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Central activation of cardiac vagal nerve by α_2-adrenergic stimulation is impaired in streptozotocin-induced type 1 diabetic rats

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ABSTRACT

To elucidate the abnormality of cardiac vagal control in streptozotocin-induced type 1 diabetic rats, we measured left ventricular myocardial interstitial acetylcholine (ACh) release in response to α_2-adrenergic stimulation as an index of in vivo cardiac vagal nerve activity. A cardiac microdialysis technique was applied to the rat left ventricle, and the effect of α_2-adrenergic stimulation by intravenous medetomidine (100 μg/kg) on myocardial interstitial ACh levels was examined in anesthetized diabetic rats (4–6 weeks after intraperitoneal streptozotocin) and age-matched control rats (protocol 1). The effect of electrical vagal nerve stimulation on ACh levels was also examined in separate rats (protocol 2). In protocol 1, medetomidine increased the ACh levels in control (from 1.76 ± 0.65 to 3.13 ± 1.41 nM, $P < 0.05$, $n = 7$) but not in diabetic rats (from 2.01 ± 0.47 to 1.62 ± 0.34 nM, not significant, n = 7). In protocol 2, electrical vagal nerve stimulation at 20 Hz significantly increased the ACh levels in both control (from 1.49 ± 0.26 to 6.39 ± 1.81 nM, $P < 0.001$, $n = 6$) and diabetic rats (from 1.77 ± 0.54 to 6.98 ± 1.38 nM, P < 0.001, n = 6). In conclusion, medetomidine-induced central vagal activation was impaired in diabetic rats, whereas peripheral cardiac vagal control of ACh release was preserved. The impairment of central vagal activation may lead to relative sympathetic predominance and promote cardiovascular complications in diabetes.

1. Introduction

Although diabetes mellitus accompanies autonomic neuropathy, abnormality in cardiac vagal control is not fully understood. In streptozotocin-induced type 1 diabetic rats, the baroreflex-mediated heart rate (HR) response is reduced, but the HR response to efferent vagal nerve stimulation (VNS) or methacholine (a muscarinic agonist) injection is enhanced (Dall'Ago et al., 2002, 2007). Similar results are reported in a mouse model of type 1 diabetes; i.e., the baroreflex-mediated HR response is impaired, but the bradycardic response to efferent VNS is augmented (Gu et al., 2008). Although the HR response to efferent VNS is mediated by acetylcholine (ACh) released from cardiac vagal nerve terminals, supersensitivity of the atrial rate response to muscarinic receptor stimulation is reported in diabetic rats (Carrier et al., 1984). Hence, the observation of the HR response alone may not allow us to determine whether the cardiac vagal ACh release is impaired in diabetes. To assess cardiac vagal nerve activity more directly, we have developed a cardiac microdialysis technique. The cardiac microdialysis enabled measurements of myocardial interstitial ACh levels from the in vivo beating heart in cats (Akiyama et al., 1994; Kawada et al., 2001), rabbits (Shimizu et al., 2009, 2012; Kawada et al., 2010), rats (Kawada et al., 2012, 2013a, 2017), and mice (Zhan et al., 2013, 2017).

The autonomic imbalance with heightened sympathetic and suppressed vagal tones may be a key component involved in both the etiology and the clinical course of cardiovascular diseases (Viki et al., 2011). Vagal tone may decline with the autonomic imbalance shifting toward increased sympathetic tone during the development from normal to impaired glucose tolerance (Wu et al., 2007). Elucidating mechanisms for reduced vagal tone in diabetes may lead to the development of a new treatment strategy for restoring autonomic balance. Using the cardiac microdialysis, we have shown that intravenous injection of an α_2-adrenergic agonist medetomidine centrally activates the cardiac vagal nerve in rabbits (Shimizu et al., 2012). We

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hypothesized that the central vagal activation by the α_2-adrenergic mechanism might be impaired in diabetic rats. To test the hypothesis, we examined medetomidine-induced central vagal activation in type 1 diabetic rats 4–6 weeks after intraperitoneal injection of streptozotocin and compared the results with those in control rats. To assess possible alteration in the peripheral vagal control, we also examined changes in myocardial interstitial ACh levels in response to electrical VNS in separate rats.

2. Materials and methods

Animal care was conducted in strict accordance with the *Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences*, which has been approved by the Physiological Society of Japan. All protocols were reviewed and approved by the Animal Subject Committee of the National Cerebral and Cardiovascular Center.

2.1. Diabetic model

Streptozotocin (Wako Pure Chemical Industries, Japan) was dissolved in physiological saline to a concentration of 20 mg/ml and administered intraperitoneally at a dose of 70 mg/kg in 12-week-old, male Wisrat–Kyoto (WKY) rats. Four to six weeks later, the rat was anesthetized by intraperitoneal injection of an anesthetic mixture of urethane (250 mg/ml) and α-chloralose (40 mg/ml) at a dose of 2 ml/kg. Under anesthesia, a droplet of venous blood was obtained by a tail vein puncture. The rat with a blood glucose level higher than 300 mg/dl was included in a diabetic group. We prepared 18 rats with intraperitoneal streptozotocin injection. As we were able to identify the successful development of diabetes based on lower body weight compared with normal rats, all 13 rats used for the present study met the above criterion of the blood glucose level. The remaining 5 rats were used for other purposes. Overnight fasting was not imposed prior to the experiment to avoid possible fasting-related stress.

2.2. Surgical preparation

Two protocols were conducted in normal control and diabetic rats, and separate rats were used for protocols 1 and 2. The normal control rats had not received an intraperitoneal injection of vehicle. Each rat was anesthetized as described above and mechanically ventilated with oxygen-enriched room air. The body temperature of the animal was maintained at around 38 °C by using a heating pad and a lamp. Venous catheters were inserted into the femoral veins bilaterally. One venous catheter was used for continuous infusion of an 18-fold diluted solution of the above anesthetic mixture (2–3 $ml \cdot kg^{-1} \cdot h^{-1}$), and the other for injections of test drugs. An arterial catheter was inserted into the right femoral artery to measure arterial pressure (AP) and HR.

