Full Paper

Losartan Normalizes Endothelium-Derived Hyperpolarizing Factor–Mediated Relaxation by Activating Ca^{2+}-Activated K^+ Channels in Mesenteric Artery From Type 2 Diabetic GK Rat

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Abstract. Ca^{2+}-activated K^+ (K_{Ca}) channels are important for endothelium-derived hyperpolarizing factor (EDHF) signaling. Since treatment with angiotensin II receptor blockers (ARBs) improves vasculopathies in type 2 diabetic patients, we asked whether the EDHF-type relaxation and its associated K_{Ca} channels [small (SK_{Ca})–, intermediate (IK_{Ca})–, and large (BK_{Ca})–conductance channels] are abnormal in mesenteric arteries isolated from Goto-Kakizaki (GK) rats at the chronic stage of type 2 diabetes (34 – 38 weeks) and whether an ARBs (losartan, $25~mg \cdot kg^{-1} \cdot day^{-1}$ for 2 weeks) might correct these abnormalities. Although the acetylcholine chloride–induced EDHF-type relaxation in mesenteric arteries from GK rats was reduced versus the Wistar controls, it was significantly restored by losartan treatment. The SK_{Ca}-blocker apamin or the IK_{Ca}-blocker 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM-34) inhibited such relaxations in the losartan-treated or -untreated Wistar groups and in the losartan-treated GK group, but not in the losartan-untreated GK group. The BK_{Ca}-blocker iberiotoxin had a significant inhibitory effect in only one of these groups, the losartan-treated GK. The relaxations induced by the SK_{Ca}/IK_{Ca} activator NS309 and the BK_{Ca} activator NS1619, which were impaired in GK rats, were normalized by losartan treatment. We conclude that losartan improves EDHF-type relaxation in GK rats at least partly by normalizing SK_{Ca}/IK_{Ca} activities and increasing BK_{Ca} activity.

Keywords: angiotensin receptor antagonist, diabetes,
endothelium-derived hyperpolarizing factor (EDHF), GK rat, potassium channel

Introduction

The endothelium plays a major role in the regulation of vascular tone. It is capable of exerting a profound relaxing influence on the underlying smooth muscle, an effect mediated by at least three different factors, depending on the vascular bed. These factors include nitric oxide (NO) and prostacyclin, both of which are diffusible factors (1). In addition, after blockade of NO- and prostacyclin-synthesis, stimulation of the endothelium is capable of evoking a vascular smooth muscle relaxation that has been attributed to a third factor, endothelium-derived hyperpolarizing factor (EDHF) (2 – 5).

The nature of EDHF and EDHF-signaling pathways is not fully understood. However, Ca^{2+}-activated K^+ (K_{Ca}) channels are known to be key players in the control of endothelium-mediated vasorelaxation, and indeed they regulate both the endothelial membrane potential and Ca^{2+} homeostasis in response to hemodynamic stresses and vasoactive factors (6). The endothelial hyperpolarization mediated by K_{Ca} channels, such as the small (SK_{Ca})– and intermediate (IK_{Ca})–conductance K_{Ca} channels, has been suggested to play a critical role in initiating EDHF-type relaxation responses in conduit and resistance-sized arteries in many species, including humans (2, 5, 7, 8). The contribution made by EDHF to relaxation is dependent on vessel size, it being more prominent in the smaller, physiologically more important arteries than in larger ones (2, 3). Since small-vessel dysfunction, such as that occurring in retinopathy, nephropathy, and

neuropathy, is one of the major complications seen in diabetes (9), an impairment of EDHF-mediated responses may have important implications for the mechanisms by which diabetes leads to vascular dysfunction. Thus, an improvement in EDHF signaling could be an interesting therapeutic target in cases involving diabetic vasculopathy.

Several lines of evidence suggest that endothelial dysfunction could play a key role in the development of both macro- and microangiopathy in diabetes patients and in animal models of diabetes (10, 11). An accumulating body of evidence indicates that endothelium-dependent relaxation is impaired in several blood vessels in type 1 and type 2 diabetes in animal models and in patients (10 – 18). Concerning EDHF-mediated responses, there is now evidence to suggest that these responses become altered both in disease states such as hypertension and as a result of ageing (3, 10). Although there are several reports suggesting that impaired EDHF-mediated responses may be present in various arteries in type 1 diabetes models (19 – 22), only a small number of reports have found an impairment of the EDHF pathway in a type 2 diabetes model, and the exact nature of its alteration in the long-term type 2 diabetic state remains unclear.

Accelerated vascular disease in type 2 diabetes is often associated with hyperglycemia, hyperinsulinemia, hypertension, and dyslipidemia (23, 24). Intensive glycemic control reduces the risk of the predominantly microvascular, and to a lesser extent macrovascular, complications seen in both type 1 and type 2 diabetes (9). A number of animal models have been used to gain more insight into the pathogenesis of the vasculopathy associated with type 2 diabetes (23, 24). Unfortunately, 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 these confounding factors to the development of diabetic vasculopathy in these models. However, the Goto-Kakizaki (GK) rat, one type 2–diabetic model, is a relatively unique strain in that it develops no obesity, hyperlipidemia, or hypertension (25). 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 (25). In the GK rat, the advent of moderate diabetes usually occurs at between 3 and 4 weeks of age and is the result of several pathomechanisms, including impaired ontogenic development of islet cells, abnormal insulin release following a glucose load, insulin resistance, a basal hyperinsulinemia, and abnormal glucose metabolism (26, 27). The GK strain thus is a valuable model for the study of type 2 diabetes *per se*, since it is without the confounding effects of obesity or hypertension.

