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ORIGINAL ARTICLE

MODIFIED PARASYMPATHETIC NERVOUS SYSTEM IN GOTO-KAKIZAKI DIABETIC RATS

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Abstract Persistent hyperglycemia is implicated in the pathogenesis of chronic diabetic complications, such as neuropathy. Therefore, we investigated Electrocardiogram (ECG)-related changes, including various ECG parameters and heart rate variability, and neuropathic changes in diabetic Goto-Kakizaki (GK) rats, a spontaneous model for type 2 diabetes. GK rats (50 weeks old) showed increased plasma glucose and decreased R-R interval variability, which was likely due to diabetic polyneuropathy in the autonomic nervous system. Interestingly, GK rats showed a decreased baroreflex compared with control Wistar rats. Furthermore, GK rats showed limited fluctuation changes in beat-to-beat dynamics. In the frequency domain analysis, the low-frequency component was similar but the high-frequency component was significantly decreased in GK rats, suggesting decreased parasympathetic nerve regulation and conserved sympathetic nerve regulation. In addition, GK rats showed a limited response to high-dose atropine, a muscarinic receptor antagonist, indicating modified parasympathetic nerve regulation in GK rats. Our data strongly suggests that GK rat could be a nice animal model for chronic cardiovascular disease related to diabetic neuropathy.

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Key words: rats; ECG; parasympathetic nerve; diabetes mellitus.

Introduction

The main damage caused by diabetes mellitus (DM) is due to various complications. Among them, peripheral neuropathy is the most common[1)] and involves somatic sensory, motor, and autonomic nerves[2)]. In the United States, the prevalence of diabetic neuropathy with symptoms varies, but electrophysiological analysis has revealed that most patients have reduced electrophysiological conductance[3)]. The presence of cardiovascular autonomic neuropathy significantly reduces patients' longevity and increases mortality[2)].

Various animal models have been established to investigate the pathogenesis of type 2 diabetes. Goto-Kakizaki (GK) rats are considered to be one of the best non-obese type 2 diabetic animal models[4)], as their characteristics resembles the majority (ca. 60 %) of Japanese diabetic patients[5)]. GK rats offer valuable tools as their abnormalities are common to and functionally present in Japanese diabetic patients. This animal model is considered appropriate to inspect various

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pathologic mechanisms of type 2 diabetes[4].

GK rats have type 2 diabetic features, such as low insulin secretion, non-obesity, and modest hyperglycemia[6]. It has been shown that GK rats exhibit early thickening of the basement membrane of renal glomeruli and a reduction in motor nerve conduction velocity (MNCV)[7], suggesting that this model is valuable for characterizing diabetic neuropathy in the autonomic nervous system.

Heart rate variability (HRV) is defined as an oscillation of R-R intervals between each heart beat that occurs as a result of autonomic nervous system sympathetic and parasympathetic activity on the sinus node[8]. Meta-analyses of published data demonstrated that reduced cardiovascular autonomic function as measured by HRV is strongly associated with an increased risk of myocardial ischemia[9]. Abnormalities in HRV and QT, as measured by electrocardiogram [ECG], serve as predictors of mortality[10]. HRV measurement is a readily available method and serves to establish a diagnosis of autonomic dysfunction.

In our previous study, Wada et al. examined peripheral nerve function in GK rats[7]. Howarth et al. reported modified QT intervals and decreased HRV in GK rats, although a detailed analysis was not conducted[11]. The relationship between diabetic neuropathy and the autonomic cardiovascular system has been described; however, the details of HRV due to diabetic neuropathy in the GK rat model have not been well characterized.

In the current study, we analyzed the influence of diabetic neuropathy in cardiovascular autonomic regulation using the GK diabetic rat model of type 2 diabetes.

Material and methods

Animals

Male GK rats (CLEA Japan, Inc., Tokyo, Japan) and normal control Wistar rats were used. A standard diet and tap water were supplied ad libitum. All experimental procedures were approved by the Institutional Animal Care and Research Advisory Committee of the Hirosaki University School of Medicine. Body weight and blood glucose levels were monitored in all animals at the age of 50 weeks. Blood glucose was measured using glucose oxidase methods on a paper strip (Toe-cho Super; Kyoto-Daiichi Kagaku, Kyoto, Japan).

