Conduction and refractory disorders in the diabetic atrium

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The number of patients with diabetes mellitus (DM) has been increasing. DM is strongly associated with increased mortality and an independent risk factor for various cardiovascular diseases (2) such as myocardial infarction and atrial fibrillation (AF) (12). Especially, DM increases the incidence of stroke and death in patients with AF (14).

Recent experimental studies reported that the increased susceptibility to AF in the diabetic animals (13, 26) was presumably due to the slowing of conduction associated with increased interstitial fibrosis (13) and the augmentation of the heterogeneity in the atrial effective refractory period (ERP) produced by sympathetic nerve stimulation (26). However, the arrhythmogenic mechanisms in the diabetic atrium remain undetermined. Especially, the underlying mechanisms for the electrical alterations of diabetic atrium in response to rapid activation need to be elucidated because most of paroxysmal AF has been known to occur as a consequence of rapid firing from pulmonary veins (8).

We thus aimed to determine whether the slowing and heterogeneity of conduction and/or the spatiotemporal dispersion of the action potential duration (APD) are increased in the diabetic atrium and these changes are augmented as the atrial activation rate becomes faster. For this purpose, we investigated the precise conduction pattern and the spatiotemporal changes in action potential (AP) at various pacing cycle lengths in the right atrium obtained from streptozotocin (STZ)-induced diabetic rats using an optical mapping system.

METHODS

Experimental Animals

The study was approved by our institutional animal research committee and conformed to the animal care guidelines for the Care and Use of Laboratory Animals in Hokkaido University Graduate School of Medicine.

Diabetes was induced by intraperitoneal injection of STZ (65 mg/kg) dissolved in citrate buffer in 8-wk-old male Wister rats (n = 27). Age-matched control rats (n = 26) received an equivalent volume of the citrate buffer solution alone. One week later, the diabetic state was confirmed by blood glucose of higher than 300 mg/dl. Sixteen weeks after the administration of STZ or the citrate buffer, the tail artery blood pressure and heart rate were measured in a conscious state by the oscillometric method (BP-98A; Softron, Tokyo, Japan). After the rats were fasted for 12 h, blood samples were collected from the internal jugular vein under anesthesia with inhalation of diethyl ether. Blood glucose was measured in both groups of rats using a glucometer (Glutest Ace R; Sanwa Kagaku Kenkyusho, Nagoya, Japan).

Langendorff Perfusion

The heart was excised from control or DM rats after anesthesia with intraperitoneal injection of pentobarbital (50 mg/kg) and heparin sodium (400 IU/kg). The heart was mounted on a Langendorff apparatus and was retrogradely perfused with Tyrode solution (37°C) containing (in mM) 143 NaCl, 5.4 KCl, 0.33 NaH2PO4, 5 HEPES, 5.5 glucose, 0.5 MgCl2, and 1.8 CaCl2 (pH 7.4 using NaOH) gassed with 100% O2 until the beating rate became stable (5–10 min). Next, optical mapping (in 8 control and 10 DM rats) or AF induction (in 12 control and 10 DM rats) was performed.

ERP and Susceptibility to Atrial Tachyarrhythmia

ERP and the duration of atrial tachyarrhythmia (AT) were measured in 12 control and 10 DM rats. Two electrodes were attached to the left atrial appendage (LAA) and right ventricle for the recording of electrocardiogram. Another electrode was attached to the right atrial appendage (RAA) for delivering programmed stimulations as a unipolar cathode. The indifferent anodal electrode was attached to the extracardiac tissue.

The intensity of the current pulse was twice that of the threshold, and the pulse duration was 0.5 ms. ERP was measured by introducing S2 extrastimulus with 5-ms decrements following eight regulatory
S₂–S₃ stimuli of 150 ms and defined as the longest S₁–S₂ interval at which S₂ failed to induce a propagated response. We measured ERP two times on each animal, and the average of them was defined as ERP of each animal. The induction of AT was performed by burst pacing five times repeatedly, at the pacing cycle length (PCL) from 70 to 30 ms in 5-ms decrements for 5 s. The duration of AT was defined as the longest time for persistence deviating from sinus rhythm for >0.1 s.