Treatment with angiotensin-converting enzyme inhibitors (ACEIs) (28) or angiotensin II (Ang II)–receptor blockers (ARBs) (29) in patients with type 2 diabetes significantly improves both macrovascular and microvascular end-points, including nephropathy, retinopathy, and neuropathy (30). Although studies on animal models of cardiovascular diseases have demonstrated disease prevention when treatment is started before the onset of complications (31), treatment of diabetic patients does not begin until after the symptoms have been diagnosed. At this time, in many instances, complications are already present. Although several studies have demonstrated that renin-angiotensin system (RAS) inhibition (e.g., by treatment with ACEIs or ARB) has beneficial effects on diabetic vasculopathy (28 – 30), little information is available to indicate whether such antagonists might correct EDHF-mediated signaling once the progression of the disease process has begun.

The aims of our study were to assess whether the EDHF-type relaxation and its associated K_{Ca} channels exhibit abnormalities in mesenteric arteries isolated from GK rats at the chronic stage of type 2 diabetes and if so, whether treatment with an ARB (losartan, for 2 weeks) might normalize such responses.

Materials and Methods

Reagents

Phenylephrine (PE), indomethacin, N^G-nitro-L-arginine (L-NNA), apamin, TRAM-34 [1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole], NS309, and NS1619 were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetylcholine chloride (ACh) was from Daiichi Pharmaceuticals (Tokyo), cromakalim was from Toronto Research Chemicals (North York, ON, Canada), and iberiotoxin was from Peptide Institute, Inc. (Osaka). Cromakalim, TRAM-34, NS309, and NS1619 were dissolved in dimethyl sulfoxide. Indomethacin 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 other drugs were dissolved in saline. Control experiments confirmed the absence of significant effects on vascular tone at the final vehicle concentration used.

Animals and experimental design

Male Wistar control rats and GK rats were obtained at the age of 4 weeks (Clea, Tokyo). All animals were allowed a standard laboratory diet (MF; Oriental Yeast Industry, Tokyo) and water ad libitum, and they were kept in an environment in which room temperature and humidity were controlled at 21°C – 22°C and 50 ± 5%,

respectively, until they were 34 – 38-week-old. Some GK and Wistar rats were given losartan (25 mg·kg^{-1}· day^{-1}, p.o., Nulotan; Banyu Co., Ltd., Tsukuba) for 2 weeks starting at 32 – 36 weeks of age. We (32, 33) and others (34) have previously reported that the above losartan dosage regimen has beneficial effects on vascular functions in a number of disease models in rats. Moreover, in a very recent study, again using the above losartan dosage regimen, we noted that Ang II–mediated contraction was largely suppressed in mesenteric arteries obtained from losartan-treated GK or Wistar rats (35). Here, we studied four groups: losartan-untreated Wistar and GK groups and losartan-treated Wistar and GK groups. This study was approved by the Hoshi University Animal Care and Use Committee, and all studies were conducted in accordance with Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and 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 and insulin, and blood pressure

Plasma glucose and insulin were measured as described previously (17, 18, 36). Briefly, the plasma glucose level was determined by the use of a commercially available enzyme kit (Wako Chemical Company, Osaka). Plasma insulin was measured by enzyme-immunoassay (Shibayagi, Gunma). After a given rat had been in a constant-temperature box at 37°C for a few minutes, its blood-pressure was measured by the tail-cuff method using a blood pressure analyzer (BP-98A; Softron, Tokyo).

Measurement of isometric force

Vascular isometric force was recorded as in our previous papers (20, 21, 36 – 40). At 34 – 38 weeks of age, rats were anesthetized with diethyl ether and then euthanized by decapitation. The superior mesenteric artery was rapidly removed and immersed in oxygenated, modified Krebs-Henseleit solution (KHS). This solution consisted of 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM NaHCO$_3$, 1.8 mM CaCl$_2$, 1.2 mM NaH$_2$PO$_4$, 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 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).

To block NO synthase (NOS) and cyclooxygenase, the tissues were equilibrated for 30 min in the presence of 100 μM L-NNA and 10 μM indomethacin before administration of PE. In our preliminary experiment, the concentration–response curves for the PE-induced contraction were not different among the four groups. For the experiments reported here, various submaximal concentrations of PE (0.1 – 0.3 μM) were used to permit amplitude-matching of the precontractions. Once the PE-induced contraction had stabilized, relaxation responses were elicited in a cumulative manner using one of the following: ACh, the K$_{ATP}$-activator cromakalim (41), the IK$_{Ca}$/SK$_{Ca}$-activator NS309 (41 – 43), or the BK$_{Ca}$-activator NS1619 (41, 42). In some experiments, concentration–response curves for the relaxant effect of ACh were generated in the combined presence of L-NNA (100 μM), indomethacin (10 μM), and one of the following: the SK$_{Ca}$-inhibitor apamin (5, 7, 8) (100 nM for 30 min), the IK$_{Ca}$-inhibitor TRAM-34 (5, 7, 8) (10 μM for 30 min), or the BK$_{Ca}$-inhibitor iberiotoxin (5, 7, 8) (100 nM for 30 min). 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. Each relaxation response was expressed as a percentage of the contraction induced by PE.

