Dysfunction of Neurotransmitter Modulation System on Adrenergic Nerves of Caudal Artery in Type 2 Diabetic Goto–Kakizaki Rats

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The Goto–Kakizaki (GK) rat is a non-obese and spontaneous model of mild Type 2 diabetes mellitus. In the present study, we compared the regulatory mechanisms of endogenous norepinephrine (NE) release from sympathetic nerves of caudal arteries of 12-week-old GK rats and age-matched normal Wistar rats. Electrical stimulation (ES) evoked significant NE release from caudal arteries of Wistar and GK rats. The amounts of NE released by ES were almost equal in Wistar and GK rats, although the NE content in caudal artery of GK rats was significantly lower than that of Wistar rats. We examined the effects of an α2-adrenoceptor agonist, clenodine (CLO), and an α2-adrenoceptor antagonist, yohimbine (YOH), on the release of endogenous NE evoked by ES. CLO significantly reduced NE release from caudal arteries of Wistar but not GK rats. On the other hand, YOH significantly increased NE release from both rats. Furthermore, we examined the effects of an A1-adenosine receptor agonist, 2-chloroadenosine (2CA), and an A1-adenosine receptor antagonist, 8-sulfophenyltheophylline (8SPT), on the release of endogenous NE evoked by ES. 2CA significantly reduced NE release from caudal arteries of Wistar but not GK rats. On the other hand, 8SPT did not affect NE release from both rats. These results suggest that the dysfunction of negative feedback regulation of NE release via presynaptic receptors on sympathetic nerves in GK rats may be involved in the autonomic nervous system dysfunction associated with diabetic autonomic neuropathy.

Key words: adrenergic nerve; diabetes mellitus; autonomic neuropathy; norepinephrine release; presynaptic receptor; rat caudal artery

Neuropathies of the central and peripheral nervous systems are known to be caused by hyperglycemia, a consequence of the deregulation of glucose in diabetes. Diabetic neuropathy is a painful complication involving progressive neuronal damage and dysfunction. Some reports indicate the appearance of peripheral neuropathy in a diabetic animal model, which affects the sensory nerves, the autonomic nervous system and even the central nervous system. Autonomic dysfunction is a particularly significant complication of diabetes that may be responsible for orthostatic hypotension, skin ulceration, arterial calcification and abnormal temperature regulation. However, most animal models of diabetic neuropathy are Type 1 diabetic models, namely, streptozotocin (STZ)-induced diabetic rats and mice.

Goto–Kakizaki (GK) rats were established as a model of inbred type 2 diabetes mellitus by selective breeding from originally non-diabetic Wistar rats using glucose intolerance. GK rats have been shown to develop Type 2 diabetes without obesity spontaneously and to exhibit higher post-prandial plasma glucose levels and lower post-prandial immunoreactive insulin levels than Wistar rats. GK rats are particularly relevant to human Type 2 diabetes because the pathogenesis of GK rats includes peripheral insulin resistance, hyperinsulinemia, hyperglycemia and glucose intolerance; these symptoms are seen as early as 4 weeks after birth. Therefore, this is a very useful model with relevance to the pathophysiology of the vasculature in human diabetes.

Yagihashi et al. reported slowing of motor nerve conduction velocity in the 2-month-old GK rats. On the other hand, Murakawa et al. reported that the 2-month-old GK rats showed a normal motor nerve conduction velocity, whereas in the 18-month-old GK rats it was reduced. Furthermore, no morphometric abnormalities were found in the 2-month-old GK rats, whereas the 18-month-old GK rats showed loss of small myelinated fibers. Although there are reports about motor nerve abnormalities in GK rat, there is still no report about an autonomic neuropathy. The progressive degeneration of the nerves of the somatic nervous system with increasing age has been reported in GK rats. However, the mechanisms underlying the development of autonomic neuropathy in GK rats remain unknown.

In the present study, therefore, we examined whether the dysfunction of the sympathetic neuroeffector mechanism is involved in Type 2 diabetic autonomic neuropathies using measurement of the content and release of endogenous norepinephrine (NE) in the caudal arteries of 12-week-old GK rats and age-matched normal Wistar rats.