General anesthesia

Anesthesia was induced by placing the rats in an anesthesia induction chamber (25 × 25 × 14 cm) containing 4 % isoflurane (Forane; Abbott Japan Co. Ltd., Tokyo, Japan) and ambient air. Subsequently, anesthesia was maintained using 2 % isoflurane inhalation anesthesia (2 L/min) for ECG recordings. All experiments were conducted between 10:00 am and 4:00 pm.

Electrophysiological characterization of nerve conduction velocity

Tibial motor nerve conduction velocity (MNCV), sensory nerve conduction velocity (SNCV), and intraepidermal nerve fiber density (IENFD) were measured in all experimental animals at 50 weeks of age, as described previously[12]. IENFD was evaluated by immunofluorescence staining of protein G product 9.5 (PGP9.5) on the skin sections obtained from experimental animals at 50 weeks of age. After dissection, the footpad skin was fixed in Zamboni's solution for 24 h. Frozen sections (50-μm thick) were incubated with an antibody against PGP9.5 (Dako, Glostrup, Denmark), and then reacted with a secondary fluorescence-labeled antibody and observed by confocal laser scanning microscopy (Olympus, Tokyo, Japan). Epidermal nerve fibers across the basement membrane of the epidermis were quantified and expressed as the number per mm distance of

basement membrane.

Evaluating the ECG

ECG recordings, HR, and R-R interval were measured simultaneously (ML846 Power Lab System; AD Instruments, Dunedin, New Zealand)[13]. An M-button (MB) connector was used as the connecting electrode[13]. For the pharmacological analyses, rats were administered either propranolol (β-adrenergic blocker, 0.03–1.0 mg/kg) as a sympathetic blockade or atropine (0.03–1.0 mg/kg) as a parasympathetic blockade. To observe the baroreflex responses, carotid arteries were ligated for 30 s with 6-0 silk sutures. Detailed information is included in the Supplemental Information.

Histological examination

The hearts of male GK rats and normal control Wistar rats were excised under ether anesthesia, fixed immediately in 4 % (v/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4℃, embedded in paraffin wax, and stained with hematoxylin-eosin (H&E) periodic acid-Schiff (PAS).

Cross-sectional area (CSA) measurements were obtained from a minimum of 50 cardiac myocytes from control Wistar and GK rats. CSA measurements were obtained using 4 images from non-overlapping regions of each tissue cross-section stained with H&E. The mean fiber CSA of the respective fiber types was determined by planimetry.

Reverse transcription polymerase chain reaction (RT-PCR)

Poly(A)$^+$ RNA was isolated from cells using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) and Oligotex-dT30 (Takara, Shiga, Japan). Reverse transcription was performed using a first-strand cDNA synthesis kit (SuperScript II™ Reverse Transcriptase, Invitrogen). PCR amplification was performed using GoTaq® Green Master Mix (Promega, Madison, WI, USA). Specific sequences of β-adrenergic receptors (β1 and β2), muscarinic M2 receptor, and β-actin were amplified by PCR[14]. For the control PCR amplification, we used the following primers for β-actin (5'-ctcttccagccttccttcct-3') and β-actin-rev (5'-cttctgcatcctgtcagcaa-3'), which resulted in amplification of a single band (165 bp). Because high levels of β-actin were expected, PCR amplification was limited to 25 cycles for comparison.

Specific sequences of β-adrenergic receptors were also amplified by PCR using Beta1 forward (5'-tcttcttcacctgtttctgggcct-3') and Beta1 reverse (5'-tgcgtgattgcagtggatcgctat-3') for Rattus norvegicus β1-adrenergic receptor gene expression (368 bp), and Beta2 forward (5'-ctatcttctgcagctgccttttgg-3') and Beta2 reverse (5'-aactctgccttcaacccgctcat-3') for Rattus norvegicus β2-adrenergic receptor gene expression (328 bp). A specific sequence of muscarinic type 2 receptors was also amplified by PCR using M2 forward (5'-tgcctccgttatgaatctcc-3') and M2 reverse (5'-tccacagtcctcacccctac-3') for Rattus norvegicus M2-muscarnic receptor gene expression.

