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Reduced expression of HCN channels in sinoatrial node of streptozotocin-induced diabetic rats

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1 **Reduced expression of HCN channels in sinoatrial node of**
2 **streptozotocin-induced diabetic rats**

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13 **Running title:** Expression of HCN channels in diabetic rat SAN

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Abstract

Diabetes mellitus (DM) is associated with an electrical remodeling of the heart, increasing risk of arrhythmias. However, knowledge of electrical remodeling in sinoatrial node (SAN) by DM is limited. We investigated the expression of HCN channel isoforms, HCN1-HCN4, in SAN from streptozotocin (STZ)-induced diabetic rats and the age-matched controls. We found that the STZ-induced diabetic rats have a lower intrinsic heart rate, a lengthened sinoatrial conduction time and rate-corrected maximal sinoatrial node recovery time in vivo as well as a longer cycle length (CL) in vitro as compared with the control. Optical mapping of the SAN demonstrated an inferior leading pacemaker site, a reduced SAN conduction velocity and diastolic depolarization slope, a longer action potential duration in the STZ-induced diabetic rats than the control. The transcripts and proteins of *HCN2* and *HCN4* in diabetic SAN were reduced. Specific blockade of HCN channels by 3 μ M ivabradine significantly prolonged CL of Langendorff heart by 18 % in the diabetic rats and 26 % in the control. The reduced expression of HCN channel isoforms in SAN of the STZ-induced diabetic rat may be an important contributor to the reduced SAN function in DM.

Keywords:

streptozotocin-induced diabetes, sinoatrial node dysfunction, HCN channels, sinoatrial node.

1 Introduction

2 Diabetes mellitus (DM) is associated with increased risk of arrhythmias, which may
3 occur in the absence of atherosclerosis, hypertension or valvular dysfunction ([Gami et](#)
4 [al. 2007](#)). Ventricular arrhythmias are the most frequent type of arrhythmia observed
5 in DM patients ([Veglio et al. 2000](#)). Electrophysiological studies have shown that the
6 action potential (AP) duration (APD) of ventricular myocytes was prolonged and the
7 transient outward K^+ current (I_{to}), L-type Ca^{2+} current (I_{CaL}), and inward rectifier
8 K^+ current (I_{K1}) were reduced in type 1 and 2 diabetic models from a variety of
9 species ([Lengyel et al. 2007](#); [Tsuchida and Watajima 1997](#); [Wang et al. 1995](#)).
10 Expression of the channel pore-forming subunits also decreased in the diabetic heart
11 ([Nishiyama et al. 2001](#); [Qin et al. 2001](#)). All these findings revealed an electrical
12 remodeling in the diabetic heart. However, knowledge of electrical remodeling in
13 SAN by DM is very limited.

14 Sinoatrial node dysfunction (SND), either tachycardia or bradycardia, is often seen in
15 both type 1 and type 2 DM ([Linnemann and Janka 2003](#)). A reduced spontaneous
16 pacemaking rate of the isolated heart and right atrial preparation was observed in
17 streptozotocin (STZ)-induced DM animal model with a lengthened APD of SAN,
18 which suggested a reduced SAN function ([Howarth et al. 2007](#); [Kofo-Abayomi and](#)
19 [Lucas 1988](#)). However, the underlying molecular mechanism of SAN electrical
20 remodeling associated with DM remains uncertain.

21 It is evidenced that the pacemaker current (I_f) carried by the
22 hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels plays an

1 important role in pacemaking and heart rate control. Mutations of HCN channel genes
2 were found to be associated with congenital SND (Milanesi et al. 2006). So far, four
3 HCN channel isoforms have been identified in mammal, that is HCN1-HCN4
4 (Ludwig et al. 1998). Considering the vital significance of HCN channels in
5 pacemaking, we hypothesized the altered expression of HCN channel isoforms may
6 be one of the molecular mechanisms of SND during DM.

