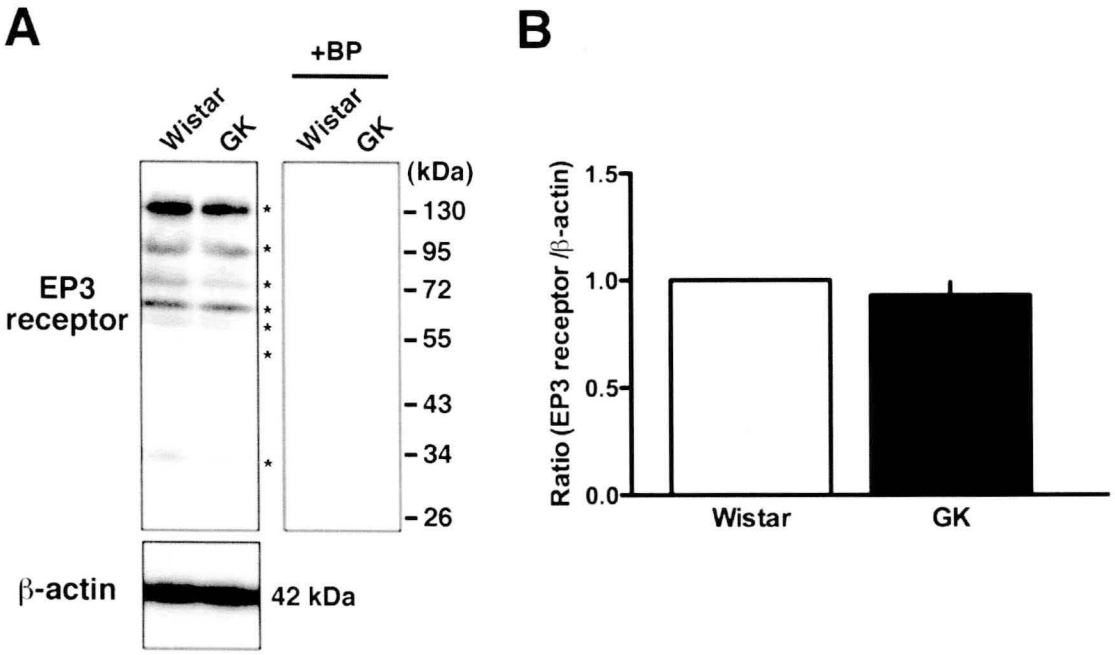


Fig. 3-5. Protein expression of EP3 receptor in superior mesenteric arteries from Wistar and diabetic GK rats. (*A*) Representative Western blots for EP3 receptor and β-actin. Asterisk indicates band disappeared after incubation of the primary antibody for EP3 receptor with the appropriate blocking peptide. BP blocking peptide. (*B*) Bands were quantified as described in "Methods", ratios being calculatedfor the optical density of EP3 receptor over that of β-actin. Data are means ± SE from six experiments.

Discussion

The principal inference to be drawn from the present study is that the contractile response to PGE_2 exhibited by the superior mesenteric artery is enhanced in type 2 diabetic GK rat when the animal is at the chronic stage of its disease. In addition, I found that: *1*) the PGE_2-induced vasocontraction is mediated by EP3, not EP1, receptors; *2*) the augmentation of the EP3 receptor-mediated vasocontraction in the GK artery is attributable to increased activation of PKCδ and increased phosphorylation of caldesmon and *3*) the superior mesenteric arterial expression of EP3 receptors did not differ between the nondiabetic Wistar and diabetic GK groups.

The GK rat, a model of type 2 diabetes without the confounding effects of hypertension or obesity [34], was used here at the later stage of diabetes [viz. 37–44 weeks old] because long-term type 2 diabetic conditions are associated with severe diabetic cardiovascular complications. In fact, numerous reports have indicated that abnormal vascular reactivity is exhibited by vessels taken from GK rats at the chronic stage of their diabetes [21-26, 48-50]. However, no previous study has investigated how PGE_2-induced vasocontraction per se, or the relevant downstream pathways or receptor expressions, might be altered in a longterm diabetic state. In the present study, I found that in a given group (viz. GK or Wistar), the PGE_2-induced vasocontraction was similar between NOS inhibitor treatment and endothelial denudation (Fig. 3-1*A* vs. *C*), and that it was enhanced in the GK artery in each condition. These findings demonstrate *a*) that the PGE_2-induced vasocontraction observed here is not endothelium-dependent and *b*) that a diabetes-related enhancement of this response can occur in the absence of endothelium and without altered NO production. The above does not, of course, exclude the possibility that NO might be a suppressor of PGE_2-mediated vasocontraction in intact arteries. In this context, it should be noted that in the same artery (superior mesenteric) isolated from the same type 2 diabetes model (GK rats), there have been shown to be impairments of the endothelium-dependent relaxations induced by various agonists [24, 25].

Receptors involved

Four subtypes of EP receptor (viz. EP1, EP2, EP3 and EP4) have been described [1, 3, 5]. Of these, the EP1 and EP3 subtypes mediate vasocontraction [1, 3, 5, 10, 12, 13]. Therefore, I focused on the contractile responses to PGE_2 and sulprostone (EP1/EP3 agonist [11, 13, 35, 36]) and on the effect of sc19220 (EP1 antagonist [11, 37]) on such responses in mesenteric arteries isolated from Wistar and GK rats. In the present study, the EP1 antagonist had almost no effect on the PGE_2- and sulprostone-induced vasocontractions. Indeed, those vasocontractions were increased in the GK (vs. Wistar) mesenteric artery both in the absence and in the presence of the EP1 antagonist. These results suggest that vasoconstrictor responses mediated by the EP3 subtype are enhanced in the GK mesenteric artery. This idea is supported by the published finding of Kobayashi et al. [12] that an EP3 agonist, but not an EP1, EP2 or EP4 agonist, induced vasocontraction in a main branch of the rat mesenteric artery.

In my Western blotting analysis, multiple bands were detected for the EP3 receptor in homogenates of superior mesenteric artery from either group. This is consistent with the results obtained in a previous study using a different vessel [11]. Multiple bands for GPCRs including EP receptors could result from oligomerization of the receptor, post-transcriptional modification of the protein or the formation of heteromers with other proteins [5, 51-54]. Although the exact conformations of the EP3 receptors present in diabetic mesenteric arteries remains unclear, I think that differences in the downstream pathway are more likely to explain the differences in the vasocontractions induced by the present agonists between GK and Wistar rats because the total expression of the EP3 receptor was similar between the two groups of rats.