In a lateral position, the left fourth to sixth ribs were partially resected to expose the heart. The pericardium was incised, and two dialysis probes were implanted into the left ventricular free wall. Details of the dialysis probe have been described previously (Akiyama et al., 1994; Kawada et al., 2012). Each dialysis probe was perfused with Ringer's solution containing a cholinesterase inhibitor eserine (100 μM) at a perfusion rate of 2 μl/min. The dialysate sampling was started from 2 h after the probe implantation. Each sampling period was 10 min with 20-μl sample volume. The ACh concentration of the dialysate sample was measured by a high-performance liquid chromatography system (Eicom, Japan). The implanted dialysis probe was broken on a few occasions due to acute changes in ventricular contractions induced by VNS or test drug injections, in which case all dialysate samples from the broken probe were discarded. When the two dialysis probes were patent throughout the protocol, ACh concentrations measured from the two dialysate samples were averaged at each sampling period. A post-mortem examination confirmed that the dialysis membrane was not exposed to the left ventricular cavity.

The rats in protocol 2 underwent bilateral vagotomy at the neck. A pair of stainless steel wire electrodes (Bioflex wire, AS633, Cooner Wire, CA, USA) was attached to the nerve. The nerve and electrodes were secured with silicone glue (Kwik-Sil, World Precision Instruments, FL, USA). The VNS was delivered bilaterally through two isolator units connected to an electrical stimulator (SEN-7203, Nihon Kohden, Japan).

2.3. Protocols

In protocol 1 ($n = 7$ for each of the control and diabetic groups), the effect of medetomidine and the effect of medetomidine combined with an α_1-adrenergic agonist phenylephrine were examined. After collecting a baseline dialysate sample for 10 min, medetomidine (100 μg/kg) was intravenously injected. Medetomidine initially elevated AP by peripheral vasoconstriction due to α_2-adrenergic stimulation, but the pressor effect waned as time elapsed. A 10-min stabilization period was allowed after the medetomidine injection; then the dialysate was sampled for 10 min. Next, to activate the arterial baroreflex, phenylephrine was administered (250 $\mu g \cdot kg^{-1} \cdot h^{-1}$) simultaneously with an additional bolus infusion of medetomidine (100 μg/kg). Another 10-min stabilization period was allowed after starting the phenylephrine administration; then the dialysate was sampled for 10 min. The AP and HR data were measured at baseline, 10 min after the medetomidine injection, and 10 min after starting the phenylephrine administration.

In protocol 2 ($n = 6$ for each of the control and diabetic groups), the effect of bilateral electrical VNS was examined. Before starting the dialysate sampling, 5-Hz VNS (5 V, 100-μs pulse width) was performed for 10 min to establish stable stimulatory conditions. After an intervening interval of 10 min, baseline dialysate was sampled for 10 min. Next, VNS was performed at 5 Hz or 20 Hz for 10 min, and the corresponding dialysate sample was collected. The intervening interval between the two stimulation trials was 10 min. The order of the 5-Hz and 20-Hz VNS trials was changed among animals. The AP and HR data were measured at baseline and 5 min after starting each VNS.

2.4. Statistical analysis

All data are presented as mean and SE values. Body weight and blood glucose levels were compared between the control and diabetic groups by unpaired *t*-test. Changes in dialysate ACh concentrations and hemodynamics were examined using two-way analysis of variance (ANOVA) with repeated measures on one factor (Glantz and Slinker, 2001). The repeated-measures factor (treatment) was the administration of drugs (protocol 1) or VNS (protocol 2). The non-repeated-measures factor was diabetes (the control versus diabetic group). When the treatment effect was significant, Tukey's test was applied for all pairwise comparisons within each group (Glantz, 2002). Because the variance of measured ACh concentrations increased with their mean, the statistical analyses on the ACh data were performed after logarithmic conversion [Snedecor and Cochran, 1989]. The magnitudes of the AP response to phenylephrine (protocol 1) and the HR response to test drugs (protocol 1) or VNS (protocol 2) were compared between the control and diabetic groups by unpaired *t*-test.

Additionally, the relationship of HR versus the logarithm of the ACh concentration was analyzed using pooled data from protocols 1 and 2. The effects of diabetes and vagotomy were examined using multiple linear regression as follows (Glantz and Slinker, 2001).

$$HR = c + b_1 \times D_{DM} + b_2 \times D_{VX} + (b_3 + b_4 \times D_{DM} + b_5 \times D_{VX}) \times log_{10} ACh \quad (1)$$

where c is the constant term or the intercept of the multiple linear regression; b_1 is the coefficient for the effect of diabetes on the intercept; b_2 is the coefficient for the effect of vagotomy on the intercept; b_3 is the slope of HR versus the logarithm of the ACh concentration; b_4 is the

coefficient for the effect of diabetes on the slope; and b_5 is the coefficient for the effect of vagotomy on the slope. D_{DM} and D_{VX} denote dummy variables encoding the effect of diabetes ($D_{DM} = 0$ for the control and $D_{DM} = 1$ for the diabetic group) and vagotomy [$D_{VX} = 0$ for intact vagi (protocol 1) and $D_{VX} = 1$ for vagotomy (protocol 2)], respectively. After confirming that neither b_4 nor b_5 was significantly different from zero, the multiple linear regression was reduced to the following equation.

$$HR = c + b_1 \times D_{DM} + b_2 \times D_{VX} + b_3 \times \log_{10} ACh \quad (2)$$

which is equivalent to the analysis of covariance (Glantz and Slinker, 2001). The multiple linear regression analysis was performed on mean data points ($3 \times 4 = 12$ data points) and individual data points [$(3 \times 7 \times 2) + (3 \times 6 \times 2) = 78$ data points]. When the multiple linear regression was performed on individual data points, dummy variables encoding individual rats and their coefficients were included in the equations to account for repeated measurements within each rat (Glantz and Slinker, 2001; Kawada et al., 2013b).