7 **Materials and methods**

8 **Animal preparation**

9 DM was induced by a single intravenous injection of STZ (65 mg/kg; Sigma, USA)
10 through tail vein to Sprague-Dawley rats (200–250 g, 3 months old). After 72 h and
11 overnight fasting, blood samples were obtained from the tail vein and the glucose
12 concentration was measured. Animals with fasting blood glucose levels on above 250
13 mg/dl were considered as diabetic. In this experiment, 35 out of 40 rats were modeled
14 successfully. Age-matched controls received an equivalent volume of the citrate buffer
15 solution alone. All animals were maintained on the same diet and water ad libitum for
16 20 weeks. Non-fasting blood glucose level was measured every 4 weeks at 11 am.
17 Blood was obtained from a tail vein, and glucose concentrations were determined
18 using an Accu-Chek Performa glucose meter (Roche Diagnostics, Switzerland).
19 All animal procedures were performed in accordance with the Guide for the Care and
20 Use of Laboratory Animals issued by the Institute of Laboratory Animal Resources
21 (NIH publication 85-23, revised 1996) and approved by the Xi'an Jiaotong University
22 Animal Care and Use Committee.

1 **Sinoatrial node function**

2 Six-lead ECGs were recorded under anesthesia with sodium pentobarbitone (40
3 mg/kg, i.p, Sigma, USA) from four leads of rats. The stimuli were applied to the right
4 atrial appendage by a bipolar stimulus through esophagus. Calculated sinoatrial
5 conduction time (SACT) was tested by a train of 8-10 stimuli at a rate of 10-20 bpm
6 above the baseline heart rate and was repeated three times ([Narula et al. 1978](#)). For
7 each rat, three SACT values were averaged. The sinus node recovery time (SNRT)
8 was obtained by overdrive pacing for 30 sec, beginning at a frequency of 6.5 Hz with
9 0.5 Hz increment until the SNRT was no longer lengthened ([Breithardt et al. 1977](#)).
10 The longest time interval from the last paced atrial depolarization to the first
11 spontaneous sinus cycle was designated as the SNRTmax. The rate-corrected maximal
12 sinoatrial node recovery time (CSNRTmax) was determined by subtracting the
13 baseline sinus cycle length (CL) from the SNRTmax.

14 **The intrinsic heart rate (IHR) in vivo**

15 The IHR was tested under complete blockade of the cardiac autonomic nervous
16 system with propranolol (3 mg/kg per ml i.v., Sigma, USA) and atropine (2 mg/kg per
17 ml i.v., Sigma, USA) as previously reported and averaged for 1 min ([Safa-Tisseront et](#)
18 [al. 1998](#)).

19 **The cycle length in Langendorff hearts**

20 Hearts were excised and perfused retrogradely through aorta on a Langendorff
21 apparatus at a constant pressure of 80 mmHg with warm ($36 \pm 0.5^{\circ}\text{C}$), oxygenated,
22 modified Krebs-Henseleit bicarbonate (KHB) buffer as described previously ([Maier et](#)

1 [al. 2003](#)). The composition of the modified KHB was as follows: (in mM) NaCl 118,
2 KCl 4.7, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 11.0, CaCl₂ 2.0, pyruvate 2.0, NaHCO₃ 25
3 and insulin 5 U/L (PH 7.4 ± 0.5). Two recording electrodes were attached to the atrial
4 appendages, and data were sampled with a BL-420 Electrophysiograph (Chengdu
5 Taimeng Technology Co., Ltd, China). Electrocardiographies were recorded at
6 baseline and at the end of 25 min perfusion of 3 µM ivabradine (Sigma, USA) which
7 has previously been demonstrated to reduce I_f by 60% without obvious effect on
8 T-type and L-type calcium current and delayed rectifier potassium current ([Bois et al.](#)
9 [1996](#)); subsequently, ivabradine was washed out with KHB buffer for 25 min. After
10 perfusion, heart samples were removed, blotted dry and weighed. The CL was
11 averaged over 3 min.

12 **Laser capture microdissection (LCM)**

13 The HE-stained, dehydrated tissue section was overlaid with a thermoplastic transfer
14 film mounted on an optically transparent CapSure HS cap (Arcturus, USA).
15 Histologically, the SAN is seen as a dense aggregation of specialized cells within a
16 highly fibrous connective tissue matrix ([Hudson 1960](#)). With HE-staining, SAN cells
17 are small interlacing cells, less darkly stained due to their poorly developed
18 microfilament cytoskeleton, with an appearance distinct from neighboring atrial
19 myocytes ([James et al. 1966](#)). The SAN cells with above typical light microscope
20 characteristics were dissected and affixed to the film by laser pulses from the PixCell
21 II LCM System (Arcturus, USA). The LCM System was set to the following
22 parameters: 7–7.5 µm laser spot size, 60 mW power, 2.5–3.0 ms duration.