Statistical analysis

Results are expressed as means ± standard error (S.E.). Statistical significance was determined by an analysis of variance (ANOVA) followed by Dunnett's post-hoc tests; p values < 0.05 were considered to indicate significant differences.

Results

Characterization of diabetes

At 50 weeks of age, GK rats were smaller than control Wistar rats (388 ± 7.0*, 510 ± 12 g, GK and Wistar rats, respectively; *$p < 0.05$ vs. Wistar rats). GK rats showed significantly higher plasma glucose levels at fasting status

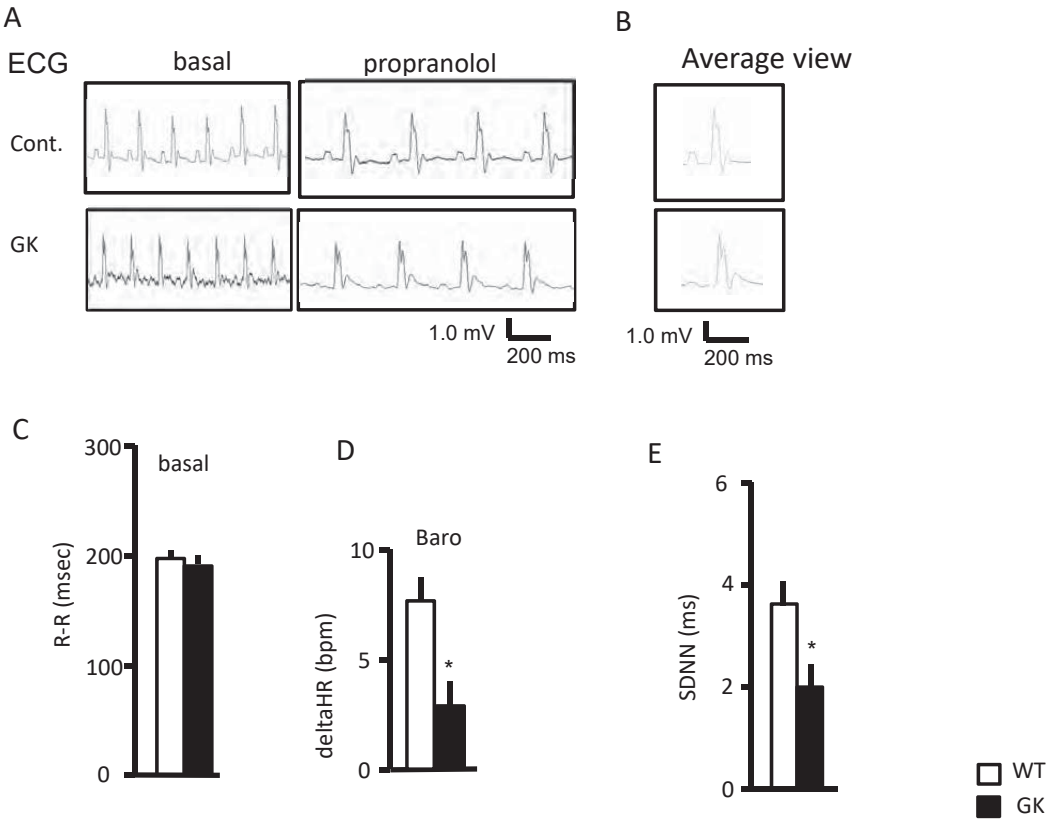

Figure 1 Decreased parasympathetic R-R regulation in the GK rat. (A) Representative ECG recordings at the basal state (left panels) and 10 min after an intraperitoneal (i.p.) propranolol injection (0.4 mg/kg body weight; right panels) of control (upper panel) and GK (lower panel) rats. (B) Computer-calculated average view of ECG waves of control (upper panel) and GK (lower panel) rats. Statistical analysis of the basal R-R interval (C), baroreflex (Baro; D), and calculated SDNN (standard deviation of the R-R interval; E). Data are expressed as the difference between baseline and each manipulation. Open bar: control (Wistar) rats; closed bar: GK rats. The physiological and pharmacological manipulations are identified above the bars. $^{*}P < 0.05$ indicates a significant difference between the control and GK rats. Each group contained at least 9 rats.