3. Results

The body weight, blood glucose level, and age at the time of the experiment did not differ between protocols 1 and 2 in control or diabetic rats. Hence, these data were pooled for protocols 1 and 2. The body weight was lower (314 ± 7 vs. 371 ± 11 g, $P < 0.001$) and the blood glucose level was higher (391 ± 18 vs. 97 ± 6 mg/dl, $P < 0.001$) in the diabetic than in the control rats. The age at the time of the experiment was not significantly different between the control and diabetic rats (control, 16.2 ± 0.8 weeks; diabetes, 17.2 ± 0.5 weeks; $P = 0.25$).

Fig. 1 illustrates the results of protocol 1. This protocol was performed on rats with intact vagi. The two-way ANOVA indicated that there was no significant difference in dialysate ACh concentrations between the control and diabetic groups. However, the significant interaction effect suggests that the drug effect on ACh was different between two groups. Although the drug effect on HR was significant, the overall effect of diabetes on HR was not significant, possibly due to the large variance of data. The two-way ANOVA indicated that AP was significantly lower in the diabetic than in the control group. The significant interaction effect suggests that the drug effect on AP was different between two groups.

In the control group (Fig. 1, left panels), intravenous medetomidine significantly increased the myocardial interstitial ACh level and decreased HR without significantly affecting AP. Medetomidine combined with phenylephrine further increased the ACh level with a significant increase in AP. The difference in HR between trials of medetomidine alone and medetomidine combined with phenylephrine did not reach statistical significance. In the diabetic group (Fig. 1, right panels), medetomidine alone did not increase ACh or decrease HR. Medetomidine combined with phenylephrine significantly increased the ACh level with a significant HR reduction and a significant AP elevation. The magnitude of phenylephrine-induced AP elevation (the difference in AP between trials of medetomidine alone and medetomidine combined with phenylephrine) did not differ between two groups (control, 50.4 ± 8.8 mm Hg; diabetes, 53.9 ± 6.1 mm Hg; $P = 0.76$). The magnitude of HR reduction induced by medetomidine was 49.9 ± 6.9 beats/min in the control group and was significantly attenuated to 1.6 ± 13.3 beats/min in the diabetic group ($P = 0.007$). The magnitudes of HR reduction induced by medetomidine combined with phenylephrine relative to the baseline were not significantly different between two groups (control, 81.1 ± 21.6 beats/min; diabetic, 71.6 ± 23.5 beats/min; $P = 0.77$).

Although changes in AP induced by medetomidine alone were not statistically significant compared with the baseline level in the control or diabetic group, mean AP after medetomidine seems to be increased in the control but not in the diabetic group (Fig. 1). To examine whether changes in ACh levels were associated with changes in AP, the relationship between changes in AP and ACh levels was checked in

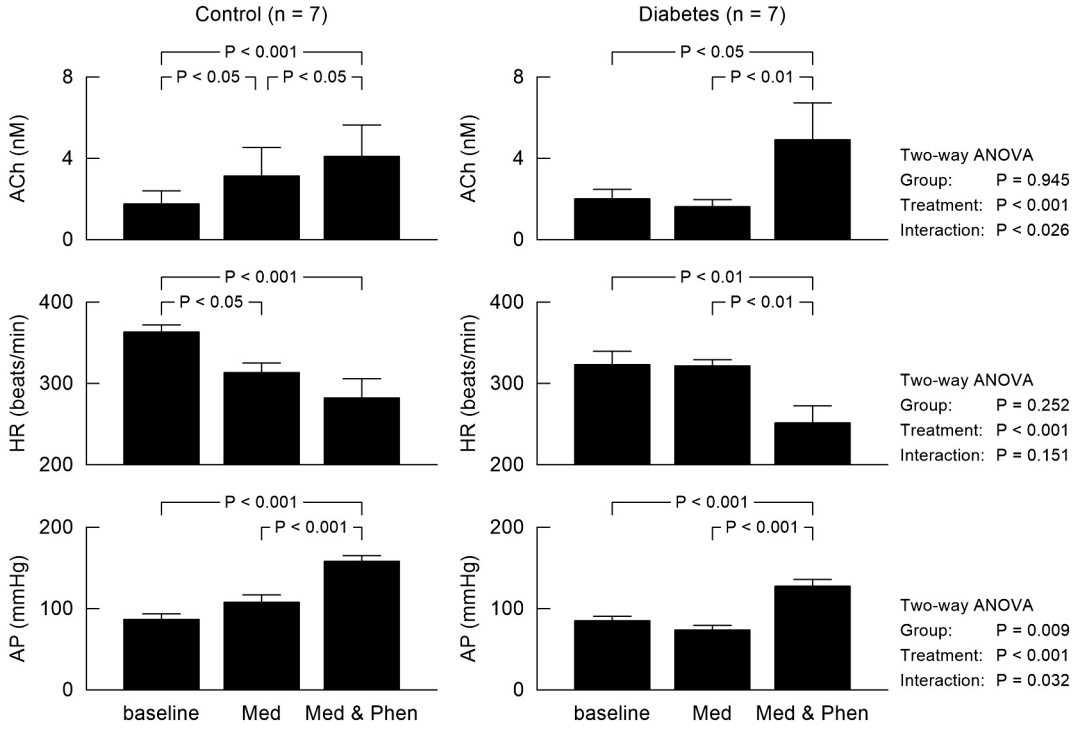

Fig. 1. Effects of intravenous medetomidine (Med) alone and medetomidine combined with phenylephrine (Med & Phen) on myocardial interstitial acetylcholine (ACh) levels, heart rate (HR), and arterial pressure (AP) in control and streptozotocin-induced diabetic rats. Data are mean and SE values. Statistical analysis was performed using two-way analysis of variance (ANOVA) with repeated measures on one factor (the drug or treatment effect). When the drug effect was significant, Tukey's test was used to detect differences within each group.