MATERIALS AND METHODS

The rats used in the present study were obtained, maintained and sacrificed in accordance with the procedures outlined in the Guidelines for Animal Experimentation of Meiji Pharmaceutical University, which was compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Sciences. As previously reported, fasting levels of serum glucose in 12-week-old GK rats were significantly higher than in age-matched Wistar rats (193 ± 12 (n = 14) vs. 126 ± 5 mg/dL (n = 14), respectively), but serum insulin levels were significantly lower in GK rats than in Wistar rats (725 ± 67 (n = 6) vs. 2227 ± 301 pg/mL (n = 8), respectively). Rats (11 to 13 weeks old) were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneal (i.p.)) and exsanguinated. As long a segment as possible (generally

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about 12 cm in length, which averages 18 mg in wet weight) of the caudal artery was removed, cleaned of connective tissue and suspended in a water-jacketed organ chamber containing 3.0 mL of a modified Krebs solution at 37°C. The composition of the Krebs solution was as follows (mmol/L): NaCl 110; KCl 4.6; CaCl₂ 2.5; NaHCO₃ 24.8; KH₂PO₄ 1.2; MgSO₄ 1.2; glucose 5.6. The solution was continuously aerated with 95% O₂ and 5% CO₂. Each artery was passed back and forth several times through a pair of platinum ring electrodes and fixed. The electrodes were positioned at a distance of approximately 1.2 cm.

The measurement of norepinephrine (NE) released by electrical stimulation (ES) from caudal arteries was achieved as previously described. Briefly, after a 60-min equilibration period, ES of the tissue was evoked by square-wave pulses of 0.5 ms duration, 50 V and a frequency of 1 Hz from a SEN-7103 stimulator (Nihon Kohden, Tokyo, Japan). After tissues were stimulated for 3 min, the bathing solution was rapidly removed by draining the organ chamber and the sample was processed for the determination of NE by HPLC-electrochemical detection (ECD) techniques as described in detail previously. A 3-min pre-stimulation sample was also collected. Tissues were stimulated twice at 30 min intervals. These stimulation periods were designated S1 and S2. The influence of drugs on the ES-evoked release of NE was assessed by exposing the tissue to clonidine (CLO) and 2-chloroadenosine (2CA) for 2 min and to yohimbine (YOH) and 8-sulfophenyltheophylline (8SPT) for 15 min before and during S2. At the end of these experiments, the NE content of the arteries was determined. Arterial preparations were homogenized in 0.4 M HClO₄ containing 1.3 mmol Na₂ ethylenediaminetetraacetic acid (EDTA) and 5.3 mmol Na₂SO₄ for deproteinization. Catecholamines in the acidified samples were isolated using batch alumina chromatography and analyzed using HPLC-ECD.

The amount of NE present in each sample was calculated by using peak area ratios relative to the internal standard 3,4-dihydroxybenzylamine (DHBA) as described by Yamamoto and Cline. In addition to the stimulation samples, 3-min pre-stimulation samples were also measured for calculation of spontaneous NE outflow. NE release, which is the difference between the amount of NE in the stimulation and pre-stimulation samples, is expressed as fmol/mg wet weight of tissue. Data pertaining to the modification of release by drugs are presented as the ratio of S2 to S1 determined in the absence (control) or presence of drugs (S2).

2-Chloroadenosine, clonidine hydrochloride and norepinephrine bitartrate salt were all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DHBA was obtained from Aldrich Chem. Co. (Milwaukee, WI, U.S.A.). Yohimbine hydrochloride was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 8-Sulfophenyltheophylline was obtained from Research Biochemical Inc. (Natick, MA, U.S.A.).

All values are presented as the mean±S.E. of at least four individual experiments. Simple comparisons of the mean and S.E. of data were performed using Student’s t-test. Post hoc multiple comparisons were made by using Turkey’s test. The 0.05 level of probability was accepted as significant. These statistical analyses were performed using a computer program (JMP 9, SAS Institute Inc., NC, U.S.A.).

**RESULTS**

**NE Release from Caudal Arteries** After the equilibration period, caudal arteries were electrically stimulated twice for 3 min at 30 min intervals. Figure 1 shows the release of NE evoked by ES at 1 Hz from Wistar and GK rats. The spontaneous outflow of NE remained approximately constant throughout the experiment (see pre-stimulation values in Fig. 1), and ES significantly increased the overflow of NE from caudal arteries of Wistar and GK rats (Fig. 1). The release of NE at each stimulation period was relatively constant in Wistar rats (Fig. 1a). However, the release of NE at the second stimulation period (S2) was significantly reduced in GK rats compared with that in the first stimulation period (S1) (Fig. 1b). The S2/S1 ratios of Wistar and GK rats were 0.984±0.092 and 0.765±0.069, respectively (Fig. 1c). The ratio of GK rats was significantly smaller than that of Wistar rats (p<0.05).

