Influences of Autonomic Nervous System on Atrial Arrhythmogenic Substrates and the Incidence of Atrial Fibrillation in Diabetic Heart

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Summary

Diabetes mellitus (DM) is clinically associated with an increased incidence of atrial fibrillation (AF), but the underlying mechanism remains unclear. We hypothesized that neural remodeling enhances AF vulnerability in diabetic hearts. Eight weeks after creating streptozotocin-induced diabetic rats (DM rats) or control rats, the hearts were perfused according to the Langendorff method. Inducibility of AF was evaluated by 5 times burst pacing from the right atrium and the atrial effective refractory period (AERP) was measured. The protocol was repeated during sympathetic nerve stimulation (SNS) or parasympathetic nerve stimulation (PNS). In tissue samples taken from the right atrium, the density of nerves positive for tyrosine hydroxylase (TH) and acetylcholinesterase (AChE) were determined. SNS significantly increased the incidence of AF in DM rats (14 ± 6 to 30 ± 8%, P < 0.01), but not in control rats (11 ± 4 to 14 ± 6%, NS). Although AERP was significantly decreased by SNS in both rats (each P < 0.01), increased heterogeneity of AERP by SNS was seen only in DM rats. PNS significantly decreased AERP and increased the incidence of AF (9 ± 5 to 30 ± 5% in control rats, 12 ± 6 to 27 ± 6% in DM rats, each P < 0.01) in both rats. The density of TH-positive nerves was heterogeneous in DM rats compared with control rats, whereas the heterogeneity of AChE-positive nerves was not different in the rats. The prevalence of AF was enhanced by adrenergic activation in diabetic hearts, in which heterogeneous sympathetic innervation was evident. These results suggest that neural remodeling may play a crucial role for increased AF vulnerability in DM. (Int Heart J 2009; 50: 627-641)

Key words: Diabetes mellitus, Atrial fibrillation, Sympathetic and parasympathetic nervous system

Atrial fibrillation (AF) is one of the most common arrhythmias and is responsible for substantial morbidity and mortality. Diabetes mellitus (DM)
is also one of the most rapidly growing chronic diseases in the world.\textsuperscript{3,4} In epidemiological studies, DM is associated with an increased incidence of AF.\textsuperscript{5-8} Because DM and AF both independently increase major serious clinical conditions such as stroke and the presence of both compounds the risk,\textsuperscript{9,10} the understanding of this pathophysiological linkage would be essential in developing a therapeutic strategy for risk reduction. However, exactly how DM facilitates AF remains unclear.

A recent study showed that intra-atrial conduction delay and increased fibrotic deposition in atria play a major role in producing atrial tachyarrhythmias in DM rats.\textsuperscript{11} Their results suggest that alterations in atrial electrophysiological properties and tissue structure may be one of the mechanisms that increases the incidence of AF in diabetic hearts. Another possible mechanism is that the abnormality of the autonomic nervous system may be related to the genesis of AF. Cardiac autonomic dysfunction is frequently found in patients with DM.\textsuperscript{12-14} In an experimental study, Schmid, \textit{et al} revealed that heterogeneous sympathetic denervation occurs in diabetic hearts.\textsuperscript{15} In addition, Olgin, \textit{et al} demonstrated that heterogeneous increases in sympathetic innervation may contribute to the promotion of AF.\textsuperscript{16} Thus, we hypothesized that alterations in the autonomic nervous system may alter the atrial electrophysiological properties, resulting in the occurrence of AF in a diabetic heart.

The aim of this study was to assess the influence of the autonomic nervous system on the occurrence of AF in the diabetic heart.

**Methods**

**Animals:** A total of 60 male Wistar rats aged 8 weeks were used in this study, which was carried out under the supervision of the Animal Research Committee in accordance with the Guidelines on Animal Experiments of Fukushima Medical University and the Japanese Government Animal Protection and Management Law (No. 115).

