Involvement of NO and MEK/ERK pathway in enhancement of endothelin-1-induced mesenteric artery contraction in later-stage type 2 diabetic Goto-Kakizaki rat

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Matsumoto T, Ishida K, Nakayama N, Kobayashi T, Kamata K. Involvement of NO and MEK/ERK pathway in enhancement of endothelin-1-induced mesenteric artery contraction in later-stage type 2 diabetic Goto-Kakizaki rat. Am J Physiol Heart Circ Physiol 296: H1388–H1397, 2009. First published March 13, 2009; doi:10.1152/ajpheart.00043.2009.—Endothelin (ET)-1 is a likely candidate for a key role in diabetic vascular complications. However, no abnormalities in the vascular responsiveness to ET-1 have been identified in the chronic stage of type 2 diabetes. Our goal was to look for abnormalities in the roles played by ET receptors (ETA and ETB) in the mesenteric artery of the type 2 diabetic Goto-Kakizaki (GK) rat and to identify the molecular mechanisms involved. Using mesenteric arteries from later-stage (32–38 wk old) individuals, we compared the ET-1-induced contraction and the relaxation induced by the selective ETB receptor agonist IRL1620 between GK rats and control Wistar rats. Mesenteric artery ERK activity and the protein expressions for ET receptors and MEK were also measured. In GK rats (vs. age-matched Wistar rats), we found as follows. 1) The ET-1-induced contraction was greater and was attenuated by BQ-123 (ETA antagonist) but not by BQ-788 (ETB antagonist). In the controls, BQ-788 augmented this contraction. 2) Both the relaxation and nitric oxide (NO) production induced by IRL1620 were reduced. 3) ET-1-induced contraction was enhanced by N\textsuperscript{\text{3}}-nitro-L-arginine (l-NNA; NO synthase inhibitor) but suppressed by sodium nitroprusside (NO donor). 4) The enhanced ET-1-induced contraction was reduced by MEK/ERK pathway inhibitors (PD-98059 or U0126). 5) ET-1-stimulated ERK activation was increased, as were the ETA and MEK1/2 protein expressions. 6) Mesenteric ET-1 content was increased. These results suggest that upregulation of ETA, a defect in ET\textsubscript{B}-mediated NO signaling, and activation of the MEK/ERK pathway together represent a likely mechanism mediating the hyperreactivity to ET-1 examined in this study.

THE WORLDWIDE PREVALENCE OF DIABETES MELLITUS, particularly type 2 diabetes, has increased significantly in recent years. Although type 2 diabetes and the associated components of the dysmetabolic syndrome are already leading causes of morbidity and mortality in societies around the world, recent estimates predict a doubling in the prevalence of type 2 diabetes by 2025 (63). Type 2 diabetes is associated with a markedly increased incidence of cardiovascular diseases (26, 56). However, the exact relationship between type 2 diabetes and cardiovascular disease is still not completely understood and, indeed, is the subject of some dispute, because type 2 diabetes is often part of an array of complex abnormalities referred to as “metabolic syndrome,” which is frequently accompanied by hypertension and obesity (56).

It is believed that an impaired ability to vasodilate and/or an enhanced sensitivity to vasoconstrictor agonists underlie the vascular dysfunction associated with diabetes (23, 26, 29, 34, 37). Endothelin (ET)-1, a peptide with potent vasoconstrictor, mitogenic, and proinflammatory properties, exerts its vascular effects by binding to endothelin type A (ETA) and type B (ETB) receptors, of which the ETA receptors mainly mediate its vasoconstrictor actions (1, 9, 15, 50, 53). There are interesting data from patients with type 2 diabetes that demonstrate increased circulating levels of ET-1 and a positive correlation between plasma ET-1 levels and vasculopathy (10). The idea that increased production of ET-1 may have functional consequences is supported by blockade of ET receptors increasing total forearm and leg blood flow in type 2 diabetic patients (5). Collectively, these observations suggest a role for ET-1 in the pathogenesis of diabetic vascular complications (3, 5, 10, 59).