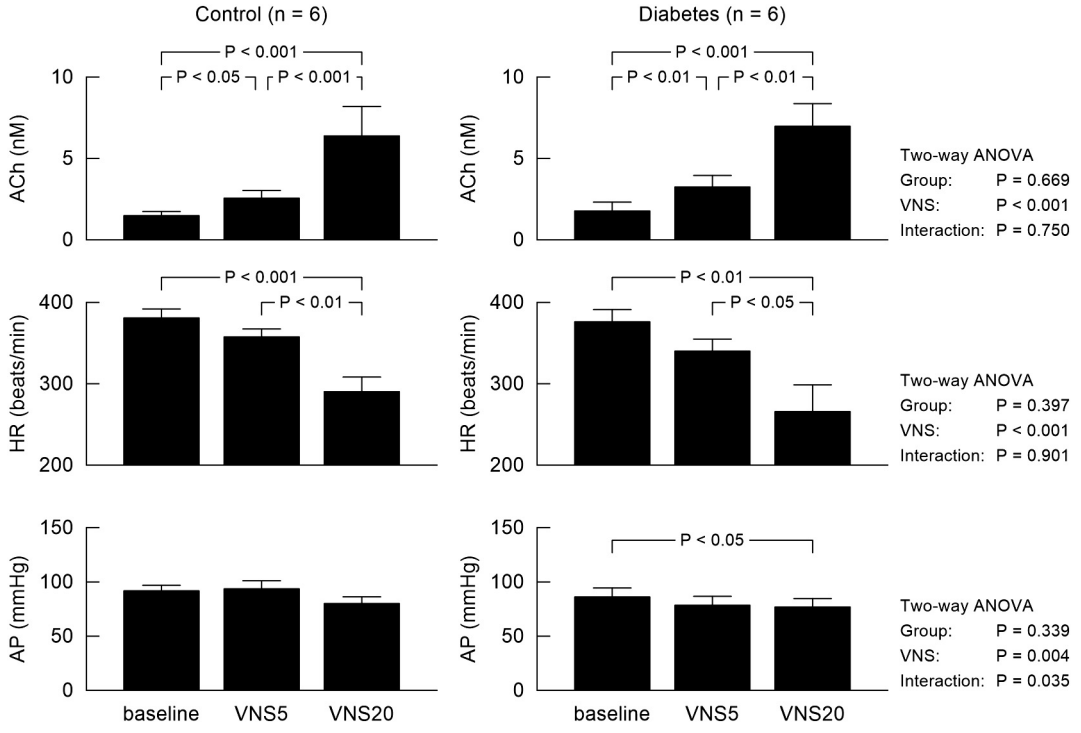

Fig. 2. Effects of electrical vagal nerve stimulation at 5 Hz (VNS5) and 20 Hz (VNS20) on myocardial interstitial acetylcholine (ACh) levels, heart rate (HR), and arterial pressure (AP) in control and streptozotocin-induced diabetic rats. Data are mean and SE values. Statistical analysis was performed using two-way analysis of variance (ANOVA) with repeated measures on one factor (the VNS effect). When the VNS effect was significant, Tukey's test was used to detect differences within each group.

individual animals. In the control group, opposite directional changes in AP and ACh (i.e., an increase in AP with a decrease in ACh or a decrease in AP with an increase in ACh) after medetomidine were observed in three rats. In the diabetic group, the opposite directional changes in AP and ACh were also observed in three rats. Hence, medetomidine-induced ACh release did not necessarily accompany an increase in AP.

Fig. 2 illustrates the results of protocol 2. This protocol was performed on rats with bilateral vagotomy. The two-way ANOVA indicated that VNS significantly increased dialysate ACh concentrations without a significant group effect (control vs. diabetes). The effect of VNS on HR was also significant without a significant group effect. Although the group effect was not significant regarding AP, a significant interaction effect suggests that the effect of VNS on AP was different between two groups.

In both the control (Fig. 2, left panels) and diabetic (Fig. 2, right panels) groups, 5-Hz VNS significantly increased the myocardial interstitial ACh level, and 20-Hz VNS further elevated the ACh level. Although the VNS decreased HR, the HR decrease was statistically significant only for the 20-Hz VNS. The magnitudes of HR reductions were not significantly different between two groups for the 5-Hz VNS (control, 23.3 ± 10.2 beats/min; diabetic, 36.0 ± 8.5 beats/min; $P = 0.56$) or the 20-Hz VNS (control, 90.7 ± 19.7 beats/min; diabetic, 110.3 ± 30.8 beats/min; $P = 0.73$). There were no significant differences in AP among the three trials in the control group. The AP was significantly lower during the 20-Hz VNS compared with the baseline level in the diabetic group.

Fig. 3 depicts the relationship of HR versus the logarithm of the myocardial interstitial ACh level obtained from protocol 1 (circles) and protocol 2 (squares). The open and filled symbols represent data from the control and diabetic rats, respectively. The multiple linear regression analysis using Eq. (1), both on mean data points and individual data points, indicated that neither diabetes nor vagotomy had a significant effect on the regression slope. These results allowed the