Pathways involving PKCδ

PKC is a well-characterized enzyme involved in myogenic constriction and agonist-induced vascular smooth muscle contraction [55-58]. In vascular smooth muscle, a number of signal transduction cascades activated by physiological agonists are regulated by

PKC and, indeed, PKC phosphorylates several substrates associated with vascular smooth muscle contraction [56, 58, 59]. The PKC isozymes are subdivided into cPKC (α, βI, βII and γ), nPKC (δ, ε, θ, and η) and aPKC (ζ, λ). Among these isozymes, PKCα, PKCδ and PKCε are known to mediate vasocontraction. Recently, Kobayashi et al. [12] demonstrated in rat mesenteric artery: *a*) that EP3 receptor-mediated vasocontraction was inhibited by pretreatment with a general PKC inhibitor or with a PKCδ inhibitor but not with a cPKC inhibitor and *b*) that EP3 agonism induced PKCδ phosphorylation. In the present study, the PGE_2- and sulprostone-induced vasocontractions were blocked by rottlerin (selective PKCδ inhibitor [12]) but not by Gö6976 (selective PKCα/β inhibitor [12, 38]). Moreover, the phosphorylation of PKCδ induced by sulprostone was increased in mesenteric arteries from GK rats vs. those from Wistar rats (Fig. 3-3). These data suggest that the enhanced EP3-induced vasocontraction observed here in diabetic arteries was due to increased PKCδ activities. However, I should point out that there is controversy in the literature over the activities of rottlerin including the selectivity of its PKC inhibition [69]. Nevertheless, on the basis of published documentation (e.g., Refs. [12, 46]), I believe that it is a sufficiently selective PKCδ inhibitor for the present purpose and indeed that a better agent is not currently available.

Pathways involving caldesmon

One of the major target proteins for PKC that is important in vascular contraction is caldesmon [47]. Two isoforms of caldesmon exist: a high molecular weight isoform, *h*-caldesmon, which is restricted to smooth muscle, and a low molecular weight isoform, *l*-caldesmon, which is expressed both in nonmuscle cells and in dedifferentiated smooth muscle cells [44, 47, 61]. Caldesmon, an actin filament-associated protein, seems to be implicated in the control of vasocontraction through inhibition of actin-activated myosin Mg^{2+}-ATPase [62, 63]. Phosphorylation of caldesmon by PKC has been shown to impair this inhibition [62, 63]. Phosphorylation of caldesmon results in vascular contraction [64] and, indeed, in the present

study, the phosphorylation of both *h*- and *l*-caldesmon induced by sulprostone was increased in arteries from GK (vs. Wistar) rats. These data suggest that the observed augmentation of EP3 receptor-mediated vasocontraction in the GK mesenteric artery may be attributable to increased phosphorylation of caldesmon. This idea is consistent with previous reports *a*) that phosphorylation of caldesmon in smooth muscle cells is associated with increased isometric force [44, 45] and *b*) that the phosphorylation of caldesmon via activation of PKCδ in cerebral arteries is associated with augmented vasocontraction [46].

Functional/physiological significance

PGE_2 is present in many tissues and has been implicated in a myriad of pathological conditions. For instance, it was reported some years ago that vascular endothelial growth factor (VEGF) is expressed at high levels in diabetic retinas and is involved in the pathogenesis of diabetic retinopathy [65]. Moreover, other studies showed that increased expression of PGE_2 leads to upregulation of VEGF expression [66] and that the retinal production of PGE_2 is increased in diabetic rats [67]. Kamata's laboratory previous studies demonstrated that production of PGE_2 is increased in diabetic arteries [26, 27] and the present data demonstrate that PGE_2-induced vasocontraction is enhanced in superior mesenteric arteries from diabetic rats. As yet, I cannot say whether or not the abnormal PGE_2 signaling present in GK arteries resulted from the long-term nature of the rats' diabetes or, indeed, whether such abnormal signaling might be the cause of such diabetic vascular complications as impairments of the peripheral/splanchnic circulation. At this point, I can only say that therapeutic regulation of PGE_2 signaling warrants further investigation as a candidate for the prevention of diabetic vascular complications. Finally, activation of PKC appears to be a key feature of diabetes mellitus and, indeed, enhanced PKC activation has been strongly implicated in the pathogenesis of diabetic vascular complications [68, 69]. The development of isozyme-specific PKC inhibitors is a feasible proposition and offers the exciting prospect of blocking pathways involved in the initiation and progression of diabetic vasculopathy.

Chapter 4

Mechanisms underlying reduced $P2Y_1$-receptor-mediated relaxation in superior mesenteric arteries from long-term streptozotocin-induced diabetic rats.

Introduction

Endothelial cells are important for the regulation of vascular tone and reactivity under both physiological and pathophysiological conditions. They perform such roles by responding to neurohumoral mediators and mechanical forces with the release of a variety of endothelium-derived factors [1, 2, 3]. Endothelial dysfunction is a phenomenon common to a number of cardiovascular diseases, including diabetes [1, 2, 4-6]. Among the vascular complications associated with diabetes is an impairment of vascular reactivity to various neurotransmitters within the macro- and microvasculature [1, 2, 7, 8]. For instance, there is an accumulating body of evidence to indicate that the relaxation responses induced by various agents are abnormally weak in diabetic patients and in animal models of diabetes, such as the streptozotocin (STZ)-induced diabetic rat [1, 7, 9-12]. Therefore, a proper understanding of the mechanisms underlying the endothelial dysfunction present at various stages and in various types of diabetes is important for preventive and therapeutic approaches against the development of diabetic vascular complications.

Nucleotides play important roles not only in intracellular nucleic acid synthesis and energy supply but also, once released into the extracellular space, in various biological responses involved in the control of cellular functions [13-18]. Extracellular triphosphate (e.g. ATP and UTP) and diphosphate (e.g. ADP and UDP) nucleotides are released into tissue fluids and plasma from aggregated platelets and from activated endothelial cells and leucocytes in response to various proinflammatory stimuli, cell death and tissue damage [13, 19]. They generate a number of responses within the vasculature, such as platelet activation and changes in smooth muscle contraction and dilatation in various arteries [15, 20, 21]. Extracellular nucleotides activate cell-surface receptors, namely purinoceptors, which can be divided into metabotropic G-protein-coupled P2Y receptors and ionotropic P2X receptors [22-25]. So far, seven P2X receptors (i.e. $P2X_{1-7}$) and eight P2Y receptors (i.e. $P2Y_{1, 2, 4, 6, 11-14}$) have been characterized [22-26]. A full understanding of signalling by purinoceptors remains elusive because receptor distribution differs among tissues [22-26] and because most purinoceptors are

able to respond to several nucleotides, resulting in a situation in which multiple receptors have overlapping ligand preferences [26]. In particular, the mechanisms that might alter purinoceptor signalling in the diabetic vasculature remain unclear at present.