Various animal models have been used to gain more insight into the pathogenesis of the vasculopathy associated with type 2 diabetes (26, 56, 63). However, many of these models exhibit features of the 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. However, the Goto-Kakizaki (GK) rat, one diabetic model, is a relatively unique strain in that it develops no obesity, hyperlipidemia, or hypertension (13, 51). The GK rat was developed from a stock of Wistar rats by selective breeding over many generations from those individuals with the highest blood glucose levels during an oral glucose tolerance test (16). In the GK rat, the advent of moderate diabetes usually occurs between 3 and 4 wk of age and is the result of several pathomechanisms, including impaired ontogenic development of islet cells (44), abnormal insulin release following a glucose load (46), insulin resistance (2), a basal hyperinsulinemia (44), and abnormal glucose metabolism (46). The GK rat thus offers a convenient model for the study of type 2 diabetes per se, without the confounding effects of obesity or hypertension. Although we (19, 27) and others (7, 54, 55) have reported that abnormalities of vascular function exist in this model, few experimental studies on reactivity to ET-1 and its associated molecular mechanisms have been conducted using GK rats at the chronic stage of diabetes.

For the present study, we designed experiments to investigate the changes in the ET-1-induced contraction of mesenteric arteries that might occur as a result of long-term diabetes. For this, we isolated mesenteric arteries from 32- to 38-wk-old GK rats and compared them with those from age-matched...
control Wistar rats, and we tried to identify some of the molecular mechanisms involved in the changes we detected.

MATERIALS AND METHODS

Reagents. Angiotensin II (ANG II), arginine vasopressin (AVP), phenylephrine (PE), Nω-nitro-L-arginine (L-NNA), IRL1620, sodium nitroprusside (SNP), cyclo(o-o-aspartyl-l-propyl-d-valyl-l-leucyl-d-tryptophyl) (BQ-123), N-[N-[1-(2,6-dimethyl-1-piperidinyl)carbonyl]-4-methyl-l-leucyl]-l-(methoxy carbonyl)-d-tryptophyl]-d-norleucine monosodium (BQ-788), and monoclonal β-actin antibody were all purchased from Sigma Chemical (St. Louis, MO). ET-1 was obtained from Peptide Institute (Osaka, Japan). PD-98059 and U0126 were obtained from Calbiochem-Novabiochem (La Jolla, CA). All drugs were dissolved in saline unless otherwise noted. All concentrations are expressed as the final molar concentration of the base in the organ bath. Horseradish peroxidase-linked secondary anti-mouse antibody was purchased from Promega (Madison, WI), whereas antibodies against extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated ERK1/2 (pT202/pY204), MEK1, and MEK2 were obtained from BD Biosciences (San Jose, CA). Antibodies against the ETA and ETB receptors were obtained from Abcam (Cambridge, UK).

Animals and experimental design. Male GK rats and Wistar control 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. This study was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health and the “Guide for the Care and Use of Laboratory Animals” adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan). At the ages of 32–38 wk, groups of rats were killed by decapitation under diethyl ether anesthesia.

Measurement of blood glucose and insulin. Plasma glucose and insulin were measured as described previously (31, 37, 38). Briefly, the plasma glucose level was determined using a commercially available enzyme kit (Wako Chemical, Osaka, Japan). Plasma insulin was measured by enzyme immunoassay (Shibayagi, Gunma, Japan).

Measurement of isometric force. Vascular isometric force was recorded as in our previous reports (31, 32, 35, 37, 39–41). At 32–38 wk of age, rats were anesthetized by 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. This weight was slightly but significantly lower in the GK group (0.82 ± 0.02 mg; n = 75) than in the Wistar group (0.90 ± 0.02 mg; n = 75, P < 0.01).

For the relaxation studies, mesenteric rings were precontracted with a submaximal concentration of PE (10⁻⁶ M, EC₆₅–₇₅). When the PE-induced contraction had reached a plateau level, IRL1620 (selective ET₆ agonist; 10⁻¹⁰–10⁻⁷ M) was added in a cumulative manner.

Measurement of nitrite and nitrate. The concentrations of nitrite (NO₂⁻) and nitrate (NO₃⁻) in the effluent from each tissue were measured using the method described previously (40). To evaluate the release of NO metabolites in mesenteric arteries, each mesenteric ring was placed in KHS at 37°C and then treated with IRL1620 (3 × 10⁻⁸ M) for 5 min. The concentrations of nitrite and nitrate in the KHS
were measured using an automated NO detector/high-performance liquid chromatography system (ENO20; Eicom, Kyoto, Japan).