Optical Mapping

We used voltage-sensitive dye imaging technology according to the methods reported previously (6). After the beating rate of the isolated heart became stable, the perfusate was switched to Tyrode solution containing 10 μM blebbistatin, a selective myosin II inhibitor, to eliminate motion artifact without any effects on the electrical parameters (7). Next, the heart was continuously perfused with Tyrode solution containing 10 μM blebbistatin at 37°C. Optical recordings were obtained during incremental stimulation in RAA at PCL of 200, 150, 100, and 80 ms and in 5-ms decrements until the pacing stimuli failed to capture one-to-one beating of the atrium.

Data Processing

Optical signals were analyzed using custom-made software (BrainVision Analyzer). To extract RA from original data, the region of interest (ROI) was selected manually. The spatial filtering procedure (7 × 7 for activation analysis and 5 × 5 for APD analysis) was conducted to remove the noise. Local activation was determined as the time point of maximum change in fluorescence over time (dF/dt) for each fluorescence signal. An activation map was constructed by calculating the time delay of the activation at each pixel relative to the pacing site.

A conduction velocity (CV) vector map was constructed from an activation map with custom-written software using an algorithm as described previously (4). Average CV was calculated by averaging CV from each vector of the ROI. To assess the difference in the conduction heterogeneity between control and DM rats, we examined the phase difference (17), which indicates the maximum difference in time points of the focal activation among the neighboring pixels. To calculate phase difference, optical signals extracted from a rectangular ROI (49 × 41 pixels, the distance between neighboring pixels ~0.13 mm) were compressed to 13 × 11 pixel area (the distance between neighboring pixels ~0.52 mm). Next, the largest difference in the activation time point between one pixel and its neighboring pixels divided by the distance between pixels in a compressed area was defined as the phase difference at each point (17). Frequency histograms were constructed for the phase difference in the compressed pixel areas. The absolute heterogeneity was calculated by subtracting the 95th percentile from the 5th percentile of the phase difference. The heterogeneity index was determined by dividing this value by the median of the phase difference (17).

APD at 80% repolarization (APD₈₀) and that at 50% repolarization (APD₅₀) were determined as the time difference between the point of activation [i.e., the time point of maximum upstroke fluorescence change over time (dF/dt)] and the point at 80 and 50% repolarization of maximum AP amplitude, respectively. To examine the spatial dispersion of APD, we measured APD₈₀ at four area as follows: RAA, right atrial free wall (Free wall), high right atrial (HRA) wall, and low right atrial (LRA) wall. HRA or LRA was defined as the area below SVC or above IVC in anatomic orientation, and the area of the Free wall was determined as one that was equally distant from RAA, HRA, and LRA (shown in Fig. 1 and see Fig. 5A).

APD restitution curves were constructed by plotting APD₈₀ against the preceding diastolic interval during burst pacing (i.e., dynamic restitution), and the data points were best fitted to a single exponential (15).

We measured pairwise difference in APD₈₀ to examine the incidence of the beat-to-beat alteration in APD, so-called “APD alternans.” APD alternans was considered to be present when there was alternating lengthening/shortening in consecutive APs and the difference of APD₈₀ in consecutive beats was >4 ms and 10%, respectively (28).

Histology

Histological analysis of RA was performed in six control and six DM rats. Right atrial tissues were dissected from the hearts and stored in neutral buffered formalin for ~1 wk. The sections of the atrial tissue were stained with Masson’s trichrome. In these stained sections, the ratio of the area occupied by interstitial fibrosis was measured using a public domain software (Image J; NIH, Bethesda, MD).