**Diabetic model:** DM was induced by administering a bolus injection of streptozotocin (STZ, Sigma Chemicals, St. Louis, MO, USA) into the tail vein at a dose of 40 mg/kg under anesthesia with an intraperitoneal administration of 40 mg/kg of sodium pentobarbital (DM rats, \(n = 30\)). One week later, using a Glucose Analyzer (Antsense II\textsuperscript{TM}, Daikin, Osaka, Japan), hyperglycemia in tail vein blood was confirmed in all rats. Identical operations without inducing DM were performed in control rats (\(n = 30\)). All STZ-induced DM rats and control rats were caged individually for 8 weeks during which they had free access to normal rat chow and water.

**Experiment protocol:** On the day of an experiment, tail artery blood pressure
and heart rate were measured by the oscillometric method (UR 5000, Ueda, Tokyo) in a conscious state. The rats were then lightly anesthetized with sodium pentobarbital (20 mg/kg, intraperitoneally) and cardiac echocardiography was performed in a supine position. After administration of a further 30 mg/kg sodium pentobarbital (50 mg/kg in total), a 0.1-mL blood sample was taken from the tail vein for blood glucose measurement. Both groups of rats (DM and control rats, \( n = 30 \) in each group) were divided into 2 groups, and used for the following experiments; ie, 16 rats in each group for electrophysiological studies using the Langendorff method, and 14 rats in each group for immunohistochemical staining.

**Langendorff-perfused heart setup:** After the chest was opened by a midline incision, a polyethylene cannula (PE 205, Clay Adams, Parsippany, NJ, USA) was inserted retrogradely into the ascending aorta for retrograde perfusion of the heart, as previously described.\(^\text{17-19}\) The heart was then perfused by a peristaltic pump (Micro Tube Pump MP-3B, Tokyo Rikakikai Co., Ltd., Tokyo) at a pressure of 90 mmHg with oxygenated modified Krebs-Henseleit solution containing (in mmol/L): NaCl 118, KCl 4.7, CaCl\(_2\) 2.5, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, NaHCO\(_3\) 2.5, glucose 5, and sodium pyruvate 5. The perfusate was continuously bubbled with 95% \( \text{O}_2 \)-5% \( \text{CO}_2 \) and was maintained at 37°C. Another polyethylene cannula (Venula 16 G, TOP, Tokyo) was inserted into the abdominal aorta for retrograde perfusion of the cervical and thoracic sympathetic nervous systems at 90 mmHg. The pulmonary artery and superior caval vein were ligated, and right atrial pressure was maintained at 5 cm H\(_2\)O by the height of the reservoir connecting to the inferior caval vein cannula (PE 205). Four bipolar electrode catheters (TT203-010, Unique Medical, Tokyo) were positioned at the upper, lower, septal, and lateral right atrium (RA) for local stimulation (Cardiac Stimulator, SANEI, Japan) and electrogram recording (EP Lab, Quinton Electrophysiology Co., USA). The interpolar distance was 1 mm, and the interelectrode distance between the upper and lower RA was approximately 10 mm.

**Sympathetic nerve stimulation:** After the spine was cut at the first level of cervical vertebrae (C1), the spinal cord from C1 to the sacral end was carefully pithed using a steel wire. A platinum bipolar electrode was inserted and placed at the first to fifth level of the thoracic vertebrae to stimulate the cardiac sympathetic nerve system.\(^\text{17,18}\) Cardiac sympathetic nerve stimulation (SNS) was performed by electrical field stimulation from the thoracic spinal canal for 5 seconds with a series of monophasic square pulses of 1-msec duration, 3 Hz, and 5 V.\(^\text{17,18}\) In a preliminary study, the reproducibility of the increased heart rate responses from 10 repeated SNSs was confirmed in both control and DM rats (data not shown, \( n = 5 \) in each group).

**Parasympathetic nerve stimulation:** After making a midline incision in the neck,
the right and left vagal nerves were carefully isolated and transected, achieving as much cranial proximity as possible. Each vagal nerve was placed on a platinum bipolar electrode for electrical stimulation. Parasympathetic nerve stimulation (PNS) was performed for 5 seconds with a series of monophasic square pulses of 1-msec duration, 3 Hz, and 5 V. In a preliminary study, the reproducibility of the decreased heart rate responses from 10 repeated PNSs was confirmed in both control and DM rats (data not shown, \( n = 5 \) in each group).