Statistical analyses

Data are expressed as means ± S.E.M. When appropriate, statistical differences were assessed by Dunnett's test for multiple comparisons after a one-way analysis of variance (ANOVA), a probability level of $P < 0.05$ being regarded as significant. Statistical comparisons between concentration–response curves were made by using a two-way ANOVA, with Bonferroni's correction for multiple comparisons being performed post hoc ($P < 0.05$ again being considered significant).

Results

General parameters

As shown in Table 1, body weight was significantly lower in GK rats than in Wistar rats, while the non-fasted blood glucose and insulin levels were significantly higher in GK rats than in Wistar rats. Treatment with losartan did not alter the above parameters in either group of rats. The systolic blood pressure was significantly lower in GK rats than in Wistar rats, while heart rate was significantly higher in GK rats than in Wistar rats. Two-week treatment with losartan tended to lower systolic blood pressure in the Wistar group and significantly lowered it in the GK group, but did not affect heart rate in either

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

	Wistar	Losartan-treated Wistar	GK	Losartan-treated GK
Body weight (g)	550.6 ± 8.2 (12)	535.6 ± 5.9 (12)	410.1 ± 5.5 (12)*	396.2 ± 6.1 (12)*
Glucose (mg·dl^{-1})	160.8 ± 4.7 (12)	171.1 ± 6.0 (12)	479.3 ± 21.1 (12)*	448.0 ± 10.0 (12)*
Insulin (ng·ml^{-1})	2.6 ± 0.2 (10)	2.6 ± 0.2 (10)	3.9 ± 0.3 (10)*	3.9 ± 0.5 (10)*
SBP (mmHg)	124.3 ± 3.7 (10)	118.8 ± 2.7 (10)	107.2 ± 2.4 (10)*	90.6 ± 2.1 (10)*,#
Heart rate (beats·min^{-1})	381.9 ± 7.0 (10)	409.0 ± 9.2 (10)	449.3 ± 4.0 (10)*	429.2 ± 10.2 (10)*

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.

group.

Effects of two-week losartan treatment on EDHF-mediated relaxation

In order to investigate the EDHF-mediated relaxation evoked by ACh in the rat mesenteric artery, we performed a series of experiments in which ACh ($10^{-9} – 10^{-5}$ M) was added cumulatively to rings precontracted by PE in the presence of 100 μM L-NNA plus 10 μM indomethacin. The ACh-induced EDHF-mediated relaxation was significantly weaker in rings from diabetic GK rats than in those from age-matched Wistar rats (Fig. 1). Losartan treatment of Wistar rats caused no significant alteration in the relaxation compared to than in the untreated Wistar group (Fig. 1). In contrast, such treatment of GK rats led to a significant enhancement of the EDHF-mediated relaxation response compared to than in the untreated GK group (Fig. 1).

Effects of various K_{Ca} inhibitors on the EDHF-mediated relaxation

In view of the published evidence that activation of K_{Ca} channels plays an important role in the regulation of EDHF-type relaxation (2, 7, 8), we examined the part played by such channels in the present EDHF-mediated relaxation. To that end, rings were incubated with various K_{Ca} channel inhibitors together with L-NNA plus indomethacin for 30 min before administration of PE.

To examine the part played by the SK_{Ca} channel in the present EDHF-mediated relaxation, rings were incubated with apamin, an SK_{Ca}-channel inhibitor. The ACh (10^{-9} – 10^{-5} M)–induced concentration-dependent relaxation was significantly weaker in apamin-treated rings than in apamin-untreated rings if the rings were from losartan-treated (Fig. 2C) or -untreated (Fig. 2A) Wistar rats, or from losartan-treated GK rats (Fig. 2D), but not if they were from losartan-untreated GK rats (Fig. 2B).

To examine the part played by the IK_{Ca} channel in the present EDHF-mediated relaxation, rings were incubated with TRAM-34, an IK_{Ca}-channel inhibitor. ACh (10^{-9} – 10^{-5} M) induced a reduced concentration-dependent re-
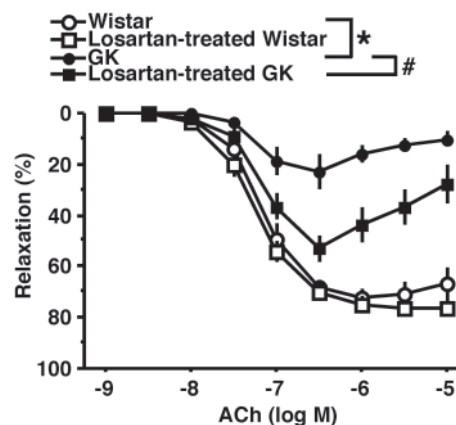

Fig. 1. Concentration–response curves for the EDHF-type relaxation evoked by ACh in isolated rings of mesenteric arteries obtained from losartan-treated and -untreated Wistar and GK rats. Ordinate shows relaxation of mesenteric arteries as a percentage of the contraction induced by PE. In this experiment, L-NNA (100 μM) + indomethacin (10 μM) was applied 30 min before the PE application and was present thereafter. Each data point represents the mean ± S.E.M. from 12 experiments; the S.E.M. is included only when it exceeds the dimension of the symbol used. *$P <$ 0.05, GK vs. Wistar. #$P <$ 0.05, losartan-treated GK vs. GK. The dose–response curves for the ACh-induced EDHF-type relaxation depicted in Fig. 1 are shown again as controls in Figs. 2 – 4.

laxation in such rings if they were from losartan-treated (Fig. 3C) or -untreated (Fig. 3A) Wistar rats or from losartan-treated GK rats (Fig. 3D), but not if they were from losartan-untreated GK rats (Fig. 3B).