125 ± 7*, 65 ± 2 mg/dL, GK, Wistar rats, respectively, $^{*}p < 0.05$ vs. Wistar rats). GK rats showed significantly reduced MNCV (54.2 ± 0.8*, 57.2 ± 0.9 m/s, GK and Wistar rats, respectively; $^{*}p < 0.05$ vs. Wistar rats), SNCV (52.9 ± 0.7*, 55.6 ± 0.7 m/s, GK and Wistar rats, respectively; $^{*}p < 0.05$ vs. Wistar rats), and IENFD (18.2 ± 0.9*, 24.8 ± 2.1 m/s, GK and Wistar rats, respectively; $^{*}p < 0.05$ vs. Wistar rats), indicating diabetic neuropathy.

ECG analysis

We assessed ECG in the control Wistar and GK rats. The ECGs of the GK rats revealed a regular pattern indicative of physiological pacemaking and excitation propagation (Fig. 1A). ECG analysis using the "average view" program revealed a normal ECG waveform pattern in the GK rats (Fig. 1B). There were no significant differences in the R-R interval or HR at baseline between the control Wistar and GK rats (Fig. 1C). In addition, there was no significant difference in other ECG parameters, such as R-R interval or QRS duration between Wistar and GK rats (Table 1).

To assess autonomic nerve function in the GK rats, we examined the carotid baroreflex function. Control Wistar rats exhibited a significant increase in HR, whereas a limited response was observed in the GK rats (Fig. 1D). Next,

Table 1. ECG parameters.

ECG parameters	PR Interval(ms)	P Duration(ms)	QRS Interval(ms)
Con	55.8 ± 1.1	25.1 ± 1.3	17.6 ± 0.8
GK	51.6 ± 2.6	21.9 ± 2.2	19.8 ± 2.5

ECG parameters (R-R interval, P duration, and QRS time) of control (Con) and GK rats. Data are expressed as the means ± S.E. of at least 9 animals.