Quantification of phosphorylated ERK2 using ELISA. The levels of phosphorylated ERK2 in mesenteric arteries were measured using the method described previously (37). Mesenteric rings were incubated with 10 nM ET-1 for 30 min and then washed with ice-cold KHS. They were then frozen in liquid N2 before being physically crushed to a fine powder in liquid N2 with a Cryo-Press (Microtech Nichion, Chiba, Japan). After lysation of these powder samples, phosphorylated ERK2 was lysed and its level quantified with the use of a quantitative assay system (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

Western blotting. The protein levels of ERK1/2, phosphorylated ERK1/2, MEK1/2, and the ET receptors were quantified using immu-noblotting procedures, essentially as described previously (28, 31, 41). Mesenteric arterial protein extracts (20 μg) were applied to 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Blots were incubated with anti-ERK1/2 (44 and 42 kDa; 1:1,000), anti-phospho-ERK1/2 (pT202/pY204; 44 and 42 kDa; 1:1,000), anti-MEK1 (1:1,000; BD Biosciences), anti-MEK2 (1:1,000; BD Biosciences), anti-ETα receptor (1:1,000; Abcam), anti-ETβ receptor (1:1,000; Abcam), or anti-β-actin (1:5,000) antibodies, with detection achieved using a horseradish peroxidase-conjugated IgG followed by enhanced chemiluminescence. Band intensity was quantified by densitometry.

Quantification of tissue ET-1 using ELISA. Analysis of mesenteric tissue ET-1 concentrations was performed as follows. 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 deoxocholate, and 0.1% SDS containing protease inhibitor cocktails (Complete protease inhibitor cocktail; 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 retained for determination of ET-1 by ELISA, which was performed according to the instructions given by the manufacturer (Assay Designs, Ann Arbor, MI). Tissue ET-1 content was calculated as content per milligram of protein in the tissue. Protein concentrations were determined by means of a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL).

Statistical analysis. The contractile force developed by mesen-teric artery rings is expressed in milligrams of tension per milliogram of tissue. Each relaxation response is expressed as a percentage of the contraction induced by PE. Data are means ± SE. Statistical differences were assessed using Dunnett’s test for multiple comparisons after a one-way ANOVA, with a probability level of P < 0.05 regarded as significant. Statistical comparisons between concentration-response curves were made using a two-way ANOVA with a Bonferroni correction performed post hoc to
RESULTS

General parameters. At the time of the experiment (when the rats were 32–38 wk old), the body weight of the GK rats (411.0 ± 5.1 g; n = 15) was lower than that of the age-matched nondiabetic control Wistar rats (551.9 ± 11.3 g; n = 15, P < 0.001). All GK rats (nonfasted) exhibited hyperglycemia (454.1 ± 13.8 mg/dl; n = 15), with blood glucose levels significantly higher than those of the Wistar rats (also nonfasted; 149.2 ± 4.4; n = 15, P < 0.001). The plasma insulin level was significantly higher in GK rats (4.5 ± 0.4 ng/ml; n = 15) than in Wistar rats (2.9 ± 0.2 ng/ml; n = 15, P < 0.001).

Contractile responses induced by ET-1, ANG II, and AVP. Exposure of mesenteric artery rings to ET-1 (10^{-10}–10^{-7} M) led to a concentration-dependent rise in tension in both GK and Wistar rats (Fig. 1A and Table 1), although the potency of ET-1 was greater in the GK group than in the Wistar group. Specifically, at intermediate concentrations of ET-1 (i.e., 3 and 10 nM), the ET-1-induced contraction was significantly stronger in rings from GK rats than in those from the Wistar controls (Fig. 1A). In contrast, although exposure of mesenteric artery rings to ANG II (10^{-9}–10^{-7} M) (Fig. 1B) or AVP (10^{-10}–10^{-7} M) (Fig. 1C) led to a concentration-dependent rise in tension in both the GK and Wistar groups, there was no significant difference between these two groups in the response to either agonist.
Effect of ET-receptor antagonist on ET-1-induced contraction.

Pretreatment of rings from either group of rats with the ETA antagonist BQ-123 inhibited the ET-1-induced contraction in a concentration-dependent manner (Fig. 2 and Table 1). It should be noted that at two concentrations of BQ-123 (0.1 and 0.3 \( \mu \)M), a stronger ET-1-induced contraction was seen in the GK group than in the Wistar group (Fig. 2 and Table 1). In addition, at intermediate concentrations of ET-1 [i.e., 3 (Fig. 2, A and B) and 10 nM (Fig. 2 C)], the ET-1-induced contraction was not significantly inhibited by 0.1 \( \mu \)M BQ-123 in the GK group, although it was completely abolished by this concentration of BQ-123 in the Wistar group. These data suggest that ETA-mediated contraction is more pronounced in GK than in Wistar mesenteric arteries.