Activation of endothelial P2 receptors is important for nucleotide-induced vasodilatation [15, 20, 21]. Three major endothelium-derived relaxing factors (EDRFs) can be involved in such responses, depending on the species and vessel-type considered: these factors are nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) [15, 27-29]. $P2Y_1$ and $P2Y_2$ receptors are thought to be responsible for adenosine 5′-diphosphate sodium salt (ADP)- and ATP/UTP-induced vasodilatation in most vessels [30-32]. I and others have found that nucleotide-induced arterial responses are altered in models of various cardiovascular diseases, such as hypertension and diabetes [14, 21, 34-37]. However, whether $P2Y_1$-receptor-mediated vasodilatation is normal or abnormal in diabetic states remains unclear.

Although diabetes can affect purinergic signalling in various tissues and cells [13], no previous study has examined $P2Y_1$-receptor-mediated vasodilatation in a long-term diabetic state. I therefore designed the present study to investigate the long-term effect of diabetes on the dilator responses to $P2Y_1$-receptor agonists in the rat isolated superior mesenteric artery. I hypothesized that $P2Y_1$-receptor-mediated relaxation would be impaired in such arteries from STZ-induced diabetic rats because of alterations in NO signalling. I also asked whether superior mesenteric arteries from control and diabetic rats might exhibit differential expressions of the $P2Y_1$ receptor.

Experiment

Reagents

Adenosine 5′-triphosphate disodium salt (ATP), ADP, phenylephrine (PE), N^G-nitro-L-arginine (L-NNA), MRS2179 and an antibody against β-actin were all purchased from Sigma Chemical (St. Louis, MO, USA), while 2-methylthio-ADP trisodium salt (2-MeSADP) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Acetylcholine chloride (ACh) was purchased from Daiichi-Sankyo Pharmaceuticals (Tokyo, Japan). Suramin was obtained from Wako Pure Chemical Industries (Osaka, Japan). All drugs were dissolved in HPLC grade water, except for suramin and MRS2179 (dissolved in DMSO). All concentrations are expressed as the final molar concentration of the base in the organ bath. Horseradish peroxidase (HRP)-linked secondary anti-mouse or anti-rabbit antibody was purchased from Promega (Madison, WI, USA). Antibody against endothelial NO synthase (eNOS) was from BD Biosciences (San Jose, CA, USA), while those against phospho-eNOS (Ser^{1177} or Thr^{495}) were from Cell Signalling Technology (Danvers, MA, USA) and that against $P2Y_1$ receptor was from Abcam (Cambridge, MA, USA).

Animals and experimental design

The experimental design 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 with 'Guide for the Care and Use of Laboratory Animals' adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan). Male Wistar rats (8 weeks old and 190–230 g body weight) received a single injection via the tail vein of STZ 65 mg kg^{-1} dissolved in a citrate buffer, as reported previously [11,12,38,39]. Age-matched control rats were injected with the buffer alone. Food and water were given ad libitum. The experiments described here were performed when the rats were 58–65 weeks old (i.e. at 50–57 weeks after the injection).

Measurement of blood glucose

Plasma glucose was measured by using a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan) as described previously [12, 39].

Functional study

Vascular isometric force was measured as in previous papers [11, 38, 39]. After killing, the superior mesenteric artery was rapidly removed and immersed in oxygenated, modified Krebs–Henseleit solution (KHS). This solution consisted of (in millimolar) 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 glucose. 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 of KHS at 37 °C. The rings were stretched until an optimal resting tension of 1.0 g was loaded, which was optimal for inducing a maximal contraction [11], then allowed to equilibrate for at least 1 h. Force generation was monitored by means of an isometric transducer (model TB-611T; Nihon Kohden, Tokyo, Japan).

Cumulative concentration-response curves were obtained to ADP (10^{-8}–10^{-5} M), 2-MeSADP [10^{-9}– 10^{-5} M; selective $P2Y_1$ agonist [40]] and ATP (10^{-8}–10^{-4} M) in superior mesenteric arteries precontracted with 10^{-6} M PE. The submaximal contraction developed in response to 10^{-6} M PE was similar between diabetic and control groups, based on preliminary experiments. To study the roles of endothelium and NO in ADP-induced relaxation, I constructed concentration-response curves to ADP *1*) after endothelial denudation and *2*) in the presence of 10^{-4} M L-NNA [NOS inhibitor [11, 35]]. To study the role of the P2-receptor pathway in ADP-induced relaxation, such relaxations were examined in the presence of 10^{-4} M suramin [nonselective P2 receptor antagonist [35]] or 10^{-5} M MRS2179 [selective $P2Y_1$ antagonist [40, 41]]. 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. When required, removal of the endothelium from arterial segments was

achieved by infusing a 3-[(3-cholamidopropyl)dimethylammonio]- 1-propanesulphonate solution (CHAPS, 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.

Measurement of NO metabolites [nitrite (NO_2^-) and nitrate (NO_3^-)]

The effluent from a given tissue was sampled and assayed by the method described previously [42]. Each superior mesenteric artery was cut into transverse rings 4 mm in length. These were placed in KHS [well-oxygenated (95% O_2–5% CO_2)] at 37 °C and then treated with ADP (3×10^{-6} M) for 15 min. The amount of NOx was calculated as follows: ADP-stimulated NOx [nmol/15 (min) g (weight of the mesenteric artery)]. The concentrations of nitrite plus nitrate (NOx) in the KHS and in the NOx standard (Eicom, Kyoto, Japan) were measured using an automated NO detector/high-performance liquid chromatography system (ENO20; Eicom).

Western blotting

The protein levels of $P2Y_1$ receptor and of phosphor-eNOS (Ser^{1177} and Thr^{495}) and total eNOS were quantified using an immunoblotting procedure, essentially as described before [42]. Superior mesenteric arteries from control and diabetic rats were isolated, cleaned of fat, dissected and frozen in liquid nitrogen. To investigate the expression of phospho-eNOS in such arteries upon ADP stimulation, mesenteric arterial rings from a given rat were incubated with KHS [well-oxygenated (95% O_2–5% CO_2)] at 37° C and then exposed to 3×10^{-6} M ADP or vehicle (water) for 15 min. For the examination of $P2Y_1$-receptor expression, we used tissues not used for drug-treatment experiments. Mesenteric arterial protein extracts (20 μg) were applied to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were incubated with anti-$P2Y_1$ (56 kDa; 1 : 500) [43], anti-phospho-eNOS (Ser^{1177}) (140 kDa; 1 : 500) [44], anti-phospho-eNOS (Thr^{495}) (140 kDa; 1 : 500) [45], anti-eNOS (140 kDa; 1 : 1000) [44] or anti-β-actin (42 kDa; 1 : 5000) antibodies, with detection being achieved using a

HRP-conjugated IgG followed by enhanced chemiluminescence. Band intensity was quantified by densitometry. Results were normalized to the b-actin expression. To assess ADP-induced eNOS phosphorylation (at Ser^{1177} or Thr^{495}), I calculated the ratio of the optical density of phosphorylated eNOS or total eNOS in ADP-stimulated or nonstimulated (basal) samples, in each case over that of the corresponding β-actin band. These values are presented as the fold increase in phosphorylated eNOS from basal. In some experiments involving detection of phospho-eNOS (Ser^{1177} or Thr^{495}), eNOS and β-actin proteins, the same membranes were stripped (phosphorylated eNOS at Ser^{1177} and phosphorylated eNOS at Thr^{495} were loaded on different gels). For $P2Y_1$-receptor and β-actin protein detection, the different samples (nondrug-treated) were loaded on to different gels.