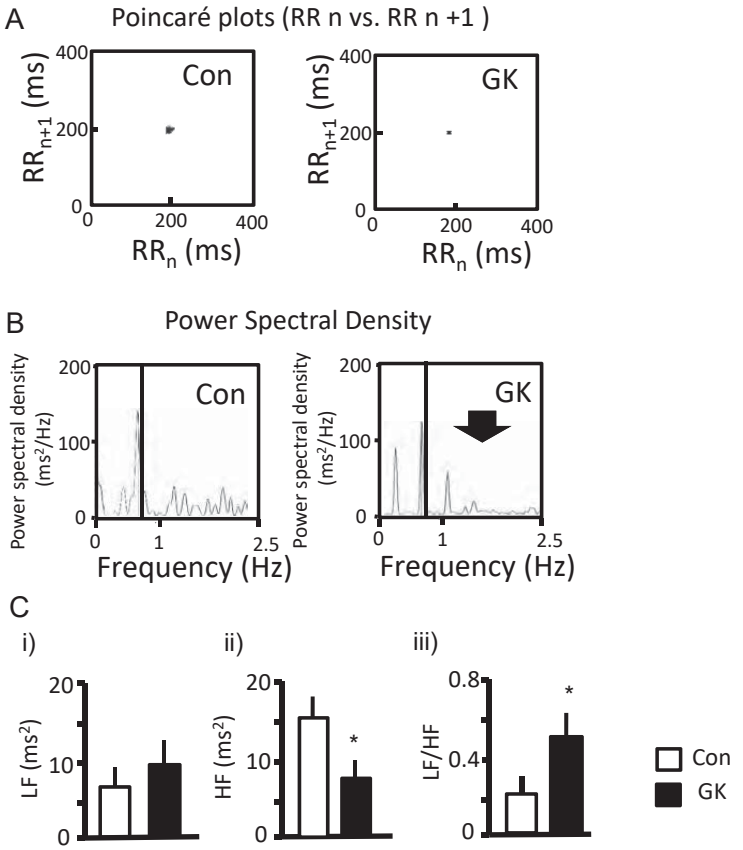

Figure 2 Modified heart rate variability (HRV). (A) Representative HRV analysis of control (Con: left panel) and GK (right panel) rats. Poincaré plots (RRn vs. RRn+1) in which consecutive pairs of R-R intervals during the control period are graphed with the n^{th}+1 R-R interval plotted against the n^{th} R-R period. Note the highly limited changes in GK rats. (B) Representative power spectral densities of control (Con: left panel) and GK (GK: right panel) rats. GK rats showed apparently limited changes in the high-frequency spectrum (arrow). (C) Statistical comparison of LF (low-frequency) (i), HF (high-frequency) (ii) components, and the LF/HF ratio (iii). $^{*}P < 0.05$ indicates a significant difference between the control and GK rats. Each group contained at least 8 rats.

we analyzed heat rate variability (HRV), which was measured as the standard deviation of the interbeat interval. Mean R-R intervals fluctuated less in the GK rats than in the control Wistar rats (Fig. 1E), indicating reduced HRV in the GK rats. Statistical analysis revealed decreased SDNN in the GK rats compared with the baseline.

HRV analysis

We further analyzed HRV in the control and GK rats. Figure 2A depicts typical results of the beat-to-beat dynamics with Poincaré plots (RR_n vs. RR_{n+1}). GK rats clearly showed limited fluctuation changes (Fig. 2A). In the frequency