Table 1 shows the NE content in caudal arterial strips and ES-induced NE release from caudal arteries of Wistar and GK rats. Percentage release of NE from caudal arteries of GK rats was significantly greater than that of Wistar rats, although the NE content in caudal artery of GK rats was significantly lower than that of Wistar rats (Table 1).

**Effects of CLO and YOH on NE Release** Figure 2 shows the effects of an α₂-adrenoceptor agonist, CLO (10 µM), and an α₂-adrenoceptor antagonist, YOH (1 µM), on the release of NE evoked by ES (1 Hz) from caudal arteries of Wistar and GK rats. CLO significantly reduced the ES-evoked NE release from Wistar but not GK rats, without change of the spontaneous overflow of NE. On the other hand, YOH significantly increased the ES-evoked NE release from Wistar and GK rats by 1.7 and 1.8 times, respectively, without change of the spontaneous overflow of NE. Furthermore, CLO reduced the...
increase of NE release from Wistar rats in the presence of YOH by 50.2%, but that from GK rats by 35.1%.

**Effects of 2CA and 8SPT on NE Release** Figure 3 shows the effects of an A1-adenosine receptor agonist, 2CA (10 μM), and an A1-adenosine receptor antagonist, 8SPT (30 μM), on the release of NE evoked by ES (1 Hz) from caudal arteries of Wistar and GK rats. 2CA reduced the ES-evoked NE release from Wistar rats without change of the spontaneous overflow of NE. However, 2CA did not significantly affect the NE release from GK rats. On the other hand, 8SPT did not significantly affect the ES-evoked NE release from both rats in the absence of 2CA without change of the spontaneous overflow of NE. However, 8SPT prevented the decrease in NE release produced by 2CA in both rats.

**DISCUSSION**

In this study, we demonstrate novel findings concerning the function of sympathetic innervation of the caudal artery of GK rats.

Although the NE content in the caudal artery of GK rats was significantly lower than that of Wistar rats, the amount of ES-induced NE release in GK rats was almost equal to that in Wistar rats. Therefore, percentage release of NE from GK rats was significantly greater than that from Wistar rats. Moreover, the ratio (S2/S1) of the amount of NE release was significantly reduced in GK rats compared with that in Wistar rats. These results suggest that the rate of decrease of NE in the nerve endings of GK rats is greater than that of Wistar rats because of the greater amount of NE release, which may account for the dysfunction of presynaptic negative feedback regulation of NE release. Previous reports have shown the reduction of NE content and stimulated overflow of endogenous NE in caudal arteries from STZ-induced diabetic rats, which reflects reduced innervation density of adrenergic nerves and/or inability to store NE. It seems that this may be responsible for the reduction of not only NE synthesis but also reuptake of NE, suggesting dysfunction of prejunctional neurotransmitter regulatory mechanisms in GK rats. The extracellular concentration of the transmitter NE, which is the primary transmitter released from sympathetic post-ganglionic nerve terminals, depends dynamically on the rate of its release and clearance.

Although the content of NE of caudal arteries in GK rats was significantly lower than that in Wistar rats, the content of NE in blood vessels is generally consistent with sympathetic innervation. The rat caudal artery is a densely innervated artery. Therefore, these results suggest that the activity of sympathetic nerves and/or sympathetic innervation density may be reduced in GK rats. Furthermore, the present results would be consistent with the suggestion that the reduction of tissue concentrations of NE leads to diabetic autonomic neuropathy. Schmidt et al. demonstrated that a deficiency in adrenergic innervation was induced in colon and spleen of chronically diabetic rats induced by STZ. Moreover, Addicks et al. have shown a decreased number of sympathetic nerve endings and innervation density in diabetic tissue, including spontaneous BB rat atria. However, we did not directly investigate the changes in the numbers of nerve fibers in caudal arteries from GK rats in the present study. Further studies are required to determine the sympathetic nerve density in caudal arteries of GK rats.