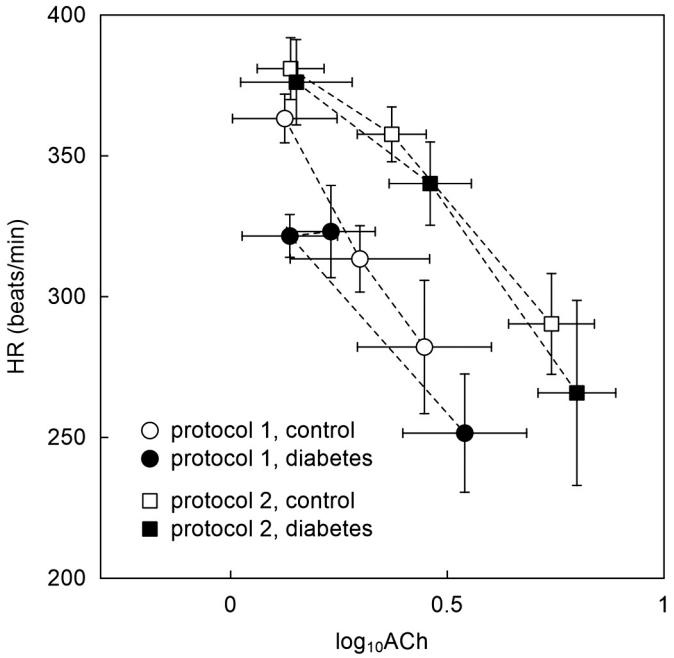

Fig. 3. Relationship of heart rate (HR) versus the logarithm of myocardial interstitial acetylcholine (ACh) concentration pooled from protocols 1 and 2. Data are mean and SE values. Circles and squares represent data points from protocols 1 and 2, respectively. Open and filled symbols represent data points from control and diabetic rats, respectively.

assumption of a common regression slope and the use of Eq. (2). When the relationship of HR versus the logarithm of the ACh concentration was analyzed using Eq. (2), vagotomy showed a significant positive effect on HR by a magnitude of approximately 50 beats/min (Table 1).

Table 1
Parameters of multiple linear regression analysis on mean data points or individual data points of heart rate versus the logarithm of myocardial interstitial acetylcholine concentration.

	c	b_1	b_2	b_3	Adjusted r^2
Mean data points	367.3 ± 7.1**	−12.4 ± 6.6	51.8 ± 6.9**	−175.1 ± 15.5**	0.931
Individual data points	368.4 ± 6.2**	−12.8 ± 6.0*	52.2 ± 6.3**	−178.0 ± 12.6**	0.787

c: constant term or the intercept of the regression; b_1: the coefficient for the effect of diabetes, b_2: the coefficient for the effect of vagotomy, b_3: the coefficient for the effect of acetylcholine on heart rate, which represents the slope of the regression. $^{**}P < 0.01$ and $^{*}P < 0.05$ indicate that the coefficient was significantly different from zero (see Eq. (2) for details).

On the other hand, diabetes showed a negative effect on HR by a magnitude of approximately −12 beats/min; however, this effect was statistically significant only for the analysis on individual data points.

4. Discussion

The present study demonstrated that medetomidine-induced central vagal activation was impaired in a rat model of streptozotocin-induced type 1 diabetes. However, when AP was increased by medetomidine combined with phenylephrine, the myocardial interstitial ACh concentration in the diabetic rats increased to a level comparable to that in the control rats, suggesting that the baroreflex pathway relating to the cardiac vagal control was not disabled in the diabetic rats. The myocardial interstitial ACh release in response to efferent VNS was not significantly impaired in the diabetic rats.

4.1. Effects of medetomidine on myocardial interstitial ACh release

It has been shown that α_2-adrenergic stimulation increases vagal nerve activity through a central mechanism (Kobinger and Walland, 1971; Kobinger and Pichler, 1974). Intravenous medetomidine, an α_2-adrenergic agonist, increases myocardial interstitial ACh levels in the rabbit right atrium (Shimizu et al., 2012, 2014) and the rat left ventricle (Kawada et al., 2012, 2013a, 2013b). The medetomidine-induced central vagal activation is impaired in spontaneously hypertensive rats (SHR) (Kawada et al., 2012), suggesting a pathological role of the central α_2-adrenergic mechanism in parasympathetic withdrawal.

In contrast to the control group, intravenous medetomidine failed to increase the myocardial interstitial ACh level in the diabetic group (Fig. 1), suggesting the impairment of α_2-adrenergic stimulation-induced cardiac vagal activation. Because the ACh release in response to electrical VNS did not differ between the control and diabetic groups (Fig. 2), the impairment of the α_2-adrenergic stimulation-induced cardiac vagal activation in the diabetic group is likely to be of central origin. In addition to the central vagal activation, α_2-adrenergic stimulation causes central sympathoinhibition (Shimizu et al., 2014). Application of an α_2-adrenergic agonist clonidine on brain slices containing the nucleus tractus solitarius (NTS) reduces electrical stimulation-induced release of [^{3}H]norepinephrine, but this effect is attenuated in streptozotocin-induced diabetic rats (Dunbar et al., 1995). Attenuation of central α_2-adrenergic action in diabetic rats is also reported regarding dexmedetomidine-induced sleep time (Guo et al., 1991). In a study using isolated brainstem from streptozotocin-induced diabetic rats, α_2-adrenergic receptors reveal a reduced affinity (Padayatti and Paulose, 1999). In sum, central neural regulations mediated by α_2-adrenergic receptors may be widely impaired in streptozotocin-induced diabetic rats.

Despite the impairment of α_2-adrenergic stimulation-induced cardiac vagal activation, medetomidine combined with phenylephrine significantly increased the myocardial interstitial ACh level with a significant HR reduction in the diabetic group (Fig. 1). According to studies by Dall'Ago et al. (2002, 2007), phenylephrine-induced reflex bradycardia was significantly impaired in diabetic rats at 10–20 days after streptozotocin but not at 30 days after streptozotocin. Hence, the preserved bradycardic response to medetomidine combined with phenylephrine in the present study does not necessarily disagree with the previous results. In contrast to the diabetic rats, SHR showed impaired ACh release in response to medetomidine alone and also medetomidine combined with phenylephrine (Kawada et al., 2012). Because the operating range of the arterial baroreflex resets to a high-pressure range in SHR (Sata et al., 2015), the phenylephrine-induced AP increase might not have reached a threshold level to evoke significant baroreflex-mediated vagal activation in SHR (Kawada et al., 2012). In contrast, the sigmoid curve describing the aortic depressor nerve activity versus mean AP does not show significant resetting in streptozotocin-induced diabetic rats (Dall'Ago et al., 2002) or mice (Gu et al., 2008). The midpoint input pressure of the sigmoid curve describing the efferent sympathetic nerve activity versus the mean baroreceptor input pressure is not different between control and streptozotocin-induced diabetic rats (Kawada et al., 2018). Hence, the phenylephrine-induced AP increase probably exceeded the threshold level for significant baroreflex activation, contributing to the myocardial interstitial ACh release in the diabetic group. The effectiveness of phenylephrine to activate the arterial baroreflex may be in line with a study by Chen et al. [2008], in which the firing rate of barosensitive NTS neurons shows a positive correlation only after phenylephrine injection in streptozotocin-induced diabetic rats.