**Electrophysiological study:** Burst pacing (1200 beats/min, 15 seconds) from the upper RA with twice diastolic threshold current was performed for the induction of AF. The induced rhythm was defined as AF when a rapid atrial irregular rhythm was maintained for more than 5 seconds. Inducibility of AF was defined as the induction rate by 5 repeated bursts. The atrial effective refractory period (AERP) was measured by a 2-msec-step decremental technique during 8 regularly paced beats at a cycle length of 200 msec at 4 different sites (upper, septal, lateral and lower) in the RA. AERP was defined as the longest S1-S2 coupling interval that failed to result in atrial capture. Mean AERP was determined as the average AERP from 4 sites in the RA. The variance of AERPs determined by \( \frac{\sum_{i=1}^{n} (\chi_i - \chi)^2}{n} \) (\( \chi_i \): each AERP) was also defined as heterogeneity. The intra-atrial conduction velocity (CV) from the upper to lower RA was obtained during upper RA pacing at a cycle length of 200 msec. These electrophysiological studies were repeated just after a 5-second SNS (\( n = 8 \) in each group) or 5-second PNS (\( n = 8 \) in each group).

**Histological examination:** After the Langendorff experiment, perfusion fixation with 4% paraformaldehyde solution at a perfusion pressure of 90 mmHg was conducted in 7 DM and 7 control rats randomly selected from each group. The RA was isolated and embedded in paraffin, sections of which (4- \( \mu \)m thick) were stained with Azan stain (Mallory Heidenhain) for observing interstitial fibrosis. In these stained sections, the ratio of the area occupied by interstitial fibrosis to the total area was determined using computer-assisted image analysis software (Lumina Vision).

**Immunohistochemical staining:** Rats (\( n = 14 \) in each group) were transcardially perfused with heparinized saline followed by a cold fixative containing 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). The RA was immediately dissected out after perfusion, and immersed in the same fixative for 24 hours at 4°C. To investigate the distribution of sympathetic innervation in the RA, we examined the density of tyrosine hydroxylase (TH)-positive nerves using an immunohistochemical method in control and DM rats (\( n = 7 \) in each group). Whole RA tissues were soaked in methanol with H\(_2\)O\(_2\) for 30 minutes. After washing in phosphate-buffered saline (PBS; 0.01M, pH 7.2), they were reacted with normal goat serum (dilution 1:20, Vector Laboratories, Burlingame,
CA, USA) for 30 minutes. They were then incubated with primary rabbit antibody against TH (Eugene Tech International, Allendale, NJ, USA) at a dilution of 1: 1000 in PBS containing 4% normal goat serum for 48 hours at 4°C. After rinsing with PBS, they were incubated with biotinylated antibody to rabbit IgG (dilution 1: 200, Vector Labaratories) for 2 hours. After rinsing with PBS, they were reacted with avidine and biotin-peroxidase complex (ABC) reagent (Vector Laboratories) for 90 minutes. Finally, they were reacted for 5-10 minutes with 0.05% 3,3’-diaminobenzidine dihydrochloride (Sigma Chemical, St. Louis, MO, USA) and 0.01% hydrogen peroxide in Tris buffer (pH 7.6).\textsuperscript{21,22} To investigate the distribution of the parasympathetic terminals in RA, we examined the density of acetylcholinesterase (AChE)-positive nerves using an enzyme-histochemical method\textsuperscript{22} in control and DM rats (n = 7 in each group). Whole RA tissues were placed in medium containing the following: acetylcholine iodide, 5 mg; 0.1 M sodium citrate, 0.5 mL; 0.04 M CuSO\textsubscript{4}; 1 mL; 0.005 M potassium ferricyanide, 1 mL; and 0.1 M sodium hydrogen malic acid buffer (pH 6.0), 6.5 mL. They were soaked in this medium at 4°C overnight after incubation at 37°C for 30 minutes.\textsuperscript{22,24} After staining, these whole tissues were mounted on a glass slide coated with gelatin (whole mount preparation), dried at room temperature, and coverslipped.\textsuperscript{21} These samples were distributed over 4 areas (upper, lower, septal and lateral), and 4 fields in each area were randomly selected. TH-positive nerves (n = 7 in each group) or AChE-positive nerves (n = 7 in each group) in these samples were manually traced by a light microscope equipped with a camera Lucida drawing tube.\textsuperscript{21,25} We calculated the average nerve density of each area (mean nerve density) using a computer analyzing system that calculated the ratio of each traced nerve area to the total area in each sample (scion image). The variations in nerve density determined by $\sum_{i=1}^{n} \frac{(x_i - \bar{x})^2}{n}$ ($\bar{x}$: average nerve density, $x_i$ : each nerve density) in all RA fields were also calculated and defined as heterogeneity.\textsuperscript{20}