ES increased NE overflow from caudal arteries of Wistar and GK rats and the amount of ES-induced NE release in GK rats was almost equal to that in Wistar rats. The amount of exocytotically released neurotransmitter is regulated by prejunctional receptors located at or near the axon terminal. In a wide variety of tissues, α2-adrenoceptors, which are classified as metabotropic autoreceptors and mediate the inhibition of NE release, are located on adrenergic nerve terminals. We examined the effects of an α2-adrenoceptor agonist, CLO, and an α2-adrenoceptor antagonist, YOH, on
the release of endogenous NE evoked by ES in caudal arteries of Wistar and GK rats. It is well known that CLO inhibits the transmitter release and YOH increases the transmitter release via presynaptic α2-adrenoceptors.24,25 We found that α2-adrenoceptor stimulation by CLO significantly reduced NE release from caudal arteries of Wistar rats, but not GK rats. On the other hand, α2-adrenoceptor blockade by YOH increased NE release from caudal arteries of Wistar and GK rats, and these effects were inhibited to the control level by CLO. However, the inhibitory effect by CLO on the increase of the NE release by YOH in GK rats was smaller than that in Wistar rats. These results suggest that the negative feedback regulation of transmitter release through autoreceptors was blunted in the caudal arteries of GK rats. Burgdorf et al. also showed the impairment of the functional α2-adrenoceptors on NE release in failing hearts from Zucker diabetic fatty rats.26 However, the increase of NE release by YOH in Wistar rats was not significantly different from that in GK rats. These results may be due to the reduction of α2-adrenergic receptor affinity for CLO, but not YOH. Dunbar et al. suggested that the α2-adrenergic receptor affinity for CLO had been reduced by nucleus tractus solitarius of STZ-induced diabetic rats.27 Furthermore, Drukarck et al. indicated that stimulation of α2-adrenoceptors led to a significantly smaller inhibition of radiolabeled NE release from the vas deferens of STZ-diabetic rats, concluding that these effects could be due to a decrease in the number of presynaptic α2-adrenoceptors in the vas deferens of diabetic rats.28 However, there are no actual reports showing such a decrease in the number or the affinity of only presynaptic α2-adrenoceptors. Moreover, there may be also a possibility that presynaptic α2-adrenoceptor mediated intracellular signal transduction system is impaired. Instead, there are some reports about adrenoceptor mRNA expression in endothelial cells and vascular smooth muscle cells in diabetes. Kobayashi et al. showed increased α2D-adrenoceptor mRNA expression in GK rat aorta endothelium.29 On the other hand, Mita et al. found no significant differences in the mRNA expression of α2-adrenoceptor subtypes between Wistar and GK rat caudal arterial smooth muscle.30 Although further studies are required, it is very difficult to quantify the number or the affinity of only presynaptic α2-adrenoceptors.

It is well known that purinoceptors, which are classified as metabotropic heteroreceptors,23 can regulate NE release from adrenergic nerve terminals in addition to α2-adrenoceptors. Studies with a large number of adrenergic neuroeffector tissues, both vascular and nonvascular, indicated that prejunctional adenosine receptors mediate the inhibition of NE release.30 Therefore, we examined the effects of an A1-adenosine receptor agonist, 2CA, and an A2-adenosine receptortagonist, 8SPT, on the release of endogenous NE evoked by ES in Wistar and GK rats. The synthetic purine 2CA is a particularly effective A2-adenosine receptor agonist. In the present study, 2CA significantly reduced NE release from caudal arteries of Wistar rats and this attenuation was prevented by 8SPT. These results are consistent with a previous report that 2CA reduced the ES-evoked release of NE from caudal arteries of Wistar-Kyoto (WKY) rats.15 However, 2CA did not significantly alter the release of NE from caudal artery of GK rats. These results indicate that prejunctional regulation of sympathetic transmitter release by adenosine is also attenuated in the caudal artery of GK rats. On the other hand, 8SPT itself had no effect on ES-evoked NE release from caudal arteries of Wistar and GK rats. Shinozuka et al. reported a similar result showing that 8SPT itself did not affect the EFS-evoked release of NE from caudal artery in Fisher-344 rats.31 Presynaptic A1-adenosine receptor is a heteroreceptor, and activation of presynaptic A1 receptors requires neuronal activity or a hypoxic condition.27 Thus, it seems that 8SPT itself does not alter transmitter release by physiological stimulation.

In summary, we indicated that presynaptic regulation via α2-adrenoceptors and A1-adenosine receptors was attenuated in GK rats. Therefore, the dysfunction of prejunctional neurotransmitter regulatory mechanisms may be responsible for the development of diabetic autonomic neuropathy. This is characterized by a reduction in the content of endogenous NE and impairment of negative feedback regulation of NE release via presynaptic receptors. Dysfunction of the autonomic nervous system is a common complication of diabetes, and functional changes in the sympathetic nervous system have been reported in several animal models of diabetes as well as in human diabetic subjects.32 Although some reports indicated changes in neurogenic contraction of caudal artery in diabetic rats,33,34 direct measurement of NE release evoked by ES from diabetic vessels has not been reported. The present study provides the first evidence in arteries that dysfunction of negative feedback regulation of NE release via presynaptic receptors in Type 2 diabetes may be attributable to the autonomic nervous system dysfunction associated with diabetic complications.

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