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

Keiko Ishida, Takayuki Matsumoto, Kumiko Taguchi, Katsu Kamata, and Tsuneo Kobayashi

Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo, Japan

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Ishida K, Matsumoto T, Taguchi K, Kamata K, Kobayashi T. 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. Am J Physiol Heart Circ Physiol 301: H1850–H1861, 2011. First published August 19, 2011; doi:10.1152/ajpheart.00502.2011.—Little is known about the vascular contractile responsiveness to, and signaling pathways for, extracellular nucleotides in the chronic stage of type 2 diabetes or whether the ANG II type 1 receptor blocker losartan might alter such responses. We hypothesized that nucleotide-induced arterial contractions are augmented in diabetic Goto-Kakizaki (GK) rats and that treatment with losartan would normalize the contractions. Here, we investigated the vasoconstrictor effects of ATP/UTP in superior mesenteric arteries isolated from GK rats (37–42 wk old) that had or had not received 2 wk of losartan (25 mg·kg−1·day−1). In arteries from GK rats (vs. those from Wistar rats), 1) ATP- and UTP-induced contractions, which were blocked by the nonselective P2 antagonist suramin, were enhanced, and these enhancements were suppressed by endothelial denudation, by cyclooxygenase (COX) inhibitors, or by a cytosolic phospholipase A2 (cPLA2) inhibitor; 2) both nucleotides induced increased release of PGE2 and PGF2α; 3) nucleotide-stimulated cPLA2 phosphorylations were increased; 4) COX-1 and COX-2 expressions were increased; and 5) neither P2Y2 nor P2Y6 receptor expression differed, but P2Y4 receptor expression was decreased. Mesenteric arteries from GK rats treated with losartan exhibited (vs. untreated GK) 1) reduced nucleotide-induced contractions, 2) suppressed UTP-induced release of PGE2 and PGF2α, 3) suppressed UTP-stimulated cPLA2 phosphorylation, 4) normalized expressions of COX-2 and P2Y4 receptors, and 5) reduced superoxide generation. Our data suggest that the diabetes-related enhancement of ATP-mediated vasoconstriction was due to P2Y receptor-mediated activation of the cPLA2/COX pathway and, moreover, that losartan normalizes such contractions by a suppressing action within this pathway.

endothelium-derived contracting factor; Goto-Kakizaki rat; purinoceptor

EXTRACELLULAR NUCLEOTIDES play important roles in physiological and pathological processes, including the regulation of vascular tone, atherosclerosis, and remodeling (10, 14). 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 (10, 14). Extracellular nucleotides act through cell surface receptors, which can be divided into P2Y and P2X receptor families (11, 44). So far, characterizations have been published of seven P2X receptor subtypes (P2X1–P2X7, ligand-gated ion channels) and eight P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, and P2Y11–P2Y14, G protein-coupled receptors) (11, 44). P2X receptors are exclusively activated by ATP, whereas P2Y receptors respond to both purine (ATP and ADP) and pyrimidine (UTP and UDP) nucleotides (10, 11, 14, 44). Specifically, ATP is a ligand for P2X1–P2X7, P2Y2, P2Y11, and P2Y13 receptors, whereas UTP is a ligand for P2Y2 and P2Y4 receptors (10, 11, 14, 44). 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 (10, 11, 14, 25, 44). 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) (10, 11, 14, 25, 44). Moreover, extracellular nucleotides release endothelium-derived contracting factors (EDCFs) in some pathophysiological states (15, 18).

Type 2 diabetes is associated with a markedly increased incidence of cardiovascular diseases (49). Various animal models have been used to gain insights into the pathogenesis of the vasculopathy associated with type 2 diabetes (49), 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 (35, 38–40, 47, 50). For example, we (35, 36, 38, 39) 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 (19). GK rats are a highly inbred strain of Wistar rats that spontaneously develop type 2 diabetes (19). 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 wk after birth (19). Although we (29, 34, 36, 37) and others (47) 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 (32). 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 (65). Moreover, although several studies have demonstrated that treatment with ARBs has beneficial effects on diabetic vasculopathy (32), 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, we 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. We 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 prostanooids signaling, and 2) the effects on these altered nucleotide-induced contractions that might be seen if rats had received short-term losartan treatment.