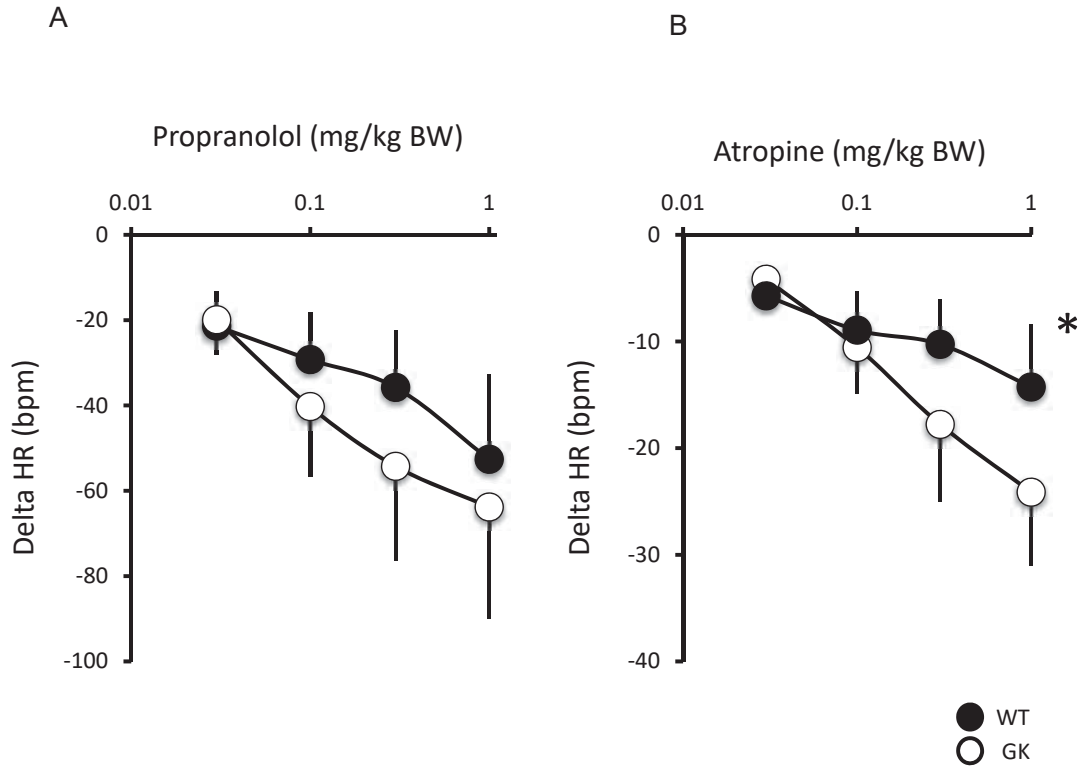

Figure 3 Pharmacological manipulation (i.p., arrow) with propranolol (A) and atropine (B). No significant difference was detected in response to various amounts of propranolol (0.03–1.0 mg/kg). Dose-dependent changes in response to various amounts of atropine (0.03–1.0 mg/kg) resulted in a decreased response in GK rats at the highest dose (1.0 mg/kg). $^{*}P < 0.05$ indicates a significant difference between the control and GK rats. Each group contained at least 7 rats.

domain analysis, low-frequency (LF; 0.2–0.75 Hz) and high-frequency (HF; 0.75–2.5 Hz) components were resolved in power spectral density (Fig. 2B). LF components were not significantly different between the 2 groups (Fig. 2Ci). On the other hand, statistical analysis of the HF components revealed a significant decrease in GK rats (Fig. 2Cii). As expected from the calculation of the HF component, GK rats showed a significantly higher LF/HF ratio than did the control Wistar rats (Fig. 2Ciii), suggesting decreased parasympathetic tone.

Pharmacological manipulation

Next, we analyzed pharmacological responses in the control Wistar and GK rats (Fig. 3). Intraperitoneal administration of propranolol (0.03 to 1.0 mg/kg, intraperitoneal [i.p.]) resulted in a prolonged R-R interval in a dose-dependent fashion (Fig. 3A). The effects of parasympathetic blockage were also analyzed using injections of atropine (0.03 to 1.0 mg/kg, i.p.), a typical muscarinic receptor antagonist. Atropine significantly reduced the R-R interval in both the control and GK rats (Fig. 3B).

Injection of propranolol, an adrenergic β-blocker, resulted in no significant differences between the control and GK rats, suggesting that sympathetic nerve regulation was relatively conserved. On the contrary, high-dose atropine (1.0 mg/kg) injection showed decreased HR responses in the GK rats compared with the control Wistar rats, suggesting that the parasympathetic nervous system is significantly modified in GK rats.