In contrast, pretreatment of arteries with the ETB antagonist BQ-788 (1 \( \mu \)M) led to no significant change in the ET-1-induced contraction in the GK group (Fig. 2 and Table 1). It should be noted that at two concentrations of BQ-123 (0.1 and 0.3 \( \mu \)M), a stronger ET-1-induced contraction was seen in the GK group than in the Wistar group (Fig. 2 and Table 1). In addition, at intermediate concentrations of ET-1 [i.e., 3 (Fig. 2, A and B) and 10 nM (Fig. 2 C)], the ET-1-induced contraction was not significantly inhibited by 0.1 \( \mu \)M BQ-123 in the GK group, although it was completely abolished by this concentration of BQ-123 in the Wistar group. These data suggest that ETA-mediated contraction is more pronounced in GK than in Wistar mesenteric arteries.

Vasodilation and NO production in response to IRL1620.

Cumulative concentration-response curves for the relaxation induced by the ETB receptor agonist IRL1620 (10^{-10} \text{ to } 10^{-7} \text{ M}) were obtained in mesenteric artery rings from Wistar and GK rats (Fig. 3A). The tension developed in response to 10^{-6} \text{ M PE} did not differ significantly between the Wistar (1.61 ± 0.1 g, \( n = 6 \)) and GK groups (1.65 ± 0.05 g, \( n = 6 \)). IRL1620 induced a concentration-dependent relaxation on this contraction that was significantly weaker in the GK than in the Wistar group (Fig. 3A). Incubation of rings with 10^{-4} \text{ M l-NNa (Fig. 3A) or endothelial denudation (data not shown) completely abolished the IRL1620-induced relaxation in each of these groups. IRL1620 (3 \times 10^{-8} \text{ M}) increased the NOx (\( \text{NO}_2^- + \text{NO}_3^- \)) level in the perfusate from mesenteric rings, but this effect was significantly weaker in rings from GK rats than in those from Wistar rats (Fig. 3B).

Effects of endothelial denudation, a NO synthase inhibitor, and a NO donor on ET-1-induced contraction. After endothelium denudation, the contractile potency of ET-1 was significantly increased in both the GK and Wistar groups (vs. endothelium-intact arteries) (Fig. 4A and Table 1). NO plays important roles in dilation and in the modulation of contractile

![Fig. 5. Effects of MEK/ERK pathway inhibitors on dose-response curves for ET-1-induced contraction of mesenteric arteries isolated from GK and Wistar rats. Data were obtained in the presence of PD-98059 (10^{-5} \text{ M; A}) or U0126 (10^{-5} \text{ M; B}). Each data point represents the mean ± SE from 6–12 experiments. \( *P < 0.05 \) vs. Wistar, \#\# \( P < 0.05 \); \#\#\# \( P < 0.01 \) vs. GK. For comparison, the dose-response curves for ET-1 depicted in Fig. 1A are shown again in A and B.](http://ajpheart.physiology.org/)

![Fig. 6. Amounts of phosphorylated ERK2 (p-ERK2) in ET-1 (10 nM)-treated mesenteric arteries from GK and Wistar rats [determined using ELISA (A) or Western blotting (B)]. A: quantification of p-ERK2 in ET-1 (10 nM)-treated mesenteric arteries. Details are given in MATERIALS AND METHODS. Each column represents the mean ± SE from 6 experiments. \* \( P < 0.05 \) vs. Wistar. B: analysis of p-ERK and ERK protein expressions in mesenteric arteries obtained from both groups. Top, representative Western blots for p-ERK2 and ERK2. The lanes are from the same gel and were noncontiguous; bottom, bands were quantified as described in MATERIALS AND METHODS. Ratios were calculated for the optical density of p-ERK2 or total ERK2 over that of the corresponding \( \beta \)-actin. Each column represents the mean ± SE from 6 experiments. \* \( P < 0.05 \) vs. Wistar.](http://ajpheart.physiology.org/)
responses in blood vessels. To help evaluate the part played by NO as a determinant of the ET-1-induced contraction in the mesenteric artery, we applied a representative NO synthase (NOS) inhibitor or NO donor to mesenteric rings. Incubation of rings with L-NNA (10^{-4} M), which inhibits both basal and agonist-induced NOS activity, significantly increased the potency of ET-1 in both the GK and Wistar groups (Fig. 4B and Table 1). On the other hand, incubation of rings with SNP (10^{-5} M) had the opposite effect: it significantly reduced the potency of ET-1 in each of these groups (Fig. 4C and Table 1). It should be noted that in the presence of L-NNA or following increased activation of the MEK/ERK pathway.