MATERIALS AND METHODS

Reagents. Phenylephrine, indomethacin, N6-nitro-L-arginine (L-NNA), ATP (disodium salt), 8,8'carbonylbis(mono,3,1-phenylene carbonyliminobis(1,3,5-naphthalenetrifulonic acid) (NF-023), suramin, α,β-methylene-ATP, nitroblue tetrazolium (NBT), and antibodies against P2Y2, P2Y4, and β-actin were purchased from Sigma Chemical (St. Louis, MO). UTP (trisodium salt) and arachidoniltriﬂuoromethyl ketone (AACOCF3) 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 A2 (cPLA2) and phospho-cPLA2 were from Cell Signaling Technology (Danvers, MA), whereas the antibody against P2Y6 was from Aitolab (Jerusalem, Israel). Drugs were dissolved in saline except for AACOCF3, U-46619, valeroyl salicylate, and NS-398 (which were dissolved in dimethyl sulfoxide (DMSO) and indomethacin (which was dissolved first in a small amount of 0.1 M Na2CO3 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 wk (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 wk old. Starting at 35–40 wk old, some GK and Wistar rats were given losartan for 2 wk (25 mg/kg·day) at 1700 hours, “Nulotan,” Banya, Ibaraki, Japan). Thus, we 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 (29, 34–38). 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 our previous studies (34–40). At 37–42 wk of age, some rats were euthanized each morning (at 0900 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 NaHCO3, 1.8 CaCl2, 1.2 NaH2PO4, 1.2 MgSO4, 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% O2-5% CO2) 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-61 IT, 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 (23, 30)], or the selective P2Y6 agonist MRS-2693 [10–8×3×10–5 M (33)] 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 P2X1 receptor antagonist (27, 48)], 10–4 M suramin [a nonselective P2 receptor antagonist (3, 45)], 10–3 M indomethacin [a nonselective COX inhibitor (38)], 3×10–6 M SQ-29548 [a TP receptor antagonist (64)], 10–4 M valeroyl salicylate [a selective COX-1 inhibitor (8)], 10–6 M NS-398 [a selective COX-2 inhibitor (52)], and 10–5 M AACOCF3 [a cPLA2 inhibitor (17)]) 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 our previous studies (38–40). 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 10–7 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

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

<table>
<thead>
<tr>
<th></th>
<th>Wistar Rats</th>
<th>GK Rats</th>
<th>Losartan-Treated GK Rats</th>
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</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>629.2 ± 11.4</td>
<td>615.7 ± 4.3</td>
<td>394.7 ± 4.6*</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>177.7 ± 6.3</td>
<td>501.5 ± 12.9</td>
<td>461.1 ± 15.8*</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>2.5 ± 0.2</td>
<td>4.7 ± 0.3*</td>
<td>4.0 ± 0.3*</td>
</tr>
<tr>
<td>Systolic blood pressure, mmHg</td>
<td>119.1 ± 2.6</td>
<td>108.5 ± 1.9*</td>
<td>98.1 ± 2.3*†</td>
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Values are means ± SE; n = 16 determinations for each group. *P < 0.05 vs. Wistar rats; †P < 0.05 vs. Goto-Kakizaki (GK) rats.
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 PGE2, thromboxane B₂ [TXB₂; a stable metabolite of thromboxane A₂ (TXA₂)], and PGF₂α, whereas one hundred time-diluted samples were used for the measurement of 6-keto PGF₁α, a stable metabolite of PGI₂. 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, cPLA₂, phospho-cPLA₂, P2Y₂, P2Y₄, and P2Y₆ were quantified using immunoblot analysis procedures, essentially as previously described (34, 35, 38–40). 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, Fig. 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 P2X₁ 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 L-N-nitro-L-arginine (l-NNA). F: ATP-induced contractions in the absence of 10⁻⁴ M l-NNA. G: concentration-response curves for P2X₁ 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; †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; ‡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.
The amount of NBT reduced (equal to the quantity of formazan) was calculated as follows: amount of NBT reduced = \( A \times V/(T \times Wt \times e \times l) \), 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, \( e \) is the extinction coefficient (0.7 l·mmol\(^{-1}\)·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).

RESULTS

General parameters. As shown in Table 1, body weight was significantly lower in GK rats than in Wistar rats, whereas nonfasted blood glucose and insulin levels were significantly higher in GK rats than in Wistar (also nonfasted). 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).

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, we 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. 1, we confirmed that in the absence of l-NNA, cumulative administration of ATP (10\(^{-5}\)–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. 1F). 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. 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. 1A). 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. 1A). 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. 1A). The existence of such endothelium-independent vasoconstriction at higher concentrations of ATP is consistent with results obtained in a previous study (18) using a different vessel, the rat aorta.