A surprising result was obtained when we examined the part played by the BK_{Ca} channel in the present EDHF-mediated relaxation, using rings incubated with iberiotoxin, a BK_{Ca}-channel inhibitor. Although the ACh-induced EDHF-type relaxation was not significantly different between iberiotoxin-treated and -untreated mesenteric rings obtained from Wistar (Fig. 4A), GK (Fig. 4B), or losartan-treated Wistar (Fig. 4C) rats, it was significantly suppressed by iberiotoxin in losartan-treated GK rats (Fig. 4D).
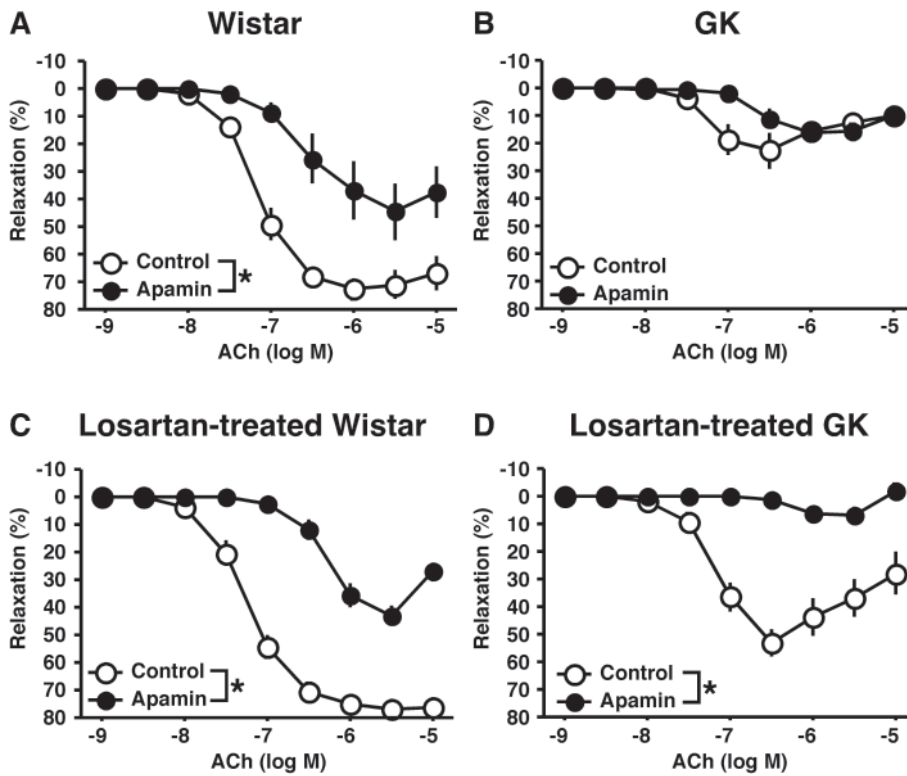
Fig. 2. Effects of the SK_{Ca} channel inhibitor apamin on the EDHF-type relaxations evoked by ACh in mesenteric arteries obtained from losartan-treated and -untreated Wistar and GK rats. In each experiment, a combination of L-NNA (100 μM), indomethacin (10 μM), and apamin (100 nM) was applied 30 min before PE application, and was present thereafter. Data are means ± S.E.M. from n = 6 (apamin-treated group) or 12 (control group) experiments. *P < 0.05 apamin vs. control.
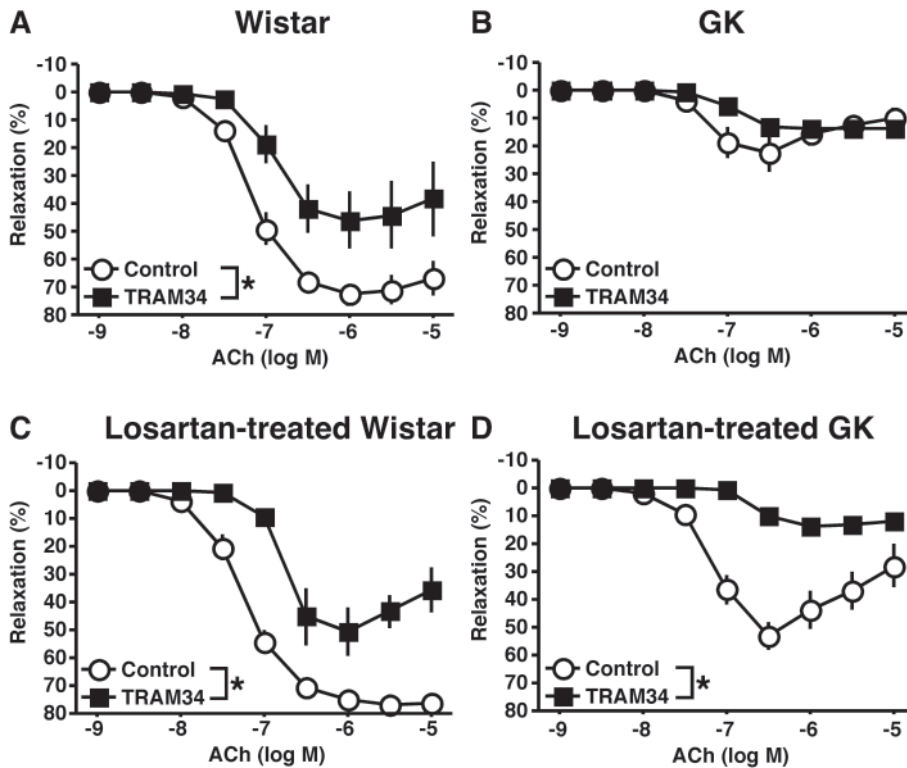