**Statistical analyses:** Data are presented as the mean ± SD. Results of the general characteristics and echocardiography parameters were analyzed using the unpaired Student’s t-test. Results of electrophysiological and histological examinations were analyzed using two-way ANOVA followed by Fisher’s post hoc test. Differences were considered significant at a level of $P < 0.05$.

**Results**

The Table shows comparisons of the general characteristics and echocardiography findings between control and DM rats. The body weight of DM rats was significantly lower than that of controls ($P < 0.01$). In contrast, the heart weight/body weight ratio of DM rats was significantly higher than that of con-
controls (P < 0.01). Blood glucose was higher in DM than in control rats (P < 0.01). There was no significant difference in systolic tail artery blood pressure and heart rate in the conscious state between control and DM rats. Furthermore, there was no significant difference in left ventricular end-diastolic diameter and
left atrial diameter between control and DM rats, although the left ventricular ejection fraction was slightly but significantly reduced in DM rats compared to control rats ($P < 0.05$).

**Figure 2.** The incidence of AF by sympathetic nerve stimulation (SNS) in control rats ($n = 8$) and DM rats ($n = 8$). SNS did not alter the incidence of AF in control rats (11 ± 4 to 14 ± 6%) but significantly increased that in DM rats (14 ± 6 to 30 ± 8%). The incidence of AF by SNS was significantly higher in DM rats than in control rats.

*a* $P < 0.01$ versus before SNS in the same group. # $P < 0.01$ versus control rats.

**Figure 3.** Comparisons of AERP at each of the 4 sites (upper, lower, septal and lateral) in the right atrium (A) and the heterogeneity of AERPs (B) with or without SNS in control rats ($n = 8$) and DM rats ($n = 8$). AERP at each of the 4 sites was significantly shortened by SNS in both rats. The heterogeneity of AERPs was markedly increased by SNS in DM rats but not in control rats, and was significantly higher in the former than in the latter. * $P < 0.01$ versus before SNS in the same group. # $P < 0.01$ versus control rats.
Electrophysiological findings: In the basal state without SNS or PNS, the incidences of AF, mean AERP, and CV were not markedly different between control and DM rats as shown in Figure 1. The influences of SNS on the incidence of AF and AERP are shown in Figures 2 and 3. SNS significantly increased the in-
The incidence of AF in DM rats (14 ± 6 to 30 ± 8%, \( P < 0.01 \)), but not in control rats (11 ± 4 to 14 ± 6%, NS) (Figure 2). AERP at each of the 4 sites was markedly and similarly shortened by SNS in both rats (each \( P < 0.01 \)) (Figure 3A). The heterogeneity of AERPs was significantly increased by SNS only in DM rats (\( P < 0.01 \)) (Figure 3B). CV was unchanged by SNS in both rats (0.81 ± 0.08 to 0.82 ± 0.08 mm/msec in control rats, 0.78 ± 0.08 to 0.82 ± 0.06 mm/msec in DM rats).

The influences of PNS on the incidence of AF and AERP are shown in Figures 4 and 5. PNS dramatically increased the incidence of AF in both rats (9 ± 5 to 30 ± 5% in control rats, 12 ± 6 to 27 ± 6% in DM rats, each \( P < 0.01 \)) (Figure 4). However, the increased rate of the incidence of AF by PNS was much higher in control than in DM rats (3.3 ± 0.8 in control rats, 2.2 ± 0.7 in DM rats, \( P < 0.05 \)). AERP at each of the 4 sites was significantly and similarly shortened by PNS in both rats (each \( P < 0.01 \)) (Figure 5A). The heterogeneity of AERPs was notably increased by PNS in control rats (\( P < 0.05 \)), but not in DM rats (Figure