To investigate the possible involvement of P2X or P2Y receptors in the ATP-induced contraction, we examined the effects of antagonists of those receptors on the ATP-induced contraction. Incubation with NF-023, a P2X receptor antagonist (\(10^{-6} \) M), reduced the ATP-induced contraction in both Wistar and GK groups (Fig. 1B). Likewise, incubation with suramin, a nonselective P2 receptor antagonist (\(10^{-4} \) M), reduced such contraction in both Wistar and GK groups (Fig. 1C). 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. 1B), whereas in the presence of suramin, this contraction was similar between the two groups (Fig. 1C). 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 (18), and since our previous reports (35–40) 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 \(\times\) \(10^{-6} \) M SQ-29548, a TP receptor antagonist, reduced the ATP-induced contraction in both Wistar and GK groups (Fig. 1, D and E). Furthermore, incubation with indomethacin abolished the difference between the two groups (Fig. 1D), as did treatment with SQ-29548 (Fig. 1E).

To investigate the difference between Wistar and GK rats in the contraction induced by P2X receptor activation, we cumulatively applied \(\alpha,\beta\)-methylene ATP, a selective P2X agonist, to rings from GK and Wistar rats (Fig. 1G). The contraction induced by \(\alpha,\beta\)-methylene ATP was greater in the GK group than in the Wistar group. Moreover, indomethacin did not affect such contractions in either group (Fig. 1G).

**Contractile responses induced by the P2Y receptor agonist.**

We next looked for differences between Wistar and GK rats in the contractions induced by a P2Y2 and P2Y4 receptor agonist. Cumulative administration of UTP (\(10^{-7}–10^{-4} \) M), a P2Y2/P2Y4 receptor agonist, induced contraction that was significantly greater in rings from GK rats than in those from Wistar in the presence of \(10^{-5} \) M l-NNA (Fig. 2A). The UTP-induced contraction was largely blocked by endothelial denudation, and this maneuver abolished the difference between the two groups (Fig. 2A). The enhanced UTP-induced contraction seen in the GK group was largely suppressed by suramin (Fig. 2B), indomethacin (Fig. 2C), or SQ-29548 (Fig. 2D). 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. 2E; note the expanded ordinate scale). The contraction induced by another P2Y2/P2Y4 agonist (UTPγS) in the presence of l-NNA was greater in the GK group than in the Wistar group (data not shown). A selective P2Y6 receptor agonist (MRS-2693) induced a very small contraction in each group (Fig. 2F; note the expanded ordinate scale).

To assess the possible involvement of the cPLA2/COX pathway in the enhanced nucleotide-induced contractions in GK rats, mesenteric rings were preincubated for 30 min with 10−5 M valeroyl salicylate (a COX-1-selective inhibitor; Fig. 3, A and D), 10−6 M NS-398 (a COX-2-selective inhibitor; Fig. 3, B and E), or 10−5 M AACOCF3 (a cPLA2 inhibitor; Fig. 3, C and F). Interestingly, the contractions induced by ATP (Fig. 3, A–C) and UTP (Fig. 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. 3).

Effect of short-term treatment with losartan on ATP- or UTP-induced contraction. We (35–37) and others (4) have demonstrated that ARBs have beneficial effects on vascular function in diabetic models. Moreover, we have previously reported that the mesenteric artery ANG II content was increased in GK rats (37) and further that short-term losartan treatment of GK rats at the chronic stage of diabetes normalized both the endothelin-1-induced contraction (37) and EDRF-mediated relaxation (36). To investigate the effect on nucleotide-induced contractions caused by blockade of the AT1 receptor, we investigated the effect of a 2-wk treatment with losartan on contractions on rings of mesenteric arteries (Fig. 4). Surprisingly, the increased ATP-induced (Fig. 4A) and UTP-induced (Fig. 4B) contractions were each strongly suppressed by such treatment in GK rats.