Fig. 3. Effects of the IK_{Ca} channel inhibitor TRAM-34 on the EDHF-type relaxations evoked by ACh in mesenteric arteries obtained from losartan-treated and -untreated Wistar and GK rats. In each experiment, a combination of L-NNA (100 μM), indomethacin (10 μM), and TRAM-34 (10 μM) was applied 30 min before PE application and was present thereafter. Data are means ± S.E.M. from n = 6 (TRAM-34–treated group) or 12 (control group) experiments. *P < 0.05 TRAM-34 vs. control.
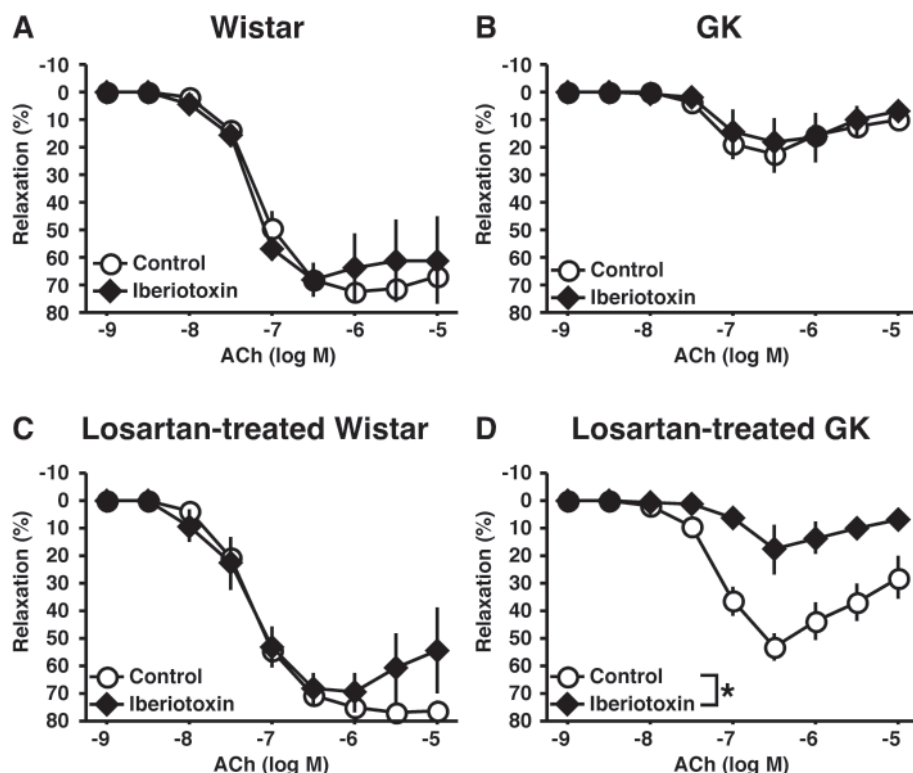
Fig. 4. Effects of the BK_{Ca} channel inhibitor iberiotoxin on the EDHF-type relaxations evoked by ACh in mesenteric arteries obtained from losartan-treated and -untreated Wistar and GK rats. In each experiment, a combination of L-NNA (100 μM), indomethacin (10 μM), and iberiotoxin (100 nM) was applied 30 min before PE application and was present thereafter. Data are means ± S.E.M. from n = 6 (iberiotoxin-treated group) or 12 (control group) experiments. *P < 0.05 iberiotoxin vs. control.

Relaxations induced by K^+-channel activators

To investigate the involvement of K_{Ca} channels or K_{ATP} channels in EDHF-type relaxation in the rat mesenteric artery, we performed a series of experiments in which NS309 (10^{-8} – 10^{-5} M, an activator of SK_{Ca}/IK_{Ca} channels), NS1619 (10^{-7} – $10^{-4.5}$ M, an activator of BK_{Ca} channels), or cromakalim (10^{-8} – 10^{-5} M, an activator of K_{ATP} channels) was added cumulatively to rings pre-contracted by PE in the presence of 100 μM L-NNA plus 10 μM indomethacin (Fig. 5, Table 2). The relaxation response induced by 3 μM NS309 was significantly weaker in the GK group than in the Wistar group (Fig. 5A), and the EC_{50} value for NS309 was significantly larger for GK than for Wistar (Table 2). Likewise, the relaxation response induced by 10 μM NS1619 was significantly weaker in GK than in Wistar (Fig. 5B), and the EC_{50} value for NS1619 was significantly larger in GK than in Wistar (Table 2). The reduced responses induced by these two K_{Ca} channel activators in the GK group were significantly improved by two-week treatment with losartan (Fig. 5: A and B, Table 2). On the other hand, the relaxation response induced by cromakalim was not significantly different among the four groups (Fig. 5C, Table 2).