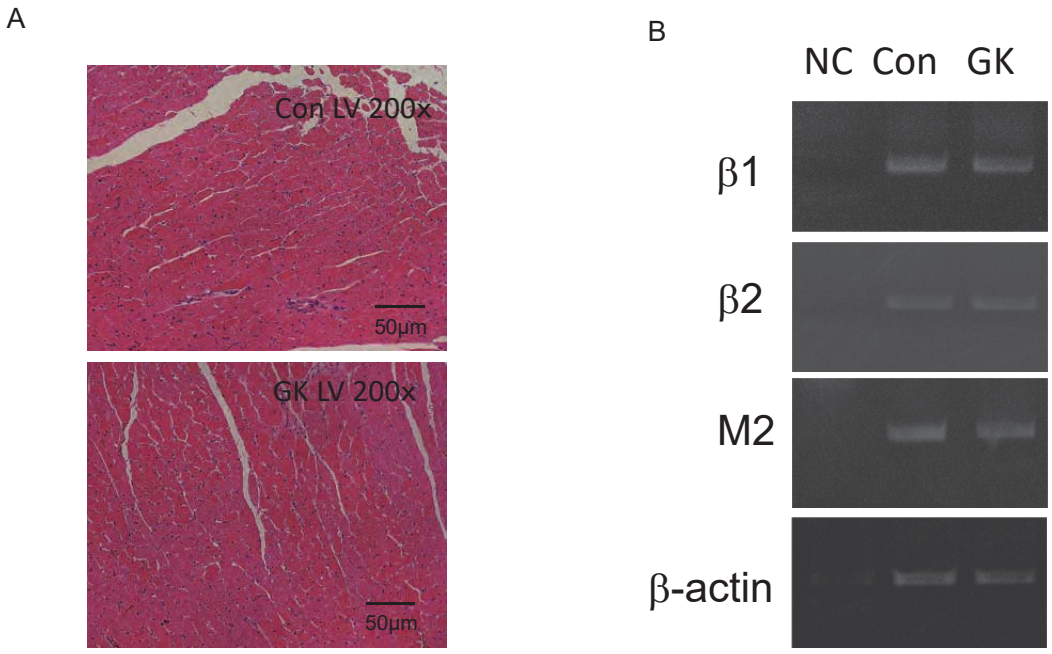

Figure 4 Conserved heart structure in GK rats. (A) Histological analysis of hearts from 50-week-old Wistar and GK rats. Hearts were sectioned transversely and stained with HE (Hematoxylin and eosin). (B) Conserved expression of adrenergic and muscarinic receptors. RT-PCR (Reverse Transcription Polymerase Chain Reaction) analysis of hearts from control Wistar and GK rats. Identification of sympathetic β-adrenergic receptor (1 and 2)-specific transcripts in the heart (NC, negative control without cDNA; Con, control Wistar rats; GK, GK rats). Expression of β-actin was evaluated as a control.

Histology

As modified autonomic nerve regulation often affects the cardiovascular system, cardiac structure was pathologically examined. The gross examinations revealed that the hearts of GK rats were similar to those of control rats (Fig. 4A). To better understand the cardiomyopathic changes observed in the hearts, a cross-sectional area (CSA) was also examined by planimetry. No significant changes were observed (data not shown), suggesting no pathological cardiac changes in the GK rats.

Expression profile

To investigate the influence of GK-related diabetes on the autonomic nervous system, we performed RT-PCR analysis of adrenergic (β1 and β2) and muscarinic (M2) receptors in the heart to probe the sympathetic and parasympathetic nervous systems, respectively (Fig. 4B). The mRNA expression levels of the β1 and β2 adrenergic receptors in the hearts of control and GK rats were not significantly different. The GK rats also showed no significant changes in M2 receptor expression. β-actin expression was examined as a control.

Discussion

In the present study, we examined the cardiovascular autonomic nervous system in GK rats, which is a model of type 2 diabetes. The ECG recordings indicated that GK rats had a decreased baroreflex and a decreased responsiveness to atropine, a parasympathetic nerve antagonist. In addition, GK rats showed decreased SDNN and unchanged R-R interval in the Poincaré plots, indicating a modified autonomic nerve regulatory system in GK rats. Furthermore, GK rats showed decreased HF

component, resulting in decreased LF/HF ratio, indicating modified parasympathetic nerve control. On the other hand, no significant changes in histological analysis were observed. The expression of autonomic nerve receptors, such as β1, β2, and M2 receptors, was not changed. Our data suggests that GK rat could be a nice animal model for chronic diabetic neuropathy.

In human, vagal tonus is the major regulator in the heart rate control. It also should be noted that diabetic patients tend to show increased heart rates. In the present study, GK rat showed modified parasympathetic nerve control, like human diabetic patients. Therefore, GK rat might be a nice animal model for diabetic autonomic neuropathy. On the other hand, as GK rat showed no significant increase in heart rate comparing with control Wistar rats, diabetic neuropathy in GK rat at 50 weeks of age might be mild. So far, neuropathic analysis with GK rat has been mainly focused on pathological changes, while little attention has been paid in ECG-related changes. In the present study, we analyzed cardiovascular autonomic nerve regulatory system in GK rat.