Western Blot Analyses

Western blot analyses for connexin 40 (CX 40) and connexin 43 (CX 43) were performed in six control and seven DM rats. The atrial tissues were snap-frozen, homogenized, and dissolved in 1 × cell lysis buffer (Cell Signaling, Danvers, MA), supplemented with 1 × Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). After centrifugation at 15,000 rpm for 20 min at 4°C, the supernatants were separated into aliquots and stored at ~80°C until the time of...
The duration of AT was significantly longer in DM rats (98.1 ± 0.5 ms was longer in DM (P < 0.05; Fig. 2, A and C). In addition, the average cycle length of AT sustained ≥0.5 ms was longer in DM (n = 7) than in control (n = 4) rats (98.1 ± 7.2 vs. 67.9 ± 6.5 ms, P < 0.05).

**Optical Mapping**

Conduction velocity. The activation maps and their CV vector maps within the RA from control and DM rats were constructed at PCL of 200 ms (Fig. 3A). In control rats, a stimulation pulse from RAA propagated RA almost uniformly (Fig. 3A, left). In contrast, isochronal crowding, indicating focal conduction delay, was observed, and the direction and magnitude of CV vector varied widely in DM rats (Fig. 3A, right). The average CVs, calculated from the conduction vector maps, were significantly slower in DM rats at any PCL (Fig. 3B).

**Phase difference.** Figure 4 shows representative activation maps (Fig. 4, left), phase difference maps (Fig. 4, middle), and phase difference histograms (Fig. 4, right) in control (Fig. 4A) and DM (Fig. 4B) rats. In control rats, focal conduction block was rarely seen, and the phase difference was distributed within 4.2 ms/mm even at the faster PCL (Fig. 4A). In contrast, it was increased in DM compared with control rats even at PCL of 200 ms (Fig. 4B). Moreover, a block line emerged at the shorter PCL, and the phase difference histogram showed wider distribution in DM rats (Fig. 4B). In addition, most of the block lines in DM rats were seen at the posterolateral portion of the atrial free wall that was close to the crista terminalis. Quantitative analysis of the phase difference demonstrated that the absolute heterogeneity was significantly greater in DM rats at any PCL (Fig. 4C). In addition, the absolute heterogeneity became larger in DM rats in accordance with the shortening of PCL. Similarly, heterogeneity index was greater at any PCL in DM rats with rate-dependent augmentation.

**ERP and Susceptibility to AT**

ERP in RAA was significantly longer in DM (n = 10) than control (n = 12) rats (49 ± 4 vs. 38 ± 2 ms, P < 0.05; Fig. 2A). The duration of AT was significantly longer in DM than control rats (2.4 ± 0.6 vs. 0.9 ± 0.3 s, P < 0.05; Fig. 2, B and C). In addition, the average cycle length of AT sustained ≥0.5 ms was longer in DM (n = 7) than in control (n = 4) rats (98.1 ± 7.2 vs. 67.9 ± 6.5 ms, P < 0.05).

**Statistical Analysis**

All data are presented as means ± SE. Simple between-group analyses were conducted by Student’s t-test. When data were analyzed across more than two cycle lengths, two-way repeated ANOVA was used. Categorical variables were compared using the Chi square test. Differences with P < 0.05 were considered significant.

### RESULTS

#### Animal Characteristics

Table 1 shows the characteristics of control and DM rats. The fasting blood glucose was significantly higher in DM rats. Body weight and heart weight were significantly lower in DM than control rats. However, the ratio of heart to body weight was significantly higher in DM rats. Systolic blood pressure and heart rate were significantly lower in DM rats.

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Values are means ± SE; n, no. of rats. DM, diabetes mellitus.
LRA wall, are shown in Fig. 5. APD in control rats was almost similar among four areas (Fig. 5A). In contrast, it varied widely among four areas in DM rats, and its duration was longer compared with control rats (Fig. 5A). The APD80 at each area was significantly longer in DM rats (Fig. 5B), and the average values in these four areas were also longer in DM rats (53 ± 2 vs. 40 ± 3 ms, P < 0.01). The coefficient of variation (SD/mean) of APD80 obtained from four areas was significantly greater in DM rats (Fig. 5C), indicating that the spatial dispersion of APD80 was increased in DM rats.