1 **Real-time quantitative reverse transcription-polymerase chain reaction**

2 The first-strand cDNAs were synthesized and amplified in real-time quantitative
3 reverse transcription-polymerase chain reactions (PCR) using the Super-Script™ III
4 Platinum® CellsDirect Two-Step qRT-PCRKit with SYBR® Green (Invitrogen, USA).
5 The internal control was *β-actin*. **Table 1** lists the primer pair sequences used in PCR.
6 Real-time PCR reactions were performed in duplicate using the ABI PRISM 7000
7 (Applied Biosystems, USA) with the following cycling programme: one step at 50 °C
8 for 2 min, one step at 95 °C for 2 min, and 45 cycles at 95 °C for 15 sec and 60 °C for
9 30 sec. The data were analyzed using the $2^{-\Delta\Delta CT}$ method ([Livak and Schmittgen](#)
10 [2001](#)).

11 **Immunohistochemistry**

12 The intact SAN preparation was isolated, fixed, dehydrated and embedded in optimal
13 cutting temperature (OCT) compound (Tissue-Tek, NL). Frozen 10-μm sections were
14 serially cut across the leading pacemaker site perpendicular to the crista terminalis.
15 Serial sections were labeled with rabbit polyclonal antibodies against connexin 43
16 (Cx43) (1:2000; Sigma, USA) and the hyperpolarization-activated cyclic
17 nucleotide-gated potassium channel 1-4 (HCN1-4) (1:200; Alomone Labs, Israel),
18 respectively. The FITC-conjugated goat anti-rabbit IgG (Zhongshan Golden Bridge
19 Biotechnology Corporation, China) was used as the secondary antibody with a
20 dilution factor of 1:100. For positive control experiments, rat brain tissue was used.
21 For the negative control experiments, the primary antibody was substituted by horse
22 serum (4%) alone. The sections were analyzed under a Leica TCS SP 2 laser scanning

1 confocal microscope (Leica, Germany).

2 **Optical mapping**

3 The high resolution optical mapping was used to investigate the pacemaking activities
4 and the patterns of electrical conduction in the atrial preparations containing the SAN
5 as described previously (Huang et al. 2007). Briefly, the atrial preparations were
6 stained by superfusion with a bolus of 2 mM di-4-ANEPPS (Molecular Probes, USA)
7 and fixed in a chamber with the endocardial side toward the high-speed
8 charge-coupled device camera (CA-D1-0128T, Dalsa, Canada). The voltage-sensitive
9 fluorescent signals were recorded from 64×64 sites simultaneously at a rate of 910
10 frames/s. The location of the leading pacemaker site was assessed in all hearts. Shifts
11 in leading pacemaker site were quantified using a grid in which the vertical axis was
12 placed parallel, and just adjacent to the crista terminalis. The horizontal axis was
13 placed along the edge of the opening of the inferior vena cava, perpendicular to the
14 vertical axis. All optical data were analyzed using custom software written in Matlab.
15 Analyses included pseudocolour electrical activation maps and isochronal contour
16 plots, which were generated from measurements of activation time at individual pixels.
17 In all cases background fluorescence was subtracted. The optical action potential was
18 generated by measuring the change in fluorescence as a function of time at the leading
19 pacemaker of the SAN. APD and diastolic depolarization (DD) slope measurements
20 were averages from 3-4 consecutive optical action potentials in one preparation. DD
21 slope was measured by fitting this phase with a straight line and determining its slope,
22 which we quantified as a relative change in fluorescence units per ms ($\Delta F/\text{ms}$). Local

1 conduction velocity (CV) was quantified specifically in the leading pacemaker site
2 using an established approach previously described (Nygren et al. 2004). Briefly,
3 activation times at each pixel from a 7×7 pixel array were determined and fit to a
4 plane using the least squares fit method. The direction on this plane that is increasing
5 the fastest represents the direction that is perpendicular to the wave front of electrical
6 propagation and the maximal slope represents the inverse of the speed of conduction
7 in that direction.