Statistical analysis

Experimental vasorelaxation values are expressed as a percentage of the maximal contraction induced by PE in a given segment. Concentration-response curves were fitted using a nonlinear interactive fitting programme (Graph Pad Prism 5.0; GraphPad Software, San Diego, CA, USA). Data are expressed as means ± SE. Statistical Statistical analysis of the concentration-response curves was performed using two-way analysis of variance (ANOVA) for comparisons between groups. For other comparisons between groups, data were analysed using ANOVA with a post hoc Bonferroni test or Student's t-test. Western blot data were analysed by 1-sample t-test, Student's t-test, or one-way ANOVA. Values of $P < 0.05$ were considered statistically significant.

Abbreviations

2-MeSADP, 2-methylthio-ADP; ACh, acetylcholine; ANOVA, analysis of varience; ADP, adenosine diphosphate; ATP; adenosine triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate solution; DMSO, dimethylsulfoxide; EC, endothelial cell; EDRF, endothelium-derived relaxing; eNOS, endothelial NOS; HRP, Horseradish peroxidase; KHS, Krebs-Henseleit solution; L-NNA, N^G-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide Synthase; NOx, nitric oxide metabolites; NBT, nitro-blue tetrazolium; P2 receptor, purinoceptor; PE, phenylephrine; PVDF, polyvinylidene difluoride; STZ, streptozotocin; SE, standard error; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SMC, smooth muscle cell,

Results

Body weight and blood glucose

At the time of the experiment (when the rats were 58–65 weeks old), the body weight of the diabetic rats (326.4 ± 19.5 g; n = 15) was lower than that of the age-matched nondiabetic control rats (690.1 ± 12.8 g; n = 18, $P < 0.001$). All STZ rats (non-fasted) exhibited hyperglycaemia (544.2 ± 24.1 mg dL^{-1}; n = 15), their blood glucose levels being significantly higher than those of the control rats (also non-fasted) (148.9 ± 4.1 mg dL^{-1}; n = 18, $P < 0.001$).

P2Y1-agonist-induced relaxation is reduced in superior mesenteric arteries from diabetic rats

When the contraction induced by PE (10^{-6} M) had reached a plateau, ADP (10^{-8}–10^{-5} M), 2-MeSADP (10^{-9}–10^{-5} M) or ATP (10^{-8}–10^{-4} M) was added cumulatively. In rings from age-matched control rats, ADP (10^{-8}–10^{-5} M) induced a concentration-dependent relaxation and this relaxation was significantly impaired in rings from diabetic rats vs. those from control rats (Fig. 4-1*A*). Likewise, the selective $P2Y_1$ agonist 2-MeSADP [40, 46] induced a relaxation that was reduced in the diabetic group vs. the control group (Fig. 4-1*B*). On the other hand, the ATP induced relaxation was similar between the two groups (Fig. 4-1*C*).

To try to confirm that the ADP-induced vasodilatation was indeed mediated by the $P2Y_1$-receptor, I investigated the effect of a selective $P2Y_1$-receptor antagonist on the ADP-induced response (Fig. 4-1*D*). MRS2179 (10^{-5} M) completely blocked the ADP-induced relaxation in each group (Fig. 4-1*D*) and therefore abolished the difference between the groups (Fig. 4-1*D*). These results suggest that $P2Y_1$-mediated vasodilatation is impaired in superior mesenteric arteries from long-term STZ-induced diabetic rats.

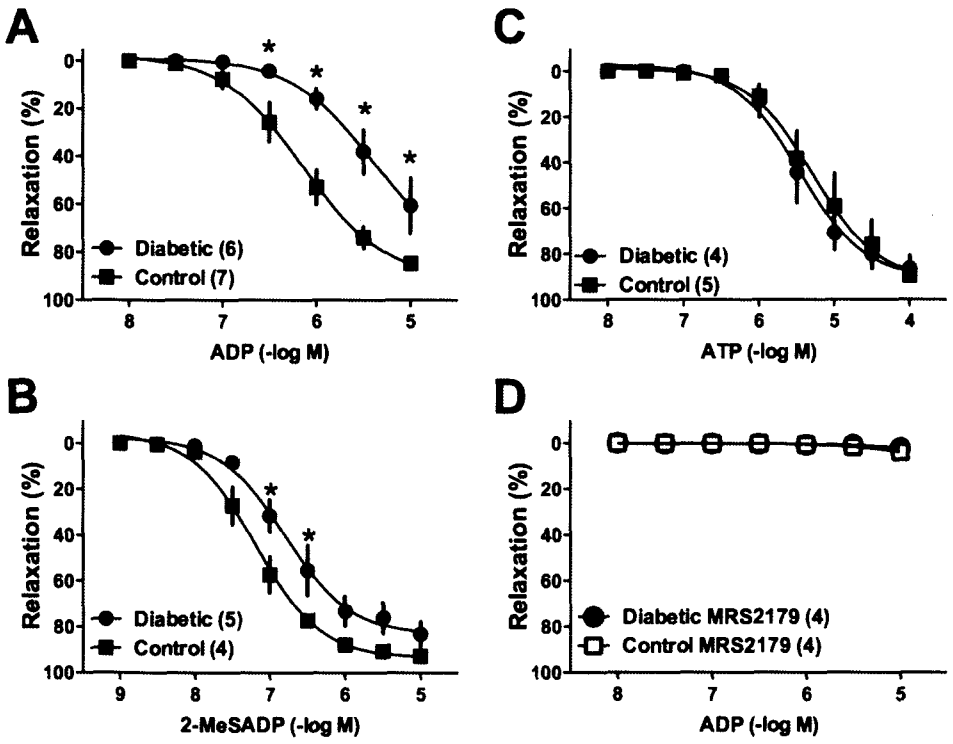


Fig. 4-1. $P2Y_1$-agonist-mediated relaxation is impaired in diabetic superior mesenteric arteries Concentration-response curves for adenosine 5′-diphosphate sodium salt (ADP) (*A*), 2-MeSADP (*B*) and adenosine 5′-triphosphate disodium salt (ATP) (*C*) and for ADP in the presence of a selective $P2Y_1$-receptor antagonist (10^{-5} M MRS2179) (*D*). Data, which are shown for superior mesenteric arteries from diabetic and control rats, are means ± SE, with the number of determinations being shown within parentheses. *$P < 0.05$ vs. control.