The data suggest that the increased mesenteric vasoconstrictor response in GK rats might be attributable not only to an endothelium-derived factor (i.e., NO) but also to endothelium-independent factor(s).

**Effect of MEK/ERK-pathway inhibitors on ET-1-induced contraction.** ET-1 modulates vascular tone through activation of mitogen-activated protein kinase (MAPK) pathways, including the ERK or MEK/ERK pathway (14, 60, 64). Pretreatment of arteries with an MEK/ERK inhibitor [PD-98059 (10^{-5} M) or U0126 (10^{-5} M)] markedly reduced the potency of ET-1 in the GK group but not in the Wistar group (Fig. 5 and Table 1). These data suggest that the increased mesenteric vasoconstriction to ET-1 exhibited by GK rats might be attributable to increased activation of the MEK/ERK pathway.

**Effects of ET-1 on ERK activation.** Collectively, the present data (Fig. 5) and previous reports (14, 28, 33, 60, 64) suggest that the ERK pathway modulates the effects of several vasoconstrictors on vascular smooth muscle. As shown in Fig. 6, ET-1-induced ERK2 phosphorylation (activation), as detected by means of ELISA (Fig. 6A) or Western blotting (Fig. 6B), was significantly greater in mesenteric arteries from GK rats than in those from Wistar rats.

**Expression of MEK.** We next examined the expressions of MEK1 and MEK2 proteins in mesenteric arteries by Western blotting (Fig. 7). MEK1 protein expression tended to be slightly (but not significantly) increased in the GK group (vs. the Wistar group) (Fig. 7A), whereas MEK2 protein expression was significantly greater in the GK than in the Wistar group (Fig. 7B).

**Expression of ET-receptor protein.** ET receptors were examined using Western blotting (Fig. 8). ETA receptor density was determined by combining data for two bands (at 54 and 39 kDa; corresponding to the native and glycosylated forms of the receptor, respectively), as described previously (11, 28). Densitometric analysis of these two bands indicated a significantly greater expression in the GK than in the Wistar group (Fig. 8A). The protein expression of the ETB receptor was also significantly increased in mesenteric arteries from GK rats (vs. Wistar rats) (Fig. 8B).

**Vascular ET-1 content.** The ET-1 content of mesenteric arteries, as measured using ELISA (Fig. 9), was significantly greater in the GK than in the Wistar group.

**DISCUSSION**

Uncertainty still surrounds the long-term effects of type 2 diabetes on the parts played by vascular ETA and ETB receptors, and their associated signaling, in vascular regulation, but evidence from a number of studies suggests an enhanced contribution of pathways mediating vasoconstriction through these receptors in several diabetic models (25, 28, 42, 55). The principal findings in the present study, conducted using mesenteric arteries isolated either from type 2 diabetic GK rats at the chronic stage (32–38 wk old) or from their Wistar controls, were that 1) ET-1-induced contraction was increased in the GK group; 2) an inhibitory effect of ETB receptor stimulation on the ET-1-induced contraction was present in the controls but not in the GK rats; 3) both ETB receptor-mediated relaxation and NO production were reduced in such GK rats; 4) in both groups, ET-1-induced contraction was modulated not only by NO signaling but also by MEK/ERK signaling; 5) activity in the MEK/ERK pathway was increased in the GK rats, and such augmented signaling enhanced the ET-1-induced contraction; 6) upregulation of ETA and ETB receptors was present in the GK group; and 7) the mesenteric ET-1 content was elevated in the GK rats.

Mesenteric arteries from 32- to 38-wk-old diabetic GK rats exhibited an enhanced vasoconstriction to ET-1 compared with their age-matched controls. ET-1 is widely known to stimulate contraction.