**ATP- or UTP-stimulated release of prostanoids.** The results described above (see Figs. 1–3) could be consistent with the enhanced nucleotide-induced contractions in GK rats being attributable to activation of the cPLA2/COX pathway. We therefore investigated the release of prostanoids from mesenteric arteries isolated from Wistar, GK, and losartan-treated GK rats (Fig. 5). In nonstimulated mesenteric rings, the production of PGE2 (Fig. 5A), PGF2α (Fig. 5B), 6-keto-PGF1α (a stable metabolite of PGI2; Fig. 5C), and TXB2 (a stable metabolite of TXA2; Fig. 5D) did not differ among the three groups. ATP (3 × 10−4 M) and UTP (10−4 M) each induced the release of PGE2 (Fig. 5E) and PGF2α (Fig. 5G), 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-PGF1α differed among the Wistar, GK, and losartan-treated GK groups (Fig. 5F). The ATP-induced release of TXB2 was greater in GK rats than in Wistar rats, but this enhancement was not affected by losartan treatment (Fig. 5H).

To examine the mechanism responsible for the nucleotide-induced release of PGE2 from GK mesenteric artery rings, we investigated the effects of endothelial denudation and COX inhibitors on ATP-induced (Fig. 5J) or UTP-induced (Fig. 5J) PGE2 production. The increased ATP-stimulated PGE2 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. 5J). The increased UTP-stimulated PGE2 production seen in the GK group was significantly reduced by each of the four maneuvers (endothelial denudation, indomethacin, valeroyl salicylate, and NS-398; Fig. 5J).

**Expression of COX-1, COX-2, phospho-cPLA2, and cPLA2.** To investigate the possible mechanisms underlying the above beneficial effects of losartan on ATP/UTP-mediated responses in mesenteric arteries from GK rats, we first examined the protein expression of COX-1, COX-2, and cPLA2 (Fig. 6). The protein expressions of COX-1 (Fig. 6A) and COX-2 (Fig. 6B) were each significantly greater in GK rats than in Wistar rats, but losartan treatment reduced only the elevated COX-2 expression.

cPLA2 is activated by a rise in cytosolic Ca2+ induced by receptor stimulation and by its own phosphorylation (Ser505) (31). We examined the expression of phospho-cPLA2 after P2Y2 stimulation. In the GK group, ATP-stimulated and UTP-stimulated cPLA2 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. 6C). Expression of total cPLA2 was not different among the Wistar, GK, and losartan-treated GK groups (data not shown).

**Expression of P2Y2, P2Y4, and P2Y6 receptors.** We measured P2Y2, P2Y4, and P2Y6 receptor expressions in mesen-

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Fig. 4. Short-term (2 wk) 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.
teric arteries by immunoblot analysis (Fig. 7). The expressions of P2Y2 (Fig. 7A) and P2Y6 (Fig. 7C) were each similar between GK and Wistar rats, whereas P2Y4 (Fig. 7B) expression was lower in GK rats than in Wistar rats. Losartan treatment of GK rats did not affect P2Y2 and P2Y6 expressions, but it increased P2Y4 expression.

**Effects of losartan on superoxide production in GK rats.** Superoxide leads to an enhancement of EDCF signaling (53, 54, 57, 58). Here, we investigated the effect of losartan treatment on superoxide generation from the nonstimulated mesenteric artery by measuring the amount of NBT reduced by superoxide (Fig. 8) (40, 60). Mesenteric artery superoxide generation was significantly greater in rings from GK rats than in those from Wistar rats and significantly weaker in the losartan-treated GK group than in the losartan-untreated GK group.

**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-cPLA2, 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 PGE2 and PGF2α and 2) the expressions of COX-2 and phospho-cPLA2. To help the reader follow the discussion of the present results and the putative underlying mechanisms, we have included a schematic summary of the pathways and interactions that may be involved (Fig. 9).
The GK rat, a model of type 2 diabetes without the confounding effects of obesity or hypertension (19), was used here at the chronic stage of diabetes (37–42 wk old) because long-term diabetic conditions are associated with severe diabetic complications, including cardiovascular dysfunction. Indeed, numerous reports (20, 22, 28, 29, 34, 36, 37, 47) 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, we first focused on the relationship between ATP-induced contraction and EDCFs (viz. vasoconstrictor prostanoids) because 1) ATP can release such factors (18); 2) we (35, 38–40) 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. 1F) and UTP-induced (Fig. 2E) contractions were smaller in the absence than in the presence of NOS inhibitor treatment. Indeed, some previous studies (18, 64) have used an NOS inhibitor (10^{-4} M L-NNA) in investigations of EDCF signaling. Here, we 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. PGE2, PGF2α, and TXA2) was increased in GK rats, and 3) COX-1

![Fig. 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-actin 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 cPLA2 phosphorylation in mesenteric arteries obtained from Wistar, GK, and losartan-treated GK rats. Ratios were calculated for the optical density of phosphorylated (p-)cPLA2 over that of cPLA2. 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; †P < 0.05, the losartan-treated GK group with UTP vs. the GK group with UTP.