In addition to the spatial dispersion of APD, the rate-dependent changes in APD were also evaluated (Fig. 6). Figure 6A shows superimposed optical recordings of AP from RAA. While no apparent changes in APD were observed among different PCLs in control rats (Fig. 6A, left), it became shorter as PCL decreased in DM rats (Fig. 6A, right). The ratios of APD50 (Fig. 6B) and APD80 (Fig. 6C) at PCLs of 150, 100, and 80 ms to those at PCL of 200 ms were significantly smaller in DM than in control rats. In analysis of APD restitution properties, the maximum slope of the APD restitution curve was >1 in two control (25%) and two DM (20%) rats [P = not significant (NS)]. There was no significant difference in the maximum restitution slope between control and DM rats (0.67 ± 0.22 in control vs. 0.81 ± 0.22 in DM rats, P = NS) (Fig. 6D).

**AP alternans.** During the continuous optical recordings, AP alternans was more frequently observed in DM than in control rats (0 vs. 100%, P < 0.001) (Fig. 7, A and B). In Fig. 7C, the relationship between APD alternans and PCL was shown. The average of the longest PCL, at which APD alternans were observed in DM rats, was 99 ± 7 ms. Moreover, in DM rats, as PCL became much shorter, AP frequently diverted to complex oscillation, and finally focal conduction block was observed (Fig. 7B).

**Histological Analysis**

Masson trichrome staining of the right atrial section demonstrated that interstitial fibrosis was more abundant in DM than in control rats (Fig. 8).

**Western Blot Analyses**

The expression of CX 40 protein in the atrial tissue was significantly lower in DM than control rats (Fig. 9, A and B). On the other hand, there was no significant difference in the expression of CX 43 protein between two groups of rats (Fig. 9, C and D).

**DISCUSSION**

The present study clearly demonstrated the important electrophysiological properties of the diabetic atrium compared with normal controls as follows. First, the susceptibility to AT was increased. Second, conduction slowing and its heterogeneity were increased. Third, APD was longer, and its spatial dispersion was increased. Fourth, the shortening of APD with the decrease in PCL was augmented. Fifth, APD alternans was more frequently observed. Sixth, interstitial fibrosis was in-
Increased. Finally, CX 40 protein expression was reduced. This is the first study that demonstrated the rate-dependent changes in CV, its heterogeneity, and AP in the diabetic atrium. These changes in electrophysiological and histological properties and gap junction protein might be the substrate for AF in the diabetic atrium.

Conduction Slowing and Heterogeneity

The present study demonstrated, for the first time, a significantly increased conduction heterogeneity and conduction slowing in the RA from DM rats using optical mapping. Recently, Kato et al. (13) examined the interatrial conduction time from RAA to the LAA during RAA pacing and reported the increase in the interatrial conduction time in the diabetic atrium (13). In their study, an interatrial conduction time was defined as a time interval for an activation to conduct from RAA to LAA, which could be influenced by the surface area of the atria, the direction of conduction, and focal conduction blocks, as well as CV. In contrast, CV from a conduction vector map represent the average of the global conduction within the atrium (4), and the heterogeneity represents focal conduction block or slowing (17). In addition, the optical mapping employed in the present study depicted the conduction pattern in the global RA with high spatial resolution (4).
Therefore, our optical mapping methods would represent the precise propagation in RA.

Previous studies reported that the conduction slowing and heterogeneity might lead to the greater propensity for AF in transforming growth factor-β1 transgenic mice (35) and the hypertensive ovine model (18). In addition, Roberts-Thomson et al. (29) demonstrated increased conduction delay and heterogeneity in the left atria from patients with mitral regurgitation.