![Fig. 7. Western blots for MEK1 (A) and MEK2 (B) in mesenteric arteries from GK and Wistar rats. Top, representative Western blots for MEK1, MEK2, and β-actin; bottom, bands were quantified as described in MATERIALS AND METHODS. Ratios were calculated for the optical density of each MEK over that of β-actin. Data are means ± SE from 8 experiments. *P < 0.05 vs. Wistar.](http://ajpheart.physiology.org/)

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ET<sub>A</sub> receptors on vascular smooth muscle cells to produce vasoconstriction and ET<sub>B</sub> receptors on endothelial cells to produce vasodilation (15, 50, 53). In the present study, the expression of the ET<sub>A</sub> receptor in mesenteric artery tissue was greater in GK rats than in control rats. Treatment with BQ-123, an ET<sub>A</sub>-receptor antagonist (18, 28, 58), inhibited the ET-1-induced contraction in arteries from both groups in a concentration-dependent manner. It should be noted that at two concentrations of BQ-123 (i.e., 0.1 and 0.3 μM), a greater ET-1-induced contraction was seen in arteries from GK rats than in those from Wistar rats. Moreover, at intermediate concentrations of ET-1 (i.e., 3 and 10 nM; at each of which a significant difference was seen between the GK and Wistar groups), the ET-1-induced contractions were not significantly inhibited by 0.1 μM BQ-123 in the GK group, but they were completely abolished by this concentration of BQ-123 in the Wistar group. These results suggest that the increased response to ET-1 displayed by our GK group was due to upregulation of ET<sub>A</sub> receptors in GK mesenteric arteries. This is consistent with previous findings of enhanced vasoconstrictor responses to ET-1 and enhanced expressions of ET receptors in various arteries either from insulin-resistant rats (25) or from a hyperinsulinemic diabetic model (28).

Endothelial ET<sub>B</sub> receptors, on the other hand, are believed to confer protective effects by mediating vasodilation in response to ET-1 or ET<sub>B</sub> agonists (although smooth muscle ET<sub>B</sub> receptors are vasoconstrictor in function) (15, 50, 53). We detected a significant increase in total ET<sub>B</sub> receptor abundance in GK rats. However, it should be noted that our immunoblotting was performed using a total vessel homogenate and therefore could not differentiate between endothelial ET<sub>B</sub> receptors and those on the vascular smooth muscle. To address this problem using a pharmacological approach, we investigated the contractile response to the ET<sub>B</sub>-specific agonist IRL1620, our prediction being that if there were an increase in smooth muscle ET<sub>B</sub> receptors, we would see a contraction rather than the relaxation expected to be conferred by the endothelial ET<sub>B</sub> receptors. In fact, we observed relaxation, and moreover, the relaxation was reduced in GK rats (vs. the control rats). Studies with endothelium-denuded vessels would be expected to help clarify the contribution made by endothelial vs. vascular smooth muscle ET<sub>B</sub> receptors, and indeed, we made the preliminary finding that IRL1620 did not induce contraction in endothelium-denuded mesenteric arteries from either 32- to 38-wk-old GK or Wistar control rats (data not shown). Moreover, treatment with BQ-788, an ET<sub>B</sub>-receptor antagonist, did not affect the ET-1-induced response in the present GK rats. On the basis of these results, we suggest that the GK mesenteric arterial ET<sub>B</sub> receptor is largely nonfunctional and that ET<sub>B</sub> receptor signaling in smooth muscle cells may play a weak or negligible role in ET-1-induced contraction in such GK rats.

Activation of endothelial ET<sub>B</sub> receptors results mainly in increased endothelial NOS activity and the release of NO (15, 53). Moreover, cross talk between ET-1 and NO may play an important role in the cardiovascular system (6, 8), and the synthesis/release of NO may play a role in the modulation of the ET-1-induced contractile response (30). Indeed, in the arteries used in the present study, we found that 1) an enhancement of the ET-1-induced contraction was induced by endothelial denudation in both the GK and control groups; and 2) in both groups, the representative NOS inhibitor L-NNA led to an enhancement of the ET-1-induced contraction, whereas the NO donor SNP suppressed such contractions. These results suggest that a suppressive effect of NO on ET-1-induced contraction is evident in mesenteric arteries from both groups of rats. However, treatment with an ET<sub>B</sub> receptor antagonist led to an
enhancement of the ET-1-induced contraction in the Wistar group but not in the GK group. There is apparent conflict among our data in that in mesenteric arteries from GK rats, an enhancing effect of L-NNA on the ET-1-induced contraction was present, but BQ-788 did not enhance this contraction. Possibly, this may be attributable to differences between these two drug treatments in NO signaling. Theoretically, as regards NO signaling, BQ-788 can only inhibit ET₂B-mediated signaling, whereas L-NNA can inhibit total NOS activity (including basal NOS activity). Actually, basal NOS activity in mesenteric arteries was similar between the GK and Wistar groups (data not shown). Moreover, the ET₂B receptor-mediated NO-dependent relaxation and NO production were greatly reduced in the present GK mesenteric arteries (vs. the Wistar group) (Fig. 3). These results indicate that the GK mesenteric artery exhibits an impairment of the vasodilator response induced by ET-1 via NO signaling following endothelial ET₂B receptor activation. Moreover, they suggest that such a defect in NO signaling might contribute to the hyperreactivity to ET-1 exhibited by such diabetic rats [since normally when mesenteric rings are exposed to ET-1, the endothelial ET₂B receptors (which mediate vasodilation) and the smooth muscle ETA receptors (which mediate vasoconstriction) are stimulated simultaneously and have a combined effect on vascular tone]. However, it should be noted that the ET-1-induced contraction was greater in mesenteric arteries from GK rats than in those from control rats even after endothelial denudation or L-NNA pretreatment. These findings suggest that other endothelium-independent modulating factor(s) may contribute to the hyperreactivity to ET-1 shown by GK mesenteric arteries.