As medetomidine combined with phenylephrine increased AP and the myocardial interstitial ACh release, there is a concern that the slight increase in AP after medetomidine alone, though it was not statistically significant, could have contributed to the increased ACh level in the control group (Fig. 1). However, two control rats showed a decreased AP with an increased ACh level after medetomidine. Hence, the increased AP was not a prerequisite for the central vagal nerve activation after medetomidine alone. This interpretation may be supported by our previous study using a similar protocol in WKY, in which medetomidine significantly increased the myocardial interstitial ACh level without accompanying an apparent increase in mean AP (Kawada et al., 2012). Changes in AP after medetomidine alone may depend on a balance of medetomidine-induced central sympathoinhibition and peripheral vasoconstriction. When prevailing sympathetic tone is high such as in the case of SHR, medetomidine causes a significant AP reduction (Kawada et al., 2012). In the present study, the diabetic rats probably had higher background sympathetic tone, which could explain a tendency of decreasing AP after medetomidine alone compared with the control rats.

One possible mechanism for the increased sympathetic tone in streptozotocin-induced diabetic rats is an enhanced cardiac sympathetic afferent reflex (CSAR) (Zhang et al., 2012). Electrical stimulation of sympathetic afferents from the left stellate ganglion suppressed medetomidine-induced central vagal activation in our previous study (Kawada et al., 2013a). Hence the enhanced CSAR may also partly explain the impairment of medetomidine-induced central vagal activation in the diabetic rats. Although changes in the central processing of CSAR contribute to the enhanced CSAR in diabetic rats (Zhang et al., 2012), further studies are required to determine whether afferent signals from the heart are also augmented in diabetic rats.

4.2. Effects of electrical VNS on myocardial interstitial ACh release

The HR response to electrical VNS was expected to be augmented because previous studies indicate an enhanced HR response to muscarinic receptor stimulation or VNS in diabetic rats (Carrier et al., 1984; Dall'Ago et al., 2002, 2007) and mice (Gu et al., 2008). The magnitude of HR reduction by VNS, however, did not differ significantly between the control and diabetic groups in the present study (Fig. 2). One factor that may explain the discrepancy is the short duration of diabetes in the present study (4–6 weeks). According to a study by Vadlamudi and McNeill (1983), the sensitivity to carbachol in diabetic rat hearts shows no change until 30 days, a reduced sensitivity at 100 days, and an enhanced sensitivity at 180, 240, and 360 days after induction of diabetes. Karakida et al. (1991) reported that the VNS-induced HR fall was significantly greater in rats with 2–3 months of diabetes than age-matched control rats but not in rats with 1–2 months of diabetes. However, the reported duration of diabetes for the enhanced chronotropic response varies among studies. For instance, the enhanced chronotropic response to VNS is observed as early as 10–20 days [Dall'Algo et al. 2002] or 30 days (Dall'Algo et al., 2007) of diabetes. Another factor that could affect the results is the analytical method. Several studies expressed the HR response to VNS or muscarinic agonists as the percent change relative to the baseline HR (Carrier et al., 1984; Dall'Ago et al., 2002, 2007; Karakida et al., 1991). A lower baseline HR in diabetes could yield a larger percent change when an absolute HR change is the same. In the present study, however, the baseline HR did not differ significantly between the control and diabetic groups in protocol 2. Hence, the HR response to VNS was not enhanced even when we calculated the percent change relative to the baseline HR.

The baseline myocardial interstitial ACh level can be detected even under the vagotomized condition (Fig. 2). The detection of the low ACh level under vagotomized condition may be explained by nonquantal ACh release that is independent of vagal nerve activity (Abramochkin et al., 2010) and the use of acetylcholinesterase inhibitor in the perfusate. In a previous study, the atrial rate and the logarithm of the atrial dialysate ACh concentration showed a negative linear relationship during VNS in rabbits [Shimizu et al., 2009]. In the present study, the myocardial interstitial ACh levels are different from those regulating the sinus node because the dialysate samples were obtained from the left ventricular interstitium. Notwithstanding this limitation, when we plotted HR versus the logarithm of the measured ACh concentration, it showed a negative correlation (Fig. 3). Vagotomy significantly moved the relationship of HR versus ACh upward by approximately 50 beats/min (Table 1), which may be explained by sympathetic activation due to the withdrawal of sympathoinhibitory vagal afferent inputs (Saku et al., 2014; Kawada et al., 2016).

Diabetes decreased the relationship of HR versus ACh by approximately 12 beats/min when individual data points were analyzed (Table 1). The negative effect of diabetes on HR may be consistent with a reduced intrinsic HR in streptozotocin-induced diabetic rats (Hicks et al., 1997). According to a study by Huang et al. (2017), expression of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels in the sinoatrial node is reduced in streptozotocin-induced diabetic rats. As the HCN channels generate the I_f current and are responsible for the early part of diastolic depolarization, the reduction of the HCN channel expression decreases the slope of the diastolic depolarization and slows HR (DiFrancesco and Borer, 2007). In the present study, the regression slope of HR versus ACh was not significantly different between the control and diabetic groups, suggesting no supersensitivity of the HR response to ACh. According to a study by Hicks et al. (1997), the enhanced sensitivity to ACh observed in diabetic rats may be partly explained by decreased acetylcholinesterase activity. Although we used an acetylcholinesterase inhibitor in the perfusate, it might not have affected the HR response because of the local administration through the dialysis probe. As already discussed, the duration of diabetes may be a key factor to explain the diverse results about the supersensitivity of the cholinergic effect.