Fig. 5. A: representative superimposed recordings of action potential from 4 areas (RAA, Free wall, HRA wall, and LRA wall) at PCL of 200 ms in control (left) and DM (right) rats. B: summary data of action potential duration at 80% repolarization (APD_{80}) at 4 areas from control (n = 8) and DM (n = 9) rats. C: the coefficient of variation (CoV, the ratio of SD to mean) in control (n = 8) and DM (n = 9) rats. *P < 0.05 and **P < 0.01 vs. control.

Fig. 6. A: representative superimposed recordings of action potential in right atrial appendage at PCL of 200 (black), 150 (red), 100 (blue), and 80 (green) ms from control (left) and DM (right) rats. The ratio of action potential duration at 50% repolarization (APD_{50}) and that of APD_{80} (C) at PCL of 150, 100, and 80 ms to that at 200 ms in control (n = 8) and DM (n = 10) rats. *P < 0.001 vs. control. D: maximum slope of action potential duration (APD) restitution curve in control (n = 8) and DM (n = 10) rats. P = not significant (NS).
and persistent AF compared with those in sinus rhythm. Therefore, increased conduction slowing and its heterogeneity in the diabetic atrium seen in this study might be one of the substrates for AF.

In this study, we, for the first time, demonstrated the reduced expression of CX 40 protein in the diabetic atrium (Fig. 9). Prior studies reported that AF is associated with the decrease in CX 40 and/or CX 43 protein, the principal atrial gap junction proteins, and/or the abnormal distribution of them (10, 16) and that those remodelings of gap junction proteins were responsible for the conduction abnormality in AF (10). In analyses of the diabetic ventricle, the abnormal distribution of CX 43, the principal ventricular gap junction protein, and the reduced conduction reserve were reported (25). Therefore, we interpret that the reduced expression of CX 40 protein, as well as the increased interstitial fibrosis (Fig. 8), was responsible for the conduction slowing and heterogeneity in the diabetic atrium.

Prolongation of APD in Diabetic Atrium

Although the prolongation of APD in the ventricle has been observed in an earlier diabetic model (33), there have been limited data regarding APD or ERP in the diabetic atrium (27). We demonstrated the prolongation of APD in the diabetic atrium at physiological conditions (Fig. 5). These findings are consistent with the previous study by Pacher et al. (27) in which the prolongation of APD was augmented in STZ-induced diabetic rats. In contrast, other studies could not find any significant changes in ERP between control and DM animals (13, 26). One of the reasons for this discrepancy might be the differences in the types of diabetic model (type 1 vs. type 2) and the duration of diabetic condition of the experimental animals (8 vs. 16 wk).

There are several potential mechanisms responsible for the prolongation of APD in the diabetic atrium. One possible mechanism is the attenuation of $K_{\text{Ca}}$ current in the diabetic atrium. Previous study in diabetic ventricular myocytes observed the attenuation of the transient outward $K_{\text{Ca}}$ current and the steady-state $K_{\text{Ca}}$ current (33), which are now thought to be major causes for the prolongation of APD. Therefore, the same mechanism might be involved also in the diabetic atrium. Another possibility is the augmentation of $Na^+/Ca^{2+}$ exchanger (NCX) current (1, 24). Cytosolic $Ca^{2+}$ ($\left[Ca^{2+}\right]_i$) is reported to be increased in ventricular myocytes from the diabetic animals due to downregulation of sarcoplasmic reticulum $Ca^{2+}$-ATPase 2a (SERCA2a) (1, 23). The increased...
[Ca\(^{2+}\)]_i is thought to augment the forward-mode NCX currents (i.e., inward currents), resulting in the prolongation of APD.

**Frequency-Dependent Shortening of APD**

In this study, we, for the first time, demonstrated the increased frequency-dependent shortening of APD in the diabetic atrium, which might be explained by the abnormal Ca\(^{2+}\) handling in diabetes. It has been reported that reuptake of [Ca\(^{2+}\)]_i is insufficient due to downregulation of SERCA2a in the diabetic ventricle (1, 23). This reduced reuptake of [Ca\(^{2+}\)]_i might be augmented as the heart rate increases (because of the reduced diastolic interval), thereby leading to a much higher [Ca\(^{2+}\)]_i level. Next, a frequency-dependent increase in [Ca\(^{2+}\)]_i would cause inactivation of the L-type calcium channel (\(I_{\text{Ca,L}}\)) and the shortening of APD.