8 **Western blot assay**

9 Membrane proteins were extracted from the leading pacemaker site of SAN, by
10 centrifugation on a sucrose buffer (including protease inhibitor cocktail) according to
11 the protocol published previously (Jia et al. 2014). The rabbit polyclonal antibodies to
12 rat HCN2 and HCN4 (1:200 dilution, respectively) or mouse monoclonal antibody to
13 rat β -actin (1:2000 dilution, Santa Cruz Biotechnology Inc., USA) were used as the
14 primary antibodies. The goat anti-rabbit IRDye[®] 800CW or goat anti-mouse IRDye[®]
15 680 LT secondary antibody (1:2000 dilution) (Li-COR Biosciences, USA) were used
16 as the secondary antibodies. Quantitative analysis of the western blot results was
17 performed with the Odyssey software 3.0 (Li-COR Bioscience, USA). For positive
18 control experiments, membrane proteins of rat brain tissue were used. For the
19 negative control experiments, the primary antibody was substituted by phosphate
20 buffered saline (PBS) alone.

21 **Statistical analysis**

22 Data are expressed as the means $\pm$ SEM. The significance of difference was

1 determined by analysis of variance (ANOVA) and a post hoc multiple comparison test
2 using the SPSS 11.0 software. Statistical significance was established at $p < 0.05$.

3 **Results**

4 **Physical characteristics of diabetic rats**

5 As illustrated in **Figure 1**, the resting heart rate (RHR) and IHR of the rats injected
6 with STZ declined continuously within the first 8 weeks after injection and then
7 maintained throughout the observation period, while the control arm was relatively
8 stable. In contrast, blood glucose level of STZ-induced rats elevated continuously
9 within the first 8 weeks after STZ injection and then maintained throughout the
10 observation period. The STZ-induced diabetic rats have higher body weight, heart
11 weight, heart-to-body weight ratio than the age-matched control group ($n = 10$ for
12 each group, each $p < 0.05$). The SACT and CSNRTmax of the diabetic rats were
13 significantly lengthened as compared to the control ($n = 10$ for each group, each $p <$
14 0.05).

15 **Effect of ivabradine on the CL in Langendorff hearts**

16 As illustrated in **Figure 2A**, the CL of Langendorff hearts from the STZ-induced
17 diabetic rats was longer than that of the age-matched controls (316 ± 5 ms vs. 247 ± 7
18 ms, $n = 5$, $p < 0.05$). Specific blockade of HCN channels by 3 μ M ivabradine
19 prolonged CL by 26% (from 247 ± 7 ms to 310 ± 8 ms) in the control but 18% (from
20 316 ± 5 ms to 372 ± 12 ms) in the diabetic rats. As compared with the control, the
21 pacemaking activity of the diabetic hearts is less sensitive to ivabradine ($n = 5$, $p <$
22 0.05) (**Figure 2B**).

1 Transcription of HCN channel isoforms in SAN

2 The cells with typical light microscope characteristics of the SAN cells were dissected
3 specifically by LCM for further analysis (**Figure 3A, B and C**). The transcripts of
4 *HCN2* and *HCN4* were abundant in the rat SAN, while neither the *HCN1* transcript
5 nor *HCN3* was detectable (data not shown). The transcripts of *HCN2* and *HCN4* in
6 SAN were reduced in the STZ-induced diabetic rats as compared with the control
7 (*HCN2*: 3.41 ± 0.32 in the diabetic rats vs. 4.13 ± 0.15 in the control, $p = 0.018$, $n = 5$
8 for each group; *HCN4*: 2.14 ± 0.23 in the diabetic rats vs. 3.40 ± 0.50 in the control; p
9 $= 0.038$; $n = 5$ for each group) (**Figure 3D**).

10 Pacemaking activity and activation pattern in SAN

11 Pacemaker activities and activation patterns were determined from voltage-sensitive
12 fluorescent signals recorded over the SAN and surrounding atrial muscle (**Figure 4A**).
13 Representative activation maps for STZ-induced diabetic rats and the control (**Figure**
14 **4B and 4C**) in sinus rhythm demonstrate that the STZ-induced diabetic rats had an
15 inferior leading pacemaker site adjacent to the crista terminalis within the right atrial
16 posterior wall and a slower conduction time than the control which had a leading
17 pacemaker site in the superior region of the right atrial posterior wall near the opening
18 of the superior vena cava. Beat to beat variability in the location of the leading
19 pacemaker site within the same heart was not observed.
20 Cycle length was longer (corresponding to a lower pacemaking rate) in STZ-induced
21 diabetic rats compared to the control (361 ± 4 ms vs. 269 ± 5 ms, $n = 20$, $p < 0.01$;
22 **Figure 4D**). SAN CV was lower in STZ-induced diabetic hearts compared to the