4.3. Limitations

First, the experiment was performed under anesthetized conditions; and thus, the results may not be directly extendable to interpret cardiac vagal nerve activity in conscious conditions. Second, we examined only a short duration (4–6 weeks) of diabetes. Further studies are required to delineate the time course of the impairment of the α_2-adrenergic stimulation-induced central vagal activation in streptozotocin-induced type 1 diabetic rats. Finally, we did not test the effect of phenylephrine alone on the myocardial interstitial ACh release because we focused on the α_2-adrenergic stimulation-induced central vagal activation. Since medetomidine enhances the phenylephrine-induced ACh release (Shimizu et al., 2012), the ACh release induced by phenylephrine alone would be lower than that in response to medetomidine combined with phenylephrine. Whether the phenylephrine-induced ACh release is altered in diabetic rats remains unknown, and this point needs to be clarified in the future.

5. Conclusion

Central vagal activation by α_2-adrenergic stimulation was impaired, but peripheral cardiac vagal nerve function including myocardial interstitial ACh release and the HR response was maintained in type 1 diabetic rats 4–6 weeks after streptozotocin injection. While the pathophysiological significance of the central vagal activation via α_2-adrenergic receptors remains to be clarified, the impairment of central vagal activation may lead to relative sympathetic predominance and promote cardiovascular complications in diabetes. Comprehensive understanding of the cardiovascular autonomic neuropathy would help develop new strategies to restore the autonomic balance and to improve the management of cardiovascular complications in diabetes.

Conflict of interest

The authors declare no conflict of interest.

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References

Abramochkin, D.V., Nurullin, L.F., Borodinova, A.A., Tarasova, N.V., Sukhova, G.S., Nikolsky, E.E., Rosenshtraukh, L.V., 2010. Non-quantal release of acetylcholine from parasympathetic nerve terminals in the right atrium of rats. Exp. Physiol. 95, 265–273.

Akiyama, T., Yamazaki, T., Ninomiya, I., 1994. In vivo detection of endogenous acetylcholine release in cat ventricles. Am. J. Physiol. 266, H854–H860.

Carrier, G.O., Edwards, A.D., Aronstam, R.S., 1984. Cholinergic supersensitivity and decreased number of muscarinic receptors in atria from short-term diabetic rats. J. Mol. Cell. Cardiol. 16, 963–965.

Chen, H.Y., Wu, J.S., Chen, J.J., Cheng, J.T., 2008. Impaired regulation in cardiovascular neurons of nucleus tractus solitarii in streptozotocin-induced diabetic rats. Neurosci. Lett. 431, 161–166.

Dall'Ago, P., Silva, V.O., De Angelis, K.L., Irigoyen, M.C., Fazan Jr., R., Salgado, H.C., 2002. Reflex control of arterial pressure and heart rate in short-term streptozotocin diabetic rats. Braz. J. Med. Biol. Res. 35, 843–849.

Dall'ago, P., D'Agord Schaan, B., da Silva, V.O., Werner, J., da Silva Soares, P.P., de Angelis, K., Irigoyen, M.C., 2007. Parasympathetic dysfunction is associated with baroreflex and chemoreflex impairment in streptozotocin-induced diabetes in rats. Auton. Neurosci. 131, 28–35.

DiFrancesco, D., Borer, J.S., 2007. The funny current. Cellular basis for the control of heart rate. Drugs 67 (Suppl. 2), 15–24.

Dunbar, J.C., Clough-Helfman, C., Barraco, R.A., Anderson, G.F., 1995. Effect of insulin and clonidine on the evoked release of norepinephrine and serotonin from the nucleus tractus solitarius of the diabetic rat. Pharmacology 51, 370–380.

Glantz, S.A., 2002. Primer of Biostatistics, 5th ed. McGraw-Hill, New York.