This hypothesis might be partially supported by the recent report by Harada et al. (9). In their study, the frequency-dependent shortening of APD was increased in the failing rabbit heart, in association with the augmented frequency-dependent reduction in \(I_{\text{Ca,L}}\). That is because, as the PCL becomes shorter, functional reduction of \(I_{\text{Ca,L}}\), due probably to Ca\(^{2+}\)-induced inactivation of \(I_{\text{Ca,L}}\), occurs in the failing heart, in which reuptake of [Ca\(^{2+}\)]_i is reduced like in the diabetic ventricle.

**Arrhythmogenic Mechanisms for Prolonged and Increased Dispersion of APD**

In the present study, we demonstrated the prolongation in the cycle length of AT as well as increased susceptibility to AT in DM rats, which would imply that the prolongation of APD was associated with the increased susceptibility to AT in DM rats. The prolongation of APD due to reduced K\(^{+}\) current has been shown to produce early afterdepolarizations (3), thereby inducing torsades des pointes in long QT syndrome. Moreover, recent studies showed AF risk was increased in patients with congenital long QT syndrome (11). In fact, the prolongation of APD produced by cesium-induced loss of K\(^{+}\) channel activities promoted AF in anesthetized dogs (32). The ablation of small-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels resulted in the prolongation of APD in mouse atrial myocytes and facilitated induction of AF with unknown electrophysiological mechanisms (20).

In addition to the prolongation of APD, spatial dispersion of APD was increased in DM rats, indicating that atrial refractoriness differed within the whole atrium. Spatial heterogeneity of APD could easily produce conduction block, thereby increasing the vulnerability to AT (5). Moreover, the susceptibility to APD alternans was increased in diabetic atrium (Fig. 7). APD alternans is considered to be one of the central mechanisms of ventricular fibrillation (30). However, limited
data have been available concerning its contribution to the arrhythmogenesis of AF (22, 34). Tsai et al. (34) recently reported that the thresholds of APD and calcium transient alternans were significantly decreased in the mechanical stretched atrial myocyte monolayer, and these observations were rescued by the overexpression of SERCA2a. SERCA2a has been shown to be downregulated in diabetic ventricular myocardium (1, 23). Therefore, the decrease in SERCA2a might account for the increased susceptibility to APD alternans in the diabetic atrium.

Heart failure (HF) is also known as a common cause of AF in clinical settings (21). Sanders et al. (31) reported the prolonged atrial ERP and increased propensity for AF in patients with HF. Lee et al. (19) reported the augmented dispersion of the left atrial ERP and significant increase in AF duration in rapid pacing-induced HF dogs. The electrophysiological alterations such as prolonged and enhanced dispersion of APD in failing atrium are similar to those of diabetic atrium observed in this study. In addition, we demonstrated the low thresholds of APD alternans in the diabetic atrium. Therefore, these repolarization abnormalities might serve as a central mechanism of the vulnerability to AT in the atrial myocardium with DM or HF, thereby contributing to the development of a novel therapeutic strategy.

Study Limitation

First, we could not examine the maximum rate of depolarization. This is due to the analysis of a calibration of the optical signals, whose change did not indicate the absolute value of the voltage change (6). Second, we could not identify the mechanisms of the prolongation of APD. It should be evaluated whether the same mechanisms as demonstrated in the diabetic ventricle (1, 24, 33) are also involved in the diabetic atrium. Third, the abnormal regulation of intracellular Ca²⁺ transient has been demonstrated in the diabetic ventricular myocardium (1, 23), which might be involved in APD alternans (34).

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REFERENCES