Effects of endothelial denudation and NOS inhibition on ADP-induced vasodilatation

Published evidence suggests that ADP-induced relaxation is mediated by the activation of the endothelium and NO signaling [46, 47]. Therefore, I assessed the relative contributions made by these factors to ADP-induced responses in diabetic superior mesenteric arteries. ADP-induced relaxation was completely blocked by endothelial denudation (Fig. 4-2*A*) and also by treatment with the NOS inhibitor L-NNA (10^{-4} M) (Fig. 4-2*B*). Consequently, each of those manoeuvres abolished the difference between the groups, suggesting that a paucity of endothelium-derived NO signalling is largely, or even solely, responsible for the defect in ADP-induced relaxation in the long-term STZ-induced diabetic superior mesenteric artery.

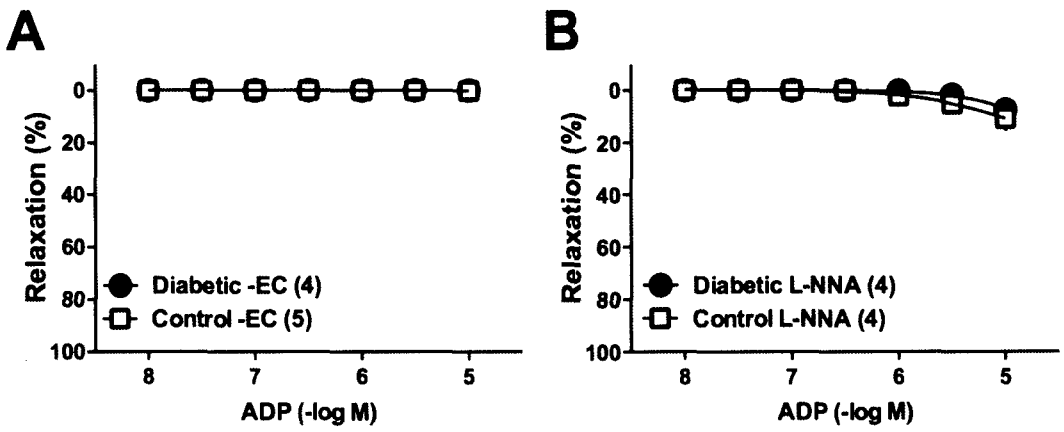


Fig. 4-2. Effects of endothelial denudation and NOS inhibition on adenosine 5′-diphosphate sodium salt (ADP)-induced relaxation in superior mesenteric arteries obtained from control and diabetic rats. Little or no ADP-induced relaxation was evident following endothelial denudation (*A*) or treatment with NOS inhibitor (10^{-4} M L-NNA) (*B*) in either group. Data are means ± SE, with the number of determinations being shown within parentheses.

ADP-induced NO production is reduced in superior mesenteric arteries from diabetic rats

Together, previous reports [31] and the present data (Fig. 4-2) suggest that the NO pathway plays an important role in ADP-mediated vasodilatation. To examine whether the difference in ADP-induced vasodilatation between the two groups of rats might be mediated by altered NO production, we measured the release of NO metabolites (i.e. NO_2^- and NO_3^-) from rings treated with ADP (Fig. 4-3). The ADP (3×10^{-6} M)-induced NOx (NO_2^- plus NO_3^-) level was significantly lower for the diabetic group than for the controls.

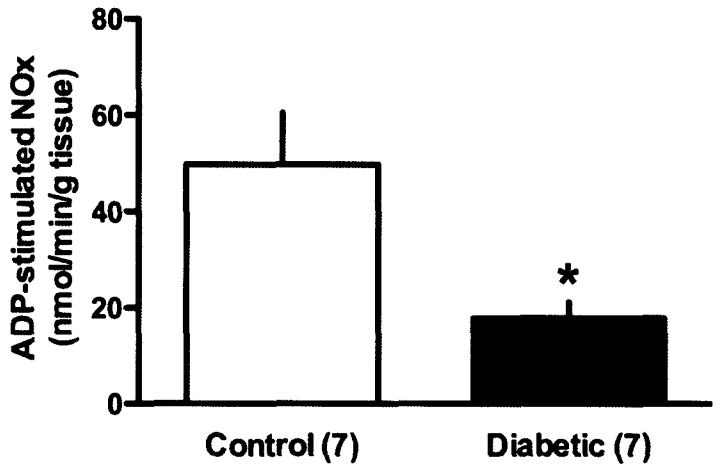


Fig. 4-3. Levels of NO metabolites (NOx; NO_2^- + NO_3^-) after stimulation of superior mesenteric artery rings with 3×10^{-6} M adenosine 5′-diphosphate sodium salt (ADP) for 15 min. Data are means ± SE, with the number of determinations being shown within parentheses. *P < 0.05 diabetic vs. control.

Expression of eNOS and ADP-induced eNOS phosphorylation in superior mesenteric arteries