Fig. 7. A–C: Western blots for P2Y2 (A), P2Y4 (B), and P2Y6 (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.

![Fig. 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.](http://ajpheart.physiology.org/)

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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 (10, 14). To investigate which receptors might be responsible for the enhancement of ATP-induced contraction in GK mesenteric arteries, we used several agonists/antagonists of these receptors. In vascular smooth muscle cells, P2X1 is the dominant P2X subtype (61), although P2X2, P2X3, P2X4, and P2X5 have also been found (41, 56). NF-023 inhibits P2X1 receptors more effectively than P2X2, P2X3, or P2X4 receptors (42), whereas α,β-methylene ATP is a relatively selective agonist for P2X1 and P2X3 receptors (42). We found that 1) the contraction induced by ATP was suppressed by both NF-023 and suramin [a nonselective P2 antagonist (1, 3, 21, 45)]; 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 (P2Y2/P2Y4 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 to increased endothelium-derived vasoconstrictor prostanoid signaling after activation of the P2Y receptor, rather than of the P2X receptor. Actually, several reports (5, 7, 46) have suggested that activation of the P2Y receptor stimulates the production of prostanoids.

Vascular endothelial cells reportedly express several subtypes of P2Y receptors (43, 61). In the present study, we found that 1) the contractions induced by P2Y2/P2Y4 agonists (UTP or UTPyS) were increased in the GK group; 2) a P2Y6 agonist induced only a very small contraction that was similar between the two groups; and 3) expressions of P2Y2 and P2Y6 were each similar between the two groups, whereas P2Y4 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 our experiments, we 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 PLA2 activation and leads to the release of AA from cellular phospholipids. Here, we found that nucleotide-induced contractions were reduced by AACOCF3, a potent and selective inhibitor of cPLA2. Moreover, both ATP- and UTP-stimulated cPLA2 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 (15, 54, 58, 62). 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 PGF2α 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 cPLA2/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 seen between GK and Wistar rats, but that indomethacin did abolish these differences. We 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, EDCC signalings are mediated not only by COX-1 activation but also by COX-2 activation (15, 38, 54, 58, 62).

Although we are 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. (55) noted attenuated purinergic receptor function in type 2 diabetic patients, whereas hypoglycemia induced P2Y4 upregulation (12). Furthermore, activation of cPLA2 can be induced by high glucose (63), or insulin (26), or high ANG II (16) [which is
increased in mesenteric arteries from GK rats (37)]. Thus, it is possible that the abnormalities observed in the P2Y receptor-dependent cPLA2/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 (10).

ARBs are very effective, safe antihypertensive drugs with pleiotropic effects (such as antiatherogenic, and anti diabetic, antiplatelet-aggregating effects) (13). A novel, intriguing, and potentially important finding of the present study is that 2-wk 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 cPLA2 phosphorylation and the UTP-induced release of PGE2 and PGF2α, 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 cPLA2 activity in vascular smooth muscle cells (16) as well as both COX-2 expression and PGE2 production in aortic fibroblasts from normotensive and hypertensive rats through AT1 receptors (6). Moreover, we (35) 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 cPLA2/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 (15, 53, 57, 59) suggesting that oxidative stress augments EDCF-mediated responses. Indeed, we have preliminary evidence showing that nucleotide-induced vasoconstrictions in mesenteric arteries from GK rats can be reduced by tempol (α-superoxide dismutase mimetic) or by apocynin [an inhibitor of NAD(P)H oxidase] (unpublished observations). Since previous reports (2, 24, 35) and the present data (Fig. 8) suggest that losartan decreases oxidative stress, the normalization of nucleotide-induced signaling by losartan may be mediated by its antioxidant 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 (11, 14, 44). Moreover, there is a possibility that release of prostanoids may be induced by nucleotides in cells other than endothelial cells. Indeed, there are reports (9, 51) of prostanoid release in vascular smooth muscle cells. Without further studies, we 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, our study demonstrates that the enhanced ATP-induced contraction seen in mesenteric arteries taken from GK rats at the chronic stage of diabetes is due to increased cPLA2/COX pathway activity and that AT1 receptor antagonism normalizes the P2Y-induced contraction in such arteries via a suppression of that pathway’s activity. We believe that our 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.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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