Discussion

The main conclusion to be drawn from the present study is that in GK rats at the chronic stage of type 2 diabetes, two-week treatment with losartan normalizes the EDHF-mediated relaxation at least partly by increasing the activities of K_{Ca} channels.

One feature of type 2 diabetes is an endothelial dysfunction related to reduced NO signaling (11, 14). However, only a small number of observational studies have reported impairments of the EDHF pathway in animal models of this condition. For instance, one laboratory demonstrated that the overall relaxation to ACh shown by sciatic nerve epineurial arterioles was impaired in diabetic ZDF rats and in a subsequent study that an ACh-induced relaxation mediated by the EDHF pathway was absent from these arteries (44). In addition, the mesenteric artery relaxation elicited by ACh-induced activation of the EDHF pathway has been shown to be impaired in fructose-fed, insulin-resistant rats (45), in type 2 diabetic Otsuka Long-Evans Tokushima Fatty rats (37 – 40), and in ZDF rats (46). Oniki et al. (47) demonstrated that the EDHF-mediated hyperpolarization and relaxation were impaired in mesenteric arteries obtained from type 2 diabetic GK rats at 18 weeks of age (an earlier stage than that studied here). However, no previous study has investigated EDHF-mediated relaxation in such rats at the *chronic* stage of diabetes.

A novel, intriguing, and potentially important finding made in this study was that the partial restoration of the EDHF-mediated relaxation that was seen in mesenteric
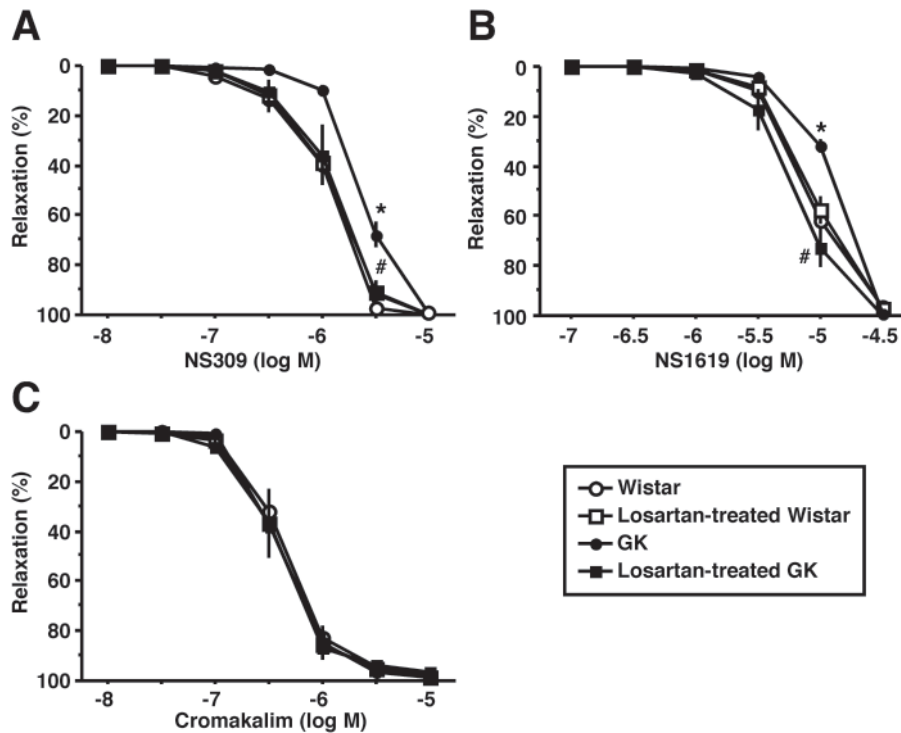
Fig. 5. Concentration–response curves for the effects of K⁺ channel activation in mesenteric arteries from losartan-treated and -untreated Wistar and GK rats. Relaxations were induced by an SK_{Ca}/IK_{Ca}-channel activator (NS309, $10^{-8} - 10^{-5}$ M) (A), a BK_{Ca}-channel activator (NS1619, $10^{-7} - 10^{-4.5}$ M) (B), or a K_{ATP}-channel activator (cromakalim, $10^{-8} - 10^{-5}$ M) (C), in each case in the presence of L-NNA (100 μM) + indomethacin (10 μM). Data are means ± S.E.M. from n = 7 (A, B) or 8 (C) experiments. *$P < 0.05$, vs. Wistar. #$P < 0.05$, vs. GK.