controls (4.8 ± 0.2 cm/s vs. 5.9 ± 0.1 cm/s, $n = 20$, $p < 0.05$; **Figure 4E**). **Figure 4F** shows representative optical APs from SAN. Quantitative analysis demonstrates that the DD slope was reduced in the SAN of diabetic rats ($0.17 \pm 0.02 \Delta F/\text{ms}$ vs. $0.21 \pm 0.01 \Delta F/\text{ms}$, $n = 20$, $p < 0.05$; **Figure 4G**). APD at 50% and 70% in SAN were 55.2 ± 3.2 and 63.5 ± 3.4 ms in diabetic rat heart compared to 44.0 ± 2.1 and 51.5 ± 2.6 ms in controls ($n = 20$, each $p < 0.05$; **Figure 4H**).

Expression of HCN channel isoform proteins in SAN

As illustrated in **Figure 5A**, SAN cells lack connexin 43 (Cx43), instead, adjacent atrial working myocytes take on positive staining of Cx43. The SAN tissues have robust HCN2 and HCN4 staining with negative HCN1 and HCN3 staining (data not shown).

Only the leading pacemaker site identified by optical mapping was harvested for further Western blot assay. As shown in **Figure 5B**, the protein expression of HCN2 was reduced by 70% from 0.56 ± 0.06 in the control to 0.17 ± 0.05 in the STZ-induced diabetic rats ($p < 0.01$; $n = 20$ for each group). And HCN4 protein was reduced by 58% by STZ (HCN4: 0.21 ± 0.04 in the diabetic rats vs. 0.50 ± 0.02 in the control; each $p < 0.01$; $n = 20$ for each group).

Discussion

The main findings of the present study is that (1) reduced SAN function in STZ-induced diabetic rats; (2) reduced contribution of HCN channel isoforms (HCN2 and HCN4) to automaticity of SAN; (3) and its down-regulating expression during DM.

1 **DM and SAN function**

2 DM is a serious public health concern with the estimated global prevalence of 4.4% in
3 2030 ([Wild et al. 2004](#)). SND is often seen in both type 1 and type 2 DM ([Linnemann](#)
4 [and Janka 2003](#)). The STZ-induced diabetic rat is a widely used experimental model
5 of type 1 DM. Its general characteristics include hyperglycaemia, hypoinsulinaemia
6 and hyperlipidaemia ([Dai et al. 1994](#)). In our study, experiments were performed 20
7 weeks after STZ-treatment. The general characteristics of the diabetic rats compared
8 with the age-matched controls included (1) a significant increase in non-fasting blood
9 glucose; (2) lengthened SACT as well as CSNRTmax, lower RHR and IHR in vivo
10 and a longer CL in vitro, (3) an inferior leading pacemaker site, a reduced SAN
11 conduction velocity, a reduced DD slope and prolonged APD, these indicated an
12 impaired SAN function in the STZ-induced diabetic rats. In accordance with our
13 findings, in vivo biotelemetry studies revealed that the heart rate is progressively and
14 rapidly reduced after administration of STZ and the reduction in heart rate can be
15 partially normalized with insulin replacement ([Howarth et al. 2006](#)). Further studies
16 demonstrated that the spontaneous beating rate of the Langendorff heart was
17 significantly reduced and APD at 50 % and 70 % from peak AP were significantly
18 prolonged in SAN from STZ-induced diabetic rat ([Hicks et al. 1998](#); [Howarth et al.](#)
19 [2007](#); [Zhang et al. 2002](#)). Some studies also found that mice with STZ-induced type 1
20 DM manifested a reduced RHR, prolonged CSNRT and an attenuated chronotropic
21 response to isoprenaline in isolated, perfused hearts ([Luo et al. 2013](#)). Similar SND is
22 present in Leptin-receptor deficient homozygous *db/db* mouse, a model of type 2 DM

1 (Soltysinska et al. 2014). In addition, the abnormalities of automaticity and
2 conduction were observed in alloxan-diabetic rabbit model, including lower sinus rate,
3 longer SNRT, inhomogeneity of atrial conduction and the occurrence of
4 atrioventricular block (Senges et al. 1980). Therefore, DM may alter the
5 electrophysiological property of SAN, and defects in the expression and/or function of
6 ion channels might underlie heart rhythm disturbances in DM.