ET-1 activates ERK1/2 and thereby promotes changes in vascular reactivity as well as vascular remodeling, inflammation, and oxidative stress (21, 28, 62, 64). Moreover, the complex signaling network that underlies MAPK activation typically requires phosphorylation by a MAPK kinase, also known as MEK; for instance, the ERK1/2 phosphorylation cascade involves MEK1/2 (48). In the present study, we observed a marked difference in the ET-1-induced contraction [at an intermediate concentration of ET-1 (i.e., 10 nM)] between the control and GK groups. Furthermore, whereas the ET-1-induced contraction in our GK group was markedly suppressed by MEK/ERK inhibitors, it was unaffected in control rats. Moreover, the ET-1 (10 nM)-stimulated ERK2 activation and MEK2 expression were both greater in mesenteric arteries from GK rats than in those from the controls. These results indicate that the MEK/ERK pathway makes a substantial contribution to the differences we observed between control and GK rats in the present ET-1-induced contraction.

Several animal models have been employed to gain more insight into the alterations in the ET system associated with diabetes (3, 22, 24). However, whereas many of these models exhibit other features of the metabolic syndrome in addition to diabetes (such as hyperlipidemia, obesity, or hypertension), the GK rat does not. Although the mechanisms underlying the present differences in ET-1-mediated responses between control and GK rats remain to be identified, long-term metabolic and/or hormonal factors (i.e., hyperglycemia and hyperinsulinemia) are prime candidates, since GK rats lack the confounding effects of obesity and hypertension. Indeed, it has been reported that high plasma glucose levels stimulate ET-1 production and signaling (47) and that such signaling is partly mediated via MAPK/ERK activation (61). In addition, there are several reports suggesting that insulin-resistant and hyperinsulinemic states cause enhanced activity in the MEK/ERK pathway (26, 52) and enhanced expressions of ET receptors (25).

Furthermore, we recently demonstrated that in the rat aorta, a high plasma insulin level and a diabetic state need to exist together to cause an augmentation of ET-1-induced contraction through the ETA receptor/phosphatidylinositol 3-kinase/MEK/ERK pathway (28). Several studies in insulin-resistant humans have reported increased circulating ET-1 concentrations that directly correlated with the level of hyperinsulinemia (12, 49). Furthermore, Ergul and colleagues (11, 17) have demonstrated that circulating and local ET-1 levels are elevated in GK rats, whereas the present study has shown that the mesenteric artery ET-1 content is elevated in such rats. Taking the above evidence and the present data together, we speculate that in long-term diabetic disease states, the above-described alterations in cellular mechanisms contribute to an increased ET-1-induced contraction (as observed in the present study in mesenteric arteries from GK rats at the chronic stage of diabetes).

The present findings indicate that upregulation of the ETA receptor, a defect in ET₂B receptor-mediated NO signaling, and activation of the MEK/ERK pathway together represent a likely mechanism mediating the hyperreactivity to ET-1 that exists in the chronic stage of type 2 diabetes in GK rats. We believe that our findings should stimulate further interest in the ET-1 system as a potential therapeutic target in diabetes-associated vascular diseases.

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**REFERENCES**

CONTRACTION TO ENDOTHELIN IN GK RAT MESENTERIC ARTERY