In the regulation of eNOS activity by agonists, signal transduction depends not only on Ca^{2+}/calmodulin but also on eNOS phosphorylation by various kinases [48-51]. Regulation of eNOS is achieved by phosphorylation of multiple sites in the protein. Phosphorylation at Ser^{1177} stimulates eNOS activity [48-51], whereas phosphorylation at Thr^{495} inhibits its activity [45]. Therefore, I examined *1*) whether ADP might promote phosphorylation of eNOS at Ser^{1177} and *2*) whether eNOS dephosphorylation at Thr^{495} might be weaker in the diabetic group than in the age-matched controls. Immunoblot analysis of ADP (3×10^{-6} M for 15 min)-stimulated and nonstimulated (basal) arteries from control and diabetic groups (using anti-phospho-eNOS and anti-eNOS antibodies) allowed detection of immunoreactive proteins (Fig. 4-4). First, I examined the expression of phosphorylated eNOS (Ser^{1177}) (Fig. 4-4*A–D*). The densitometric ratio of eNOS to β-actin tended (i.e. not significantly) to be increased in mesenteric arteries from diabetic rats in both ADP-stimulated and nonstimulated conditions (Fig. 4-4*B*). In arteries from the control group, the densitometric ratio of phosphorylated eNOS (at Ser^{1177}) to β-actin was significantly increased by ADP-stimulation (vs. basal) (Fig. 4-4*C*). However, there was no such difference between ADP-stimulated and basal in mesenteric arteries from diabetic rats (Fig. 4-4*C*). As the apparent level of total eNOS protein differed between the groups (Fig. 4-4*B*), I compared the groups in terms of their fold increase from the basal (i.e. nonstimulated) level (Fig. 4-4*D*). The value of the fold increase in phosphorylated eNOS (at Ser^{1177}) was significantly smaller in arteries from the diabetic group than in those from the control group (Fig. 4-4*D*). I next evaluated the ADP-stimulated eNOS phosphorylation (Thr^{495}) levels (Fig. 4-4*E–H*). The densitometric ratio of eNOS to β-actin tended (i.e. not significantly) to be increased in the diabetic arteries (vs. their controls) (Fig. 4-4*F*). Furthermore, the phosphorylated eNOS (Thr^{495}) expression (Fig. 4-4*G*) and the value of the fold increase in phosphorylated eNOS (Thr^{495}) (Fig. 4-4*H*) did not differ between the diabetic and age-matched control groups in either the ADP-stimulated or ADP-nonstimulated

condition.

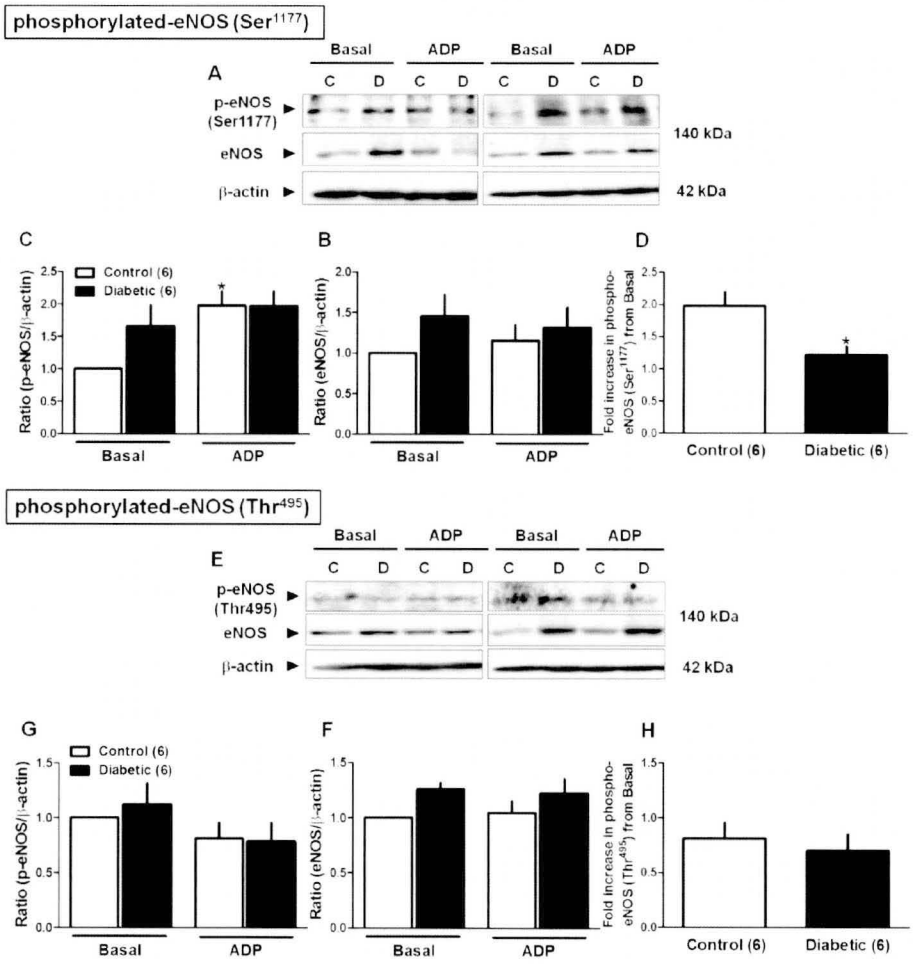


Fig. 4-4. Phosphorylation of endothelial NO synthase (eNOS) at Ser^{1177} by adenosine 5′-diphosphate sodium salt (ADP) is reduced in superior mesenteric arteries from diabetic rats. Phospho-eNOS (Ser^{1177} and Thr^{495}) and total eNOS protein contents were assessed by Western blotting in superior mesenteric rings treated with ADP (3×10^{-6} M) or vehicle (basal) for 15 min. (*A, E*) Representative blots are shown for phosphorylated eNOS (*A*: Ser^{1177}, *E*: Thr^{495}), as well as for eNOS, in control and diabetic arterial rings, in each case with ('ADP') or without ('Basal') stimulation with ADP. (*B, F*) Bands for eNOS and β-actin quantified as described in Materials and Methods, ratios being calculated for the optical density of eNOS over that of β-actin. (*C, G*) Quantification of eNOS phosphorylation at Ser^{1177} (*C*) and at Thr^{495} (*G*). Ratios were calculated for the optical density of phosphorylated p-eNOS over that of β-actin. (*D, H*) Quantification of eNOS phosphorylation (*D*: Ser^{1177}, *H*: Thr^{495}, see Materials and Methods): y-axis shows fold increase (vs. corresponding basal). Data are means ± SE, with the number of determinations being shown within parentheses. *P < 0.05 vs. corresponding control.

Expression of $P2Y_1$ receptor in superior mesentericarteries

Alterations in $P2Y_1$ receptors have been reported in various pathophysiological states and during ageing [52, 53]. To investigate the possible mechanism underlying the reduced ADP-mediated vasodilatation seen here in diabetic arteries, I used Western blotting. In superior mesenteric arteries, the protein expression of $P2Y_1$ receptors did not differ between the diabetic and age-matched control groups (Fig. 4-5).