7 **HCN channel and SAN function**

8 The HCN channels activate early in phase 4 when the cell is hyperpolarized and thus
9 contribute to early diastolic depolarization. The mammalian genome encodes four
10 HCN channels, that is HCN1-HCN4, which share ~80 % of homology but distinct
11 biophysical properties and expression profiles (Biel et al. 2002; Clapham 1998;
12 Kaupp and Seifert 2001; Ludwig et al. 1998; Stieber et al. 2004). *HCN4* is the
13 dominant HCN isoform in adult SAN of rabbit, murine and canine, accounting for
14 80% of the total HCN transcript (Moosmang et al. 2001; Shi et al. 1999). *HCN4*
15 knock-out mice demonstrated 50 % reduction in heart rate (Baruscotti et al. 2011), or
16 recurrent sinus pauses (Herrmann et al. 2007). *HCN1* takes up 18% of the total HCN
17 mRNA in rabbit SAN but only at very low level in mouse SAN (Moosmang et al.
18 2001). The main cardiac phenotype of the *HCN1* knock-out mice is a pronounced
19 SND characterized by impaired impulse formation and sinoatrial conduction, resulting
20 in bradycardia, sinus arrhythmia, and recurrent sinus pauses (Fenske et al. 2013). The
21 *HCN2* transcripts are also expressed in mouse SAN. However, they are equally
22 distributed among atria and SAN at a low to moderate level (Moosmang et al. 2001).

1 *HCN2*-null mice also displayed cardiac sinus dysrhythmia, a shift of the maximum
2 diastolic potential to hyperpolarized values. Mice with cardiomyocyte-specific
3 deletion of *HCN2* displayed the same dysrhythmia as mice lacking *HCN2* globally,
4 indicating that the dysrhythmia is indeed caused by dysfunction of SAN (Ludwig et al.
5 2003). By contrast, *HCN3*-deficient mice display normal cardiac pacemaker activity
6 with an abnormality in the ventricular repolarization (Fenske et al. 2011).

7 The expression HCN channel isoforms is dynamic during different physiological and
8 pathophysiological conditions. Our previous study revealed that the age-associated
9 impairment of SAN function was accompanied with a reduced expression of HCN
10 channels (Huang et al. 2007; Huang et al. 2016). In spontaneously beating fetal and
11 neonatal ventricular myocytes, a prominent I_f is detectable. During maturation, I_f
12 density decreases, and ventricular myocytes lose their ability to generate spontaneous
13 action potentials (Cerbai et al. 1999; Robinson et al. 1997). Re-expression of I_f in
14 adult ventricular myocytes was also found in ventricular hypertrophy and heart failure
15 (Cerbai et al. 1996; Fernandez-Velasco et al. 2003). This disease-dependent alteration
16 of I_f is mirrored by an increased expression of HCN channels. Examination of mRNA
17 levels in hypertrophied ventricular myocytes revealed an upregulation of *HCN2* and
18 *HCN4* in different animal models (Fernandez-Velasco et al. 2003). In addition,
19 analysis of human myocytes obtained from patients with end-stage heart failure shows
20 an increase in *HCN4* mRNA (Borlak and Thum 2003). It was observed that the
21 upregulation of I_f in diseased myocytes results in the occurrence of a diastolic
22 depolarization in ventricular myocytes (Sridhar et al. 2006). Experiments with

1 adenoviral *HCN2* constructs support these finding. Infected myocytes develop a
2 pronounced phase 4 depolarization and spontaneous activity as a result of *HCN2*
3 overexpression (Qu et al. 2001). Taken together, HCN channels may contribute
4 significantly to the formation of arrhythmias in certain cardiac disease states, leading
5 to the hypothesis that an altered expression of HCN channel isoforms may trigger, at
6 least in part, the declined function of diabetic SAN.

7 **Reduced expression of HCN channel isoforms in diabetic SAN**

8 This study indicated that in the STZ-induced diabetic rat, the expression of *HCN2* and
9 *HCN4* in SAN was reduced at both transcription and protein levels. In order to
10 correlate the expression and function, a blocker of HCN channels, ivabradine was
11 used to perfuse in Langendorff hearts. Ivabradine is not an isoform-specific blocker.
12 Experiments investigating the affinity of ivabradine for homomeric HCN channels do
13 not reveal any substantial isoform-specificity. Ivabradine blocked the slow inward
14 current through expressed human HCN1, HCN2, HCN3, and HCN4 channels with
15 mean half maximal inhibitory concentration of 2.25 μ M (Stieber et al. 2006).
16 Although our study could not define the specific contribution of each HCN isoform,
17 the prolonged CL led by ivabradine perfusion at least suggests that blockade of HCN
18 channels contributes to the reduction in spontaneous pacemaking rate. The reduced
19 effect of ivabradine in the STZ-induced diabetic rat heart in turn may be caused by
20 reduced expression of HCN channels.