Table 2. EC_{50} values for K⁺ channel activators-induced relaxations of mesenteric rings from losartan-treated and -untreated Wistar and GK rats

EC_{50} (μM)	Wistar	Losartan-treated Wistar	GK	Losartan-treated GK
NS309	1.15 ± 0.14 (7)	1.25 ± 0.22 (7)	2.30 ± 0.23 (7)*	1.27 ± 0.17 (7)#
NS1619	7.67 ± 0.96 (7)	8.30 ± 0.99 (7)	13.40 ± 0.33 (7)*	6.83 ± 1.17 (7)#
Cromakalim	0.50 ± 0.09 (8)	0.47 ± 0.08 (8)	0.46 ± 0.10 (8)	0.44 ± 0.08 (8)

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

arteries from losartan-treated GK rats was associated with evidence of a marked change in the roles played by K_{Ca} channels in these arteries.

First, we need to discuss SK_{Ca} and IK_{Ca} channels. Activation of these channels in the arterial endothelium is a key step leading to smooth muscle hyperpolarization and relaxation, independently of both NO and prostacyclin (2, 6). Activation of endothelial SK_{Ca} and IK_{Ca} channels is also of crucial importance in the initiation of the EDHF signal following agonist stimulation (2, 7, 8). Consequently, a defective EDHF-mediated relaxation, as well as an impaired endothelial hyperpolarization–mediated relaxation, would be expected to be caused by any significant loss of these hyperpolarizing K_{Ca} channels from the endothelium. Indeed, in mice, modulation of the expression of endothelial SK3 and/or IK1 channels affects the arterial tone of the isolated mesenteric artery, the diameter of these arteries in situ, and the blood pressure of

the animal (48). A decreased expression of the SK3 channel would be expected to cause adverse effects (3). Indeed, Kohler et al. (49) demonstrated that experimental chronic renal failure leads to a loss of EDHF-type relaxation that is caused at least in part by an impaired functional expression of the endothelial hyperpolarizing K_{Ca} channels. Later, Burnham et al. (46) demonstrated that the EDHF pathway, especially the SK_{Ca}-mediated one, is impaired in type 2 diabetic ZDF rats without a reduction in channel gene expression. In the present study, we found that 1) the ACh-induced EDHF-mediated relaxation was significantly reduced following apamin or TRAM-34 treatment in mesenteric arteries from Wistar rats, but not in those from GK rats, and 2) the relaxation induced by NS309 was significantly impaired in GK rats. These results suggest that in type 2 diabetic mesenteric arteries the activities of certain endothelial K_{Ca} channels (i.e., SK_{Ca} and IK_{Ca}) are impaired and that the contribu-

tions made by these channels to the EDHF-mediated response are reduced. Moreover, we found that in rings from losartan-treated GK rats, but not in those from losartan-untreated GK rats, the ACh-induced EDHF-mediated relaxation was significantly reduced by the above two specific inhibitors of SK_{Ca} or IK_{Ca} and also that the NS309-induced relaxation was normalized by losartan treatment in GK rats. These results strongly suggest that in GK rats, losartan normalizes the EDHF-mediated relaxation at least partly by restoring the activities of SK_{Ca} and/or IK_{Ca} channels.

Now, we need to discuss BK_{Ca} channels. These channels are expressed in mesenteric arteries (50), and this channel activity has been shown to be impaired in the mesenteric microvessels of insulin-resistant rats (51) as well as in the coronary arterial myocytes of diabetic fatty rats (52). In the present long-term type 2 diabetic model, a reduced relaxation response to the synthetic BK_{Ca}-channel opener NS1619 was observed in the mesenteric artery. This impaired relaxation in GK mesenteric arteries was significantly normalized by treatment with losartan. These results suggest that although the relaxation induced by activation of BK_{Ca} channels is impaired in mesenteric arteries from GK rats, it can be improved by losartan treatment. Previous studies have suggested that activation of BK_{Ca} contributes to EDHF-mediated signaling (5, 8). Here, we found that although the ACh-induced EDHF-mediated relaxation was not affected by iberiotoxin in the losartan-treated Wistar, losartan-untreated Wistar, or losartan-untreated GK groups, it was inhibited by iberiotoxin in the losartan-treated GK group. These results suggest that opening of the BK_{Ca} channel plays a minor or no role in EDHF-type vascular relaxation in the normal mesenteric artery, but interestingly it does make such a contribution in the losartan-treated diabetic GK mesenteric artery. What mechanisms might underlie the emergence of a BK_{Ca}-related component of the EDHF-type relaxation in losartan-treated diabetic GK rats? Several reports have suggested that diffusible EDHFs such as NO, epoxyeicosatrienoic acids, H_2O_2, and C-type natriuretic peptide can activate BK_{Ca} channels in various arteries (2 – 5). In our recent study on GK rats, two-week losartan treatment, at the dosage used here, normalized plasma NO metabolism and increased the basal level of NO formation in the mesenteric artery (35). Although we did not investigate the possible involvement of these diffusible EDHFs in ACh-induced EDHF-type relaxation in mesenteric arteries from losartan-treated GK rats, we speculate that the residual NO synthesis that persists after an apparent block of NOS might contribute to the ACh-mediated EDHF-mediated relaxation via activation of BK_{Ca} in smooth muscle cells. Another mechanism by which the increased BK_{Ca} channel activity might