In clinical analysis, cardiovascular autonomic neuropathy has been linked to cardiac arrhythmias, postural hypotension, and increased incidence of asymptomatic ischemia. Furthermore, it correlates with myocardial infarction and reduced likelihood of survival after myocardial infarction[15]. The autonomic nervous system closely integrates vital processes, such as HR, blood pressure, and myocardial contractility. Therefore, the autonomic nervous system plays a pivotal role in the regulation of the cardiovascular system. Myocardial infarction is the primary cause of death in diabetic patients. A large longitudinal study presented the first evidence that HRV is a pivotal predictor of cardiac mortality after myocardial infarction[16]. The vagal nerve plays a crucial role in mediating HRV. The ability to augment vagal activity can be quantified by baroreflex sensitivity. Therefore, considering the importance of HRV in diabetic patients, nice animal model for HRV analysis is important. In this sense, our present study of modified parasympathetic nerve control in GK rat seems to be important. GK rat might be a nice animal model for diabetic cardiovascular neuropathy.

The duration of diabetes has been associated with a high incidence of neuropathy[17]. The Diabetes Control and Complications Trials (DCCT) confirmed the beneficial effects of meticulous control of blood glucose on the incidence of chronic complications[18]. In type 2 diabetic patients, the Kumamoto study showed that intensive insulin treatment for 7 years improved nerve conduction velocity (NCV) and the vibration perception threshold (VPT) compared with those treated conventionally[19]. In contrast, the UK prospective diabetes study (UKPDS) on 3,867 type 2 diabetic patients did not identify the effects of glucose control on the prevalence of neuropathy, although there was a significant reduction in the risk for retinopathy and nephropathy[20]. Thus, it might be interesting to analyze intensive insulin treatment or other therapies to prevent diabetic neuropathy in conjunction with the evaluation of ECG and HRV with GK rats.

Characteristic findings of the peripheral nervous system in diabetic patients include distal and sensory predominant nerve fiber degeneration, axonal loss, and endoneurial microangiopathy[2]. Dyck et al. proposed that microvascular injury might be the most probable factor for the diffuse neuropathy in diabetes[21]. However, this hypothesis does not explain the importance of hyperglycemia and the duration of diabetes in the occurrence of diabetic neuropathy. There also emerges a controversy, such as the predominance of the involvement of small fibers, in early diabetic neuropathy. As nerve fiber loss has not been demonstrated universally, microangiopathy might not always account for the diabetic fiber loss. In addition, Malik et al.

demonstrated that patients who showed high-grade microangiopathic changes following overt neuropathy did not have clinically evident neuropathy at the time of nerve biopsy[22]. The extent of microangiopathic changes correlated well with subsequent nerve fiber loss in diabetic nerves[23]. In this sense, GK rat might be a useful for evaluation of various types of treatments, as nerve biopsy seems to be limited in the clinic.

In the present study, we focused on heart rate changes in GK rats. Sanyal et al. reported decreased parasympathetic nerve regulation in Spontaneously Diabetic Bio-Breeding (Kob) rats, another diabetic animal model. In their analysis, Kob rats had lower HF, while no significant changes in the LF /HF ratio were observed[24]. In the current study, GK rats also showed lower HF, while GK rats showed increased LF/HF ratio, which might be due to different experimental conditions. Nevertheless, combined with Sanyal's study, our findings indicate that HRV analysis is an effective method for evaluating diabetic neuropathy. Zao et al., has analyzed TRH (thyrotropin-releasing hormone)-related changes in GK rats, such as food intake, hormone releases (ghrelin, insulin, peptide YY, and others), while no electrophysiological changes were analyzed[25]. As nerve control is mainly regulated by ion channels, electrophysiological analysis might be more preferable to analyze diabetic neuropathy, while little electrophysiological studies have been reported with GK rats. Nevertheless, further analysis will be required to evaluate autonomic neuropathy in GK rats.

Rosengren et al. reported that GK rats have high expression of α2A-adrenergic receptors in pancreatic islets, which might be related to lower insulin secretion[26]. Likewise, we anticipated decreased sympathetic tonus due to α2A-adrenergic receptor overexpression in GK rats. Indeed, the current study revealed decreased parasympathetic nerve tonus. Nevertheless, a detailed analysis of α2A receptor expression in the sympathetic nerve system in GK rats should be considered in the future study.

In conclusion, the GK rat showed decreased parasympathetic nerve tone, suggesting the importance of this rat as a typical model to analyze cardiovascular autonomic nerve changes in diabetic neuropathy.

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