**Figure 6.** Atrial immunohistochemical staining of tyrosine hydroxylase-(TH) positive nerves at the 4 right atrial sites (upper, lower, septal and lateral). A: The tracing samples of TH-positive nerves at the 4 right atrial sites. B: Histograms of mean TH-positive nerve density at all 4 atrial sites. Although the mean nerve density of TH-positive nerves tended to be lower in DM rats (\( n = 7 \)) than in control rats (\( n = 7 \)) at each of the 4 sites, this difference reached statistical significance only for the lower site. C: Graph shows the heterogeneity of TH-positive nerves. The density of TH-positive nerves was more heterogeneous in DM rats than in control rats. + \( P < 0.01 \) versus control rats.
CV was unchanged by PNS in both rats (0.79 ± 0.06 to 0.72 ± 0.08 mm/msec in control rats, 0.80 ± 0.07 to 0.75 ± 0.07 mm/msec in DM rats).

**Histological findings:** The quantified relative areas of interstitial fibrosis in DM rats evaluated by Azan staining did not differ from those in controls (7.1 ± 0.8% in control rats, 7.6 ± 0.9% in DM rats, each NS). Figure 6A shows the tracing of TH-positive nerves in both rats. Although the mean density of TH-positive nerves seemed to be lower in DM than in control rats at each of the 4 sites, this difference reached statistical significance only for the lower site (Figure 6B). Thus, the density of TH-positive nerves was more heterogeneous in DM rats than in controls (P < 0.01), as shown in Figure 6C. Figure 7A shows the tracing of AChE-positive nerves in both rats. Although the mean density of the AChE-positive nerves was significantly lower in DM rats than in controls at each of the 4 sites (P < 0.01) (Figure 7B), no significant heterogeneity in their density was observed between the rats (Figure 7C).
DISCUSSION

This is the first study to evaluate the influence of the autonomic nervous system on the vulnerability leading to AF in diabetic hearts. The main findings obtained with STZ-induced diabetic rat hearts are as follows: First, SNS significantly increased the incidence of AF in DM but not in control rats. AERP was shortened by SNS in both rats, but the heterogeneity of AERP by SNS was increased in DM rats compared with that in controls. Second, PNS significantly decreased AERP and increased the incidence of AF in both rats. Third, the density of TH-positive nerves was heterogeneous in DM compared with control rats, whereas that of AChE-positive nerves decreased homogeneously in DM rats. Thus, the prevalence of AF was enhanced by adrenergic activation in diabetic hearts, in which heterogenous sympathetic innervation was evident. These findings suggest that neural remodeling may play a crucial role in increased AF vulnerability in DM.

Various arrhythmogenic mechanisms, ie, autonomic dysfunction,\textsuperscript{12-14} conduction delay, and repolarization abnormalities\textsuperscript{26,27} have been proposed in the diabetic heart. However, these abnormalities have mainly been observed at the ventricular level. In other words, there is a lack of evidence needed to determine whether these arrhythmogenic substrates exist at the atrial level and relate to the vulnerability leading to AF in diabetic hearts. A recent study showed that intra-atrial conduction delays and increased fibrotic depositions in Type 2 DM rat atria play a principle role in producing atrial tachyarrhythmias.\textsuperscript{11} These results suggest that alterations in atrial electrophysiological properties and tissue structure may be one of the mechanisms for inducing AF in diabetic hearts. On the contrary, in this study using Type 1 DM rats, the inducibility of AF, AERP, and CV without autonomic nerve stimulation was unchanged in DM compared to control rats. In addition, dilatation of the left atrium and interstitial fibrotic change were not indicated in DM rats compared to controls. Our results suggest that electrophysiological and structural remodeling do not occur, at least at this stage (16 weeks of age), in Type 1 DM rats. Accordingly, when considering the difference between other results and ours, whether or not fibrotic change is present in the atrium seems to be critical. Several experimental studies have documented that atrial fibrosis is closely associated with a propensity toward AF through slowing down the atrial conduction velocity,\textsuperscript{11,28} especially in aged rats.\textsuperscript{20} However, the results of studies of myocardial interstitial fibrosis in DM have varied widely.\textsuperscript{11,29,30} Our previous study has revealed that interstitial fibrosis does not appear in the Otsuka Long-Evans Tokushima Fatty (OLETF) rat type 2 DM model.\textsuperscript{30} Further study is needed to determine how fibrotic change could be observed in a DM animal model, and to clarify the relevance of atrial fibrosis to
AF vulnerability in DM.