be achieved is activation of the AT_2 receptor. Indeed, Dimitropoulou et al. (53) demonstrated that Ang II could induce vasodilation by opening BK_{Ca} channels via stimulation of the AT_2 receptor. Our recent study (35) demonstrated that production of Ang II was increased in GK mesenteric arteries and that this was not affected by two-week losartan treatment. A possible scenario is therefore that in GK mesenteric arteries, the BK_{Ca} channel is activated, via the AT_2 receptor, by an elevated level of Ang II. However, the vasodilator effect of this activation may be evident only when it is unmasked by a sustained blockade of the AT_1 receptor by an agent such as losartan. At present, the notion that the above phenomenon exists is speculative, and the extent to which such an effect might have contributed to the improvement of the EDHF-mediated response seen in mesenteric arteries from losartan-treated GK rats remains unclear because the levels of the channel proteins were not determined. This was because insufficient materials could be obtained to allow mRNA and protein studies to be performed alongside the functional studies. Nevertheless, our results suggest that the impaired EDHF-mediated relaxation seen in mesenteric arteries from established type 2 diabetic GK rats is improved by chronic losartan treatment through normalization effects on the activities of SK_{Ca} and/or IK_{Ca} channels, together with an enhancement effect on the activity of the BK_{Ca} channel.

In the present study, cromakalim, a K_{ATP}-channel activator, induced a relaxation in the presence of L-NNA and indomethacin that did not differ between mesenteric arteries from Wistar and GK rats and was unaffected by two-week losartan treatment. On the other hand, in the seminal study of Oniki et al. (47), on mesenteric artery rings precontracted with 10 μM norepinephrine, the concentration–response curve obtained for levcromakalim was shifted to the right, without a change in the maximal relaxation, in rings obtained from GK rats vs. those from Wistar rats at 18 weeks of age. Possibly, the discrepancy between the two studies may be explained by differences between the agonist used to precontract the rings [10 μM norepinephrine (47) vs. 100 – 200 nM PE (the present study)], by the absence (47) vs. the presence (the present study) of L-NNA plus indomethacin, or by the difference between the duration of the diabetes. Future experiments will need to address these issues. Moreover, a limitation of the present study should be mentioned. Although EDHF signaling is mediated by membrane hyperpolarization in vascular smooth muscle cells, this study did not seek to evaluate the hyperpolarization in mesenteric arteries using the electrophysiological technique. In contrast, Oniki et al. (47) did evaluate EDHF-mediated hyperpolarization. They demonstrated that it was weaker in mesenteric arteries from GK rats at

18 weeks of age than in those from age-matched Wistar rats. Although some reports have suggested that the presence of a reduced EDHF-mediated hyperpolarization in diabetic arteries is associated with an impaired relaxation (19, 47), future experiments will be required to confirm or deny this association.

Although the mechanism underlying the impairment of the EDHF-mediated response seen in type 2 diabetic states remains unclear, Ang II needs to be considered as a causal factor. In mesenteric arteries obtained from spontaneously hypertensive rats (SHRs), both a markedly reduced EDHF-mediated relaxation and a reduced EDHF-mediated hyperpolarization have been observed, by comparison with those in arteries from age-matched normotensive Wistar-Kyoto rats (54). The finding that these responses could be restored by an ACEI, by an ARB, or by a combination of the two has been taken as an indication of the negative influence of the RAS on EDHF-mediated responses (54, 55). Indeed, in a very recent study, Dal-Ros et al. (56) demonstrated in the rat that Ang II–induced hypertension is associated with selective impairments of EDHF-mediated relaxation and hyperpolarization in the mesenteric artery. In the present study, two-week treatment with losartan improved the EDHF-mediated relaxation in mesenteric arteries from GK rats even though the treatment was begun when the rats were already in the established phase of type 2 diabetes (i.e., at 32 – 36-week-old). Furthermore, in our GK diabetic model, Ang II levels are significantly elevated in mesenteric arteries (35). To judge from the above evidence and the present findings, a suppression of Ang II signaling might have potential not only as a way of preventing the diabetes-related deterioration in EDHF-mediated relaxation, but also as a therapeutic strategy for EDHF-mediated responses in established type 2 diabetic states.

Previous studies have suggested that impairments of EDHF-mediated responses are present in disease states, indicating the potential for therapeutic interventions (3, 4). For example, chronic treatment with an ACEI, with an ARB, or with a diuretic can normalize EDHF-mediated responses in SHRs (3). In addition, dietary supplements and exercise have beneficial effects on EDHF responses (3). However, the mechanisms underlying these drug-induced and adjuvant-induced improvements in EDHF responses remain poorly understood. EDHF-mediated responses are clearly affected in a variety of pathological conditions, and use of the above therapeutic or adjuvant interventions to restore EDHF-mediated responses might be beneficial for the patient(s). Especially intriguing is the possibility that because EDHF plays important roles in the microvasculature, an enhancement of EDHF-mediated responses might contribute to improvements in diabetic microvascular complications, such as those involved in retinopathy, nephropathy, and neuropathy. Although losartan is already used clinically in several cardiovascular diseases (29, 30), the present study provides evidence of its potential as a therapeutic drug for the improvement of EDHF-mediated responses in non-hypertensive type 2 diabetic states.

In conclusion, our study suggests that losartan improves EDHF-mediated responses in GK rat mesenteric arteries by enhancing certain K_{Ca}-channel activities. We believe that our findings should stimulate further interest in losartan as a potential therapeutic drug for use against diabetes-associated vasculopathy.

Acknowledgments

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