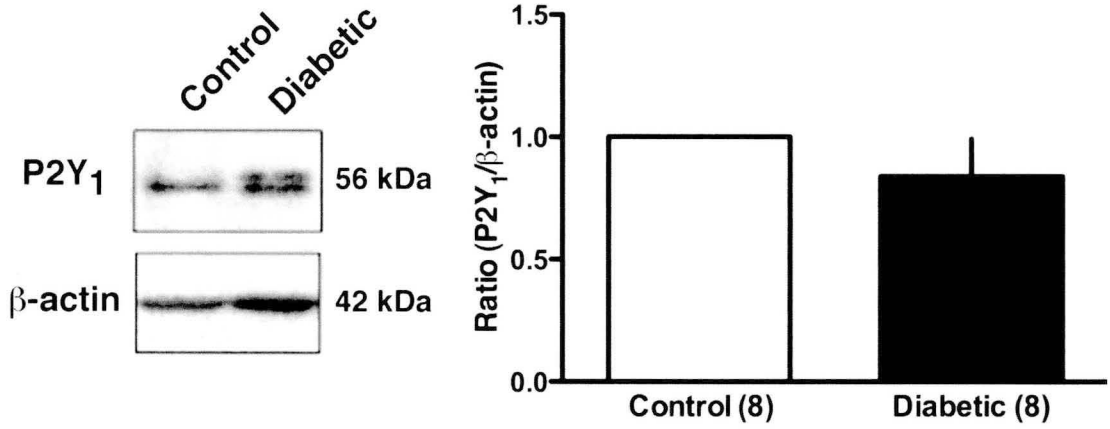


Fig. 4-5. Protein expression of $P2Y_1$ receptor in superior mesenteric arteries from control and diabetic rats. Left: representative Western blots for $P2Y_1$ receptor and b-actin. Right: bands quantified as described in Materials and Methods, ratios being calculated for the optical density of $P2Y_1$ receptor over that of β-actin. There was no difference between the groups. Data are means ± SE, with the number of determinations being shown within parentheses.

Discussion

The principal, fundamental finding made in the present study was that in superior mesenteric arteries isolated from STZ-induced diabetic rats, the relaxation response to ADP is impaired, at least when the animals have had a disease state of long duration. In addition, I found that: *1*) the ADP-induced vasodilatation in that artery was mediated by the $P2Y_1$ receptor and NO signalling; *2*) the ADP-induced NO production was reduced in the diabetic mesenteric arteries (vs. the age-matched controls); *3*) the ADP-induced stimulation of eNOS is dysfunctional in the diabetic mesenteric arteries; and *4*) the protein expression of the $P2Y_1$ receptor did not differ between the control and diabetic groups.

Among the purinoceptors, the $P2Y_1$ and $P2Y_2$ receptors are thought to be responsible for nucleotideinduced vasodilatation in most tissues [20, 30]. The $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2Y_4$ and $P2Y_{11}$ receptors evidently mediate NO production and subsequent vasodilatation [20, 54-56]. In the present study, the vasodilator responses to the preferential $P2Y_1$-receptor agonist ADP [23, 25] and the selective $P2Y_1$-receptor agonist 2-MeSADP [23, 25, 40] were each impaired in the diabetic mesenteric arteries. The ADP-induced vasodilatation was completely blocked in each group – and the intergroup difference in this response therefore completely abolished – by the selective $P2Y_1$-receptor antagonist MRS2179 [23, 25, 41]. These results strongly suggest that a defect in $P2Y_1$-receptormediated relaxation is present in superior mesenteric arteries in long-term diabetic rats. By contrast, the relaxation induced by ATP did not differ between the control and diabetic groups, and MRS2179 had no significant effect on the ATP induced relaxation in either group (data not shown). ATP may activate endothelial purinoceptors related to vasodilatation in addition to the $P2Y_1$ receptor [20, 54-56]. Therefore, the apparent lack of a difference in the ATP induced relaxation between the two groups may be attributable to compensatory changes mediated by other purinoceptors and/or EDRFs.

Endothelial NO synthase, a key determinant of vascular homoeostasis [2, 49, 57,58], is a Ca^{2+}/calmodulin-dependent enzyme that is activated in response to stimulation of a number of different Ca^{2+}-mobilizing cell-surface receptors [2, 49, 57,58]. Some studies have indicated that

activation of the $P2Y_1$ receptor in vascular endothelial cells induces Ca^{2+}-independent NO production. [31, 59]. Regulation of eNOS is known to be achieved via phosphorylation of multiple sites within the protein [2, 49, 57, 58]. The two most thoroughly studied sites are the activation site Ser^{1177} and the inhibitory site Thr^{495} [60]. As activation of $P2Y_1$ receptors on endothelial cells leads to phosphorylation and activation of eNOS [50, 51, 60], I focused on effects on both of those sites in the ADP-stimulated superior mesenteric artery. I found that in the long-term diabetic superior mesenteric artery, although the expressions of eNOS protein and nonstimulated eNOS phosphorylation (at Ser^{1177}) tended (nonsignificantly) to be increased, the ADP-stimulated fold increase in phosphorylated eNOS (at Ser^{1177}) was reduced. In contrast, the ADP-stimulated phosphorylation of eNOS at Thr^{495} did not differ between the two groups. Whether the finding that the nonstimulated Ser^{1177} phosphorylation tended to be increased in the diabetic arteries is of any importance remains unclear. What these results do suggest is that ADP-induced stimulation of eNOS is dysfunctional, not eNOS activity per se, in the present diabetic arteries. Indeed, ADP-stimulated NO production was reduced in the diabetic arteries (vs. the age-matched controls) despite an unchanged $P2Y_1$-receptor expression. Kamata's laboratory and others have suggested that in diabetes, the vasodilator response to an agonist such as ACh is impaired as a consequence of endothelial dysfunction (e.g. reduced phosphorylation of eNOS at Ser^{1177}) [1, 44]. In superior mesenteric arteries from STZ-induced diabeticWistar rats (58–65 weeks old) and their age-matched controls, I found that the ACh-induced relaxation was similar between the two groups (unpublished observation), suggesting that eNOS activation by ACh in that artery was not affected by the diabetes. On the other hand, the ACh-induced EDHF-type relaxation was impaired in the aged diabetic group vs. the age-matched control group (unpublished observation). Therefore, in aged diabetic rats, the contributions made by NO- and EDHF-type relaxations appear to differ by agonist, and we believe that the present findings may be important for the elucidation of the pathophysiological roles played by extracellular nucleotides in later-stage diabetes. The regulation of eNOS by phosphorylation at Ser^{1177} has been studied extensively. Many protein

kinases–including AMP-activated protein kinase [60], cyclic AMP-dependent protein kinase/protein kinase A [62], Akt [48, 63] and PLC [64], as well as cyclic GMP-dependent protein kinase [65] – act, at least in part, through this Ser^{1177} phosphorylation site. However, the precise mechanisms by which such kinases are associated with $P2Y_1$-mediated eNOS phosphorylation in long-term diabetic states remain unclear at present. Consequently, future studies should seek to identify the molecular mechanisms by which eNOS phosphorylation is induced by $P2Y_1$-receptor stimulation in diabetic mesenteric arteries.