Many clinical observations have suggested that the autonomic nervous system is involved in the genesis of at least some forms of AF. Coumel, et al have described different types of paroxysmal AF resulting from an increased vagal tone or a rise in adrenergic tone in clinical settings. However, there has been little direct evidence to support an important role for adrenergic action in producing AF. Rensma, et al demonstrated that isoproterenol or propranolol administration failed to change AERP or CV, and Liu and Nattel showed that the induction of AF was increased by PNS but remained unchanged by SNS in normal dogs. Contrary to previous reports, the present study showed that SNS significantly increased the incidence of AF and the variability in AERP in DM rats. This is the first report indicating that adrenergic activation could create an atrial milieu for producing AF in the diabetic heart. Furthermore, we demonstrated that the density of TH-positive nerves was more heterogeneous in DM than in control rats. This result suggests that heterogeneous cardiac sympathetic denervation is present in DM rats. Olgin, et al showed that heterogeneous sympathetic denervation of the atria by topical phenol application results in the heterogeneity of refractoriness and promotes AF. Jayachandran, et al used positron emission tomography (PET) imaging to document that dogs with AF showed inhomogeneous changes in atrial sympathetic innervation. Therefore, it is speculated that increased heterogeneous sympathetic denervation, regional adrenergic activation, and heterogeneity of refractoriness might be partly responsible for the occurrence of AF in diabetic hearts.

In one clinical study, the autonomic tone before the onset of AF has been used to identify so-called vagally mediated AF. In addition, experimental studies have shown that PNS dramatically shortens the AERP and increases its dispersion, which in turn contributes to the vulnerability of AF in the normal dog heart. In the present study, PNS significantly reduced AERP, increased the heterogeneity of AERP, and enhanced the incidence of AF in control rats, findings consistent with previous observations of PNS. However, the role of the parasympathetic nervous system in a diabetic heart is less clear. Several studies have shown that parasympathetic activity is suppressed in diabetic hearts. Moreover, Uehara, et al demonstrated that defects in parasympathetic innervation were more frequent and occurred relatively earlier in DM than defects in sympathetic innervation. In this study, the density of AChE-positive nerves decreased homogeneously in DM rats compared to controls. In addition, the heterogeneity of AERP by PNS did not appear in DM rats, and the rate of an increased incidence of AERP by PNS was lower in DM than in control rats. Thus, our results suggest that PNS may be much less effective than SNS in promoting AF in a diabetic heart, in which parasympathetic denervation is progressive
compared to that in a control heart.

**Study limitations:** There are some limitations in the present study. First, it remains unknown whether the alterations in sympathetic and parasympathetic innervation observed in this study also occur in other types of DM model or in the old stage of DM. Therefore, further study using a different DM model is needed to clarify these issues. Second, we measured variables under an open chest and anesthetized conditions. Since it is reported, on the basis of heart rate variability studies, that an imbalance between sympathetic and parasympathetic activity influences the heart in DM, we need to investigate these influences under conscious conditions in the future. Third, we did not investigate the cellular ionic currents of atrial myocytes. The possible influence of autonomic nerve activation on membrane ionic channels should also be examined.

**Clinical implications:** If heterogeneous increases in sympathetic innervation contribute to the development of AF, then creating a homogeneous sympathetic milieu might be antifibrillatory. In fact, Yano, *et al* have reported that bilateral stelllectomy is effective in the prevention of AF in a dog rapid atrial pacing model. This suggests that β-adrenoreceptor blockade might be expected to prevent AF promotion in a diabetic heart. The present study indicates that the future development of antiarrhythmic interventions in DM should target not only the prevention of electrophysiological remodeling, but also that of neural remodeling, especially via sympathetic nerve activation.

**References**

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