Glantz, S.A., Slinker, B.K., 2001. Primer of Applied Regression & Analysis of Variance, 2nd edn. McGraw-Hill, New York.
Gu, H., Epstein, P.N., Li, L., Wurster, R.D., Cheng, Z.J., 2008. Functional changes in baroreceptor afferent, central and efferent components of the baroreflex circuitry in type 1 diabetic mice (OVE26). Neuroscience 152, 741–752.
Guo, T.Z., Maze, B., Maze, M., 1991. Attenuation of central alpha 2 adrenergic action in diabetic rats. Pharmacol. Biochem. Behav. 39, 383–387.
Hicks, K.K., Seifen, E., Stimers, J.R., Kennedy, R.H., 1997. Diabetes with and without ketoacidosis on right atrial pacemaker rate and autonomic responsiveness. Am. J. Phys. 273, H1888–H1893.
Huang, X., Zhong, N., Zhang, H., Ma, A., Yuan, Z., Guo, N., 2017. Reduced expression of HCN channels in the sinoatrial node of streptozotocin-induced diabetic rats. Can. J. Physiol. Pharmacol. 95, 586–594.
Karakida, T., Yamazaki, Y., Homma, S., 1991. Blood pressure and heart rate changes in streptozotocin diabetic rats, with special reference to postural hypotension. Jpn. J. Physiol. 41, 589–603.
Kawada, T., Yamazaki, T., Akiyama, T., Shishido, T., Inagaki, M., Uemura, K., Miyamoto, T., Sugimachi, M., Takaki, H., Sunagawa, K., 2001. In vivo assessment of acetylcholine releasing function at cardiac vagal nerve terminals. Am. J. Physiol. Heart Circ. Physiol. 281, H139–H145.
Kawada, T., Akiyama, T., Shimizu, S., Kamiya, A., Uemura, K., Sata, Y., Shirai, M., Sugimachi, M., 2010. Large conductance Ca^{2+}-activated K^{+} channels inhibit vagal acetylcholine release at the rabbit sinoatrial node. Auton. Neurosci. 156, 149–151.
Kawada, T., Akiyama, T., Shimizu, S., Kamiya, A., Uemura, K., Sata, Y., Shirai, M., Sugimachi, M., 2012. Central vagal activation by alpha$_2$-adrenergic mechanism is impaired in spontaneously hypertensive rats. Acta Physiol. (Oxf.) 206, 72–79.
Kawada, T., Akiyama, T., Shimizu, S., Kamiya, A., Uemura, K., Turner, M.J., Shirai, M., Sugimachi, M., 2013a. Sympathetic afferent stimulation inhibits central vagal activation induced by intravenous medetomidine in rats. Acta Physiol. (Oxf.) 209, 55–61.
Kawada, T., Li, M., Shimizu, S., Kamiya, A., Uemura, K., Turner, M.J., Mizuno, M., Sugimachi, M., 2013b. High-frequency dominant depression of peripheral vagal control of heart rate in rats with chronic heart failure. Acta Physiol. (Oxf.) 207, 494–502.
Kawada, T., Li, M., Zheng, C., Sugimachi, M., 2016. Acute effects of vagotomy on baroreflex equilibrium diagram in rats with chronic heart failure. Clin. Med. Insights Cardiol. 10, 139–147.
Kawada, T., Akiyama, T., Shimizu, S., Fukumitsu, M., Kamiya, A., Sugimachi, M., 2017. Desipramine increases cardiac parasympathetic activity via α_2-adrenergic mechanism in rats. Auton. Neurosci. 205, 21–25.
Kawada, T., Shimizu, S., Hayama, Y., Yamamoto, H., Saku, K., Shishido, T., Sugimachi, M., 2018. Derangement of open-loop static and dynamic characteristics of the carotid sinus baroreflex in streptozotocin-induced type 1 diabetic rats. Am. J. Physiol. Regul. Integr. Comp. Physiol. 315, R553–R567.
Kobinger, W., Pichler, L., 1974. Evidence for direct α-adrenergic stimulation of effector neurons in cardiovascular centers by clonidine. Eur. J. Pharmacol. 27, 151–154.
Kobinger, W., Walland, A., 1971. Involvement of adrenergic receptors in central vagus activity. Eur. J. Pharmacol. 16, 120–122.
Padayatti, P.S., Paulose, C.S., 1999. Alpha2 adrenergic and high affinity serotonergic receptor changes in the brain stem of streptozotocin-induced diabetic rats. Life Sci. 65, 403–414.
Saku, K., Kishi, T., Sakamoto, K., Hosokawa, K., Sakamoto, T., Murayama, Y., Kakino, T., Ikeda, M., Ide, T., Sunagawa, K., 2014. Afferent vagal nerve stimulation resets baroreflex neural arc and inhibits sympathetic nerve activity. Physiol. Rep. 2, e12136.
Sata, Y., Kawada, T., Shimizu, S., Kamiya, A., Akiyama, T., Sugimachi, M., 2015. Predominant role of neural arc in sympathetic baroreflex resetting of spontaneously hypertensive rats. Circ. J. 79, 592–599.
Shimizu, S., Akiyama, T., Kawada, T., Shishido, T., Yamazaki, T., Kamiya, A., Mizuno, M., Sano, S., Sugimachi, M., 2009. In vivo direct monitoring of vagal acetylcholine release to the sinoatrial node. Auton. Neurosci. 148, 44–49.
Shimizu, S., Akiyama, T., Kawada, T., Sata, Y., Mizuno, M., Kamiya, A., Shishido, T., Inagaki, M., Shirai, M., Sano, S., Sugimachi, M., 2012. Medetomidine, an α_2-adrenergic agonist, activates cardiac vagal nerve through modulation of baroreflex control. Circ. J. 76, 152–159.
Shimizu, S., Akiyama, T., Kawada, T., Kamiya, A., Turner, M.J., Yamamoto, H., Shishido, T., Shirai, M., Sugimachi, M., 2014. Medetomidine suppresses cardiac and gastric sympathetic nerve activities but selectively activates cardiac vagus nerve. Circ. J. 78, 1405–1413.
Snedecor, G.W., Cochran, W.G., 1989. Statistical Methods, 8th ed. Iowa State, Iowa, pp. 290–291.
Vadlamudi, R.V., McNeill, J.H., 1983. Effect of alloxan- and streptozotocin-induced diabetes on isolated rat heart responsiveness to carbachol. J. Pharmacol. Exp. Ther. 225, 410–415.
Viki, A.I., Maser, R.E., Ziegler, D., 2011. Autonomic imbalance: prophet of doom or scope for hope? Diabet. Med. 28, 643–651.
Wu, J.S., Yang, Y.C., Lin, T.S., Huang, Y.H., Chen, J.J., Lu, F.H., Wu, C.H., Chang, C.J., 2007. Epidemiological evidence of altered cardiac autonomic function in subjects with impaired glucose tolerance but not isolated impaired fasting glucose. J. Clin. Endocrinol. Metab. 92, 3885–3889.
Zhan, D.Y., Du, C.K., Akiyama, T., Sonobe, T., Tsuchimochi, H., Shimizu, S., Kawada, T., Shirai, M., 2013. In vivo monitoring of acetylcholine release from cardiac vagal nerve endings in anesthetized mice. Auton. Neurosci. 176, 91–94.
Zhan, D.Y., Du, C.K., Akiyama, T., Morimoto, S., Shimizu, S., Kawada, T., Shirai, M., Pearson, J.T., 2017. Cardiac vagal control in a knock-in mouse model of dilated cardiomyopathy with a troponin mutation. Auton. Neurosci. 205, 33–40.
Zhang, L., Xiong, X.Q., Fan, Z.D., Gan, X.B., Gao, X.Y., Zhu, G.Q., 2012. Involvement of enhanced cardiac sympathetic afferent reflex in sympathetic activation in early stage of diabetes. J. Appl. Physiol. 113, 47–55.