Recently, the ecto-nucleotidase NTPDase 1 has been reported to be important in controlling endothelial P2Y-receptor-dependent relaxation [66]. Extracellular nucleotides are metabolized by NTPDase 1 and this enzyme could promote desensitization of the $P2Y_1$ receptor. The protein expression of total NTPDase 1 between diabetic rats in superior mesenteric arteries did not differ and age-matched control rats (data not shown). Although previous studies on diabetes have reported increased NTPDase 1 activity [67, 68], the extent of any NTPDase 1 contribution to the promotion of $P2Y_1$-receptor desensitization remains unclear. In this context, further research will be required to establish the extent to which this enzyme might be associated with the impaired ADP-mediated vasodilation present in long-term diabetes.

A number of previous studies using STZ-induced diabetic rats have observed abnormalities of responses to various vasoactive substances in a variety of blood vessels [1, 69, 70]. In such studies, diabetes is normally induced at a relatively young age in rats by giving STZ, and the effects of STZ-induced diabetes are generally observed for a period of 8–20 weeks. In such studies, Ralevic and colleagues found that *1*) at 8 weeks after the induction of STZ diabetes in rats, sensory-motor-nerve-evoked vasodilatation was severely attenuated, although sympathetic nerve and endothelial functions were normal [71] and *2*) at 12 weeks after the induction of STZ diabetes in rats, there was both pre-junctional impairment of sympathetic neurotransmission and impaired endothelial function in mesenteric arterial beds [72]. On the other hand, vascular reactivity to agonists in long-term diabetic states has seldom been investigated [73-75]. Some reports have suggested that in long-term diabetic states, the dynamic

equilibrium for various nucleotides (i.e. between generation and metabolism) undergoes alteration both systemically and locally [15, 76, 77]. Although the present study has clearly demonstrated that $P2Y_1$-mediated vasodilatation is impaired in superior mesenteric arteries in long-term STZ diabetic rats, further studies will be required focusing, for instance, on the time-course of changes in the reactivity to $P2Y_1$ agonists and in NO signaling in the diabetic state to establish causal relationships.

Conclusions

In the present study, I have investigated the mechanisms underlying the diabetes-related changes in the extracellular nucleotides-induced responses in superior mesenteric arteries from rats. I found that the COX-mediated and endothelium-dependent contraction could be enhanced by not only ACh but also by extracellular nucleotides. I also found that the enhancement of extracellular nucleotide-induced contraction was attribute to the increased release of PGE_2 from endothelial cells and to the enhancement of EP3 receptor–mediated vasoconstriction via activation of the PKCδ pathway in smooth muscle cells. I believe that these findings play an important role in make a great contribution to the exploration of therapeutic targets for diabetes-associated vascular diseases.

The above findings lead to the following conclusions;

(Chapter 1) I found that pravastatin treatment (viz. 4-week treatment of OLETF rats already at the chronic stage) normalized EDCF-induced contraction by suppressing Rho-kinase activity and by promoting antioxidant activity in the superior mesenteric arteries from rats at the chronic stage of type 2 diabetes. The vasculoprotective actions of pravastatin in OLETF mesenteric arteries can be largely attributed to the suppression of EDCF signalling by a cholesterol-independent pleiotropic effect.

The present study provides a credible explanation for the beneficial effects of statins on the vascular system in type 2 diabetes.

(Chapter 2) The findings of this study indicate *1*) abnormal release of prostanoids from endothelium and *2*) increases in the expressions of phospho-$cPLA_2$, COX-1, and COX-2 in superior mesenteric artery from diabetes rats. The aberrant nucleotide-induced contractions and the levels of PGE_2 and $PGF_{2\alpha}$ release in diabetes rats could be normalized by losartan

treatment via suppression of $cPLA_2$/COX activity. I suggest that the diabetes-related enhancement of nucleotides-mediated vasoconstriction is due to P2Y receptor-mediated activation of the $cPLA_2$/COX pathway and that losartan normalizes such contractions by suppressing this pathway.

(Chapter 3) I investigated the mechanisms underlying the enhancement of PGE_2-induced contraction in superior mesenteric arteries from type 2 diabetic rats. Among the PGs, PGE_2, one of the important factors in EDCF, has been reported to be a vasoconstrictor mediated by the EP1 and EP3 receptors in smooth muscle. In superior mesenteric arteries, *1*) PGE_2-induced vasocontraction was mediated by EP3, but not by EP1 receptor; *2*) the augmentation of the EP3 receptor-mediated vasocontraction in the GK artery was attributable to activation of PKCδ and increased phosphorylation of caldesmon and *3*) no difference observed in the expression of EP3 receptor between diabetic and control groups. My data suggest that the diabetes-related enhancement of EP3 receptor-mediated vasocontraction results from activation of the PKCδ pathway. Alterations in EP3 receptor-mediated vasocontraction may play an important role in the abnormal vascular functions in chronic diabetic states.

(Chapter 4) I found that *1*) the ADP-mediated vasodilation in superior mesenteric artery was induced by the $P2Y_1$ receptor and NO signalling, *2*) the ADP-induced NO production was reduced by dysfunction of eNOS activity (as evidenced by reduced the fold increase in eNOS phospholation at Ser^{1177} with no difference in fold increase in eNOS phospholation at Thr^{495}) in the STZ-induced diabetic superior mesenteric arteries and *3*) the protein expression of $P2Y_1$ receptor was not different between the control and diabetic groups. These results suggest that $P2Y_1$-receptor-mediated vasodilatation is impaired in superior mesenteric arteries from long-term type 1 diabetic rats.

List of publications

1) Pravastatin normalizes EDCF-mediated response via suppression of Rho-kinase signalling in mesenteric artery from aged type 2 diabetic rat. : **Ishida K**, Matsumoto T, Taguchi K, Kamata K, Kobayashi T. **Acta Physiol**, 205, 255-265 (2012).

2) Mechanisms underlying altered extracellular nucleotide-induced contractions in mesenteric arteries from rats in later-stage type 2 diabetes: effect of ANG II type 1 receptor antagonism. : **Ishida K**, Matsumoto T, Taguchi K, Kamata K, Kobayashi T. **Am J Physiol Heart Circ Physiol,** 301, H1850-61 (2011).

3) Protein kinase C delta contributes to increase in EP3 agonist-induced contraction in mesenteric arteries from type 2 diabetic Goto-Kakizaki rats. : **Ishida K**, Matsumoto T, Taguchi K, Kamata K, Kobayashi T. **Pflugers Arch**, 463, 593-602 (2012).

4) Mechanisms underlying reduced $P2Y_1$-receptor-mediated relaxation in superior mesenteric arteries from long-term streptozotocin-induced diabetic rats. : **Ishida K**, Matsumoto T, Taguchi K, Kamata K, Kobayashi T, **Acta Physiol,** 207, 130-41 (2013).

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Chapter 4

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