21 In summary, the expression of HCN channel isoforms in SAN was reduced in
22 STZ-induced diabetic rat, which may be an important contributor to the reduced SAN

1 function in DM. This needs further functional test by electrophysiology to verify.

2

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2 **Table 1. Primer pairs used in real-time quantitative RT-PCR.**

Gene	Gene loci	Primer sequences	Amplicon (bp)
<i>HCN1</i>	<i>NM-053375</i>	F: 5'-ACACCCACCTGACCAGAGAAG-3' R: 5'-CCCGAGGACATCTGTCTGAAC-3'	217
<i>HCN2</i>	<i>NM-053684</i>	F: 5'-TCAACGAGGTGCTGGAGGAGT-3' R: 5'-CTCTGCCTGCTGCACCATCT-3'	206
<i>HCN3</i>	<i>NM-053685</i>	F: 5'-CGTTCCCAGGTGTCTCTGTTG-3' R: 5'-ACTGGCTCAGGCACTGATGAC-3'	227
<i>HCN4</i>	<i>NM-021658</i>	F: 5'-CTCCAGGAGCCTGCAGTTCTC-3' R: 5'-GCAAGAAGTGCTCCAGGAGTC-3'	228
<i>β-actin</i>	<i>NM-031144</i>	F: 5'-GACAGGATGCAGAAGGAGATTACTG-3' R: 5'-CACAGAGTACTTGCGCTCAGGA-3'	87

3 **F, forward primer; R, reverse primer**

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Figure legends

Figure 1. Changes of physical characteristics and SAN function in STZ-induced diabetic rats. RHR, resting heart rate; IHR, intrinsic heart rate; SACT, sinoatrial conduction time; CSNRTmax, rate-corrected maximal sinoatrial node recovery time. * $p < 0.05$ vs. the control; # $p < 0.01$ vs. the control; $n = 10$.

Figure 2. Effect of ivabradine on the cycle length (CL) in Langendorff hearts from STZ-induced diabetic rats. (A) Representative electrocardiogram recorded in two groups. (B) Reduced effect of ivabradine on CL in the STZ-induced diabetic group.

Figure 3. Expression of HCN channel transcripts. (A and B) HE-stained sections of SAN tissues before capture. (C) The remaining tissue after laser capture of the SAN cells. Asterisks mark the SAN artery as a landmark. (D) Transcription of *HCN2* and *HCN4* in laser-captured SAN cells. * $p < 0.05$ vs. the control; $n=5$.

Figure 4. Pacemaker activity and activation pattern in SAN. (A) Rat atrial preparation containing SAN used for optical mapping experiments. SVC, opening of the superior vena cava; IVC, opening of the inferior vena cava; IAS, interatrial septum; CT, crista terminalis. (B) Representative activation maps in atrial preparations from a STZ-induced diabetic rat and a control. Time interval between isochrones is 1.5 ms. (C) Location of the leading pacemaker sites of the STZ-induced diabetic rats and the

1 control group. Orange axes were placed adjacent to the crista terminalis and along the
2 opening of the inferior vena cava. Blue points represent the leading pacemaker sites of
3 the STZ-induced diabetic SAN. Yellow points represent the leading pacemaker sites
4 of the control. (D) Comparison of the cycle length between the two groups. (E)
5 Comparison of SAN conduction velocity between the two groups. (F) Representative
6 optical APs from a diabetic heart and a control heart. Optical APs were measured in
7 the region of the leading activation site in the SAN. (G) Comparison of DD slope
8 between the two groups. (H) Comparison of APD50 and APD70 between the two
9 group. * $p < 0.05$ vs. the control; $n=20$. # $p < 0.01$ vs. the control; $n=20$.

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11 **Figure 5.** Expression of HCN channel proteins. (A) Confocal images of serial
12 sections of rat SAN tissues labeled with Cx43, HCN2, and HCN4 from the
13 STZ-induced diabetic rats. SNA, sinus node artery; IAS, interatrial septum; endo,
14 endocardium; epi, epicardium. Scale bar = 300 μm . (B) Western blot assays of HCN2
15 and HCN4 for two groups. # $p < 0.01$ vs. the control; $n=20$.

A

B