Roles of Impaired Intracellular Calcium Cycling in Arrhythmogenicity of Diabetic Mouse Model

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Abstract

Background: Diabetes mellitus is associated with an increased risk of ventricular arrhythmias (VA), but the underlying electrophysiological mechanisms are not fully explored. This study was aimed to test whether dynamic factors and Ca\textsubscript{i} handling play roles in arrhythmogenesis of a diabetic animal model.

Methods: We used 26 db/db type 2 diabetes mice and 28 control mice in this study. VA inducibility was evaluated in vivo under isoflurane general anesthesia. The intracellular Ca\textsuperscript{2+} (Ca\textsubscript{i}) and membrane voltage (V\textsubscript{m}) signals of the Langendorff-perfused mouse hearts were simultaneously recorded using the optical mapping technique. Action potential duration (APD), Ca\textsubscript{i} dynamics conduction velocity (CV), and arrhythmogenic alternans were analyzed. Western blot was conducted to examine expressions of calcium handling and associated ion channels proteins.

Results: The diabetic db/db mice showed significantly increased VA inducibility and severity. Longer APD and Ca\textsubscript{i} transient duration, and slower Ca\textsubscript{i} decay and CV in the db/db mice than these in the control ones were observed. Dynamic pacing showed increased incidence of spatially discordant alternans leading to more VA inducibility in the db/db mice. Western blot analyses revealed increased phosphorylated-Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II protein expression and decreased ryanodine receptor protein expression, which probably underlay the molecular mechanisms of enhanced arrhythmogenicity in db/db mice.
**Conclusions:** The type 2 diabetic mouse hearts show impaired repolarization, Ca$_i$ handling homeostasis, and cardiac conduction reserve, leading to vulnerability of spatially discordant alternans development and induction of VA. Altered Ca$_i$-handling protein expressions probably underlie the molecular mechanisms of arrhythmogenicity in the type 2 diabetes animal model.

**Key words:** arrhythmia; conduction velocity; db/db mouse; intracellular calcium dynamics; type 2 diabetes

**Introduction**

Cardiovascular diseases are the leading causes of death in the diabetic population,$^{1,2}$ and diabetic patients are at an increased risk of cardiac arrhythmias in addition to susceptibility to ischemic injury.$^3$ Although multitudinous clinical and experimental evidences posit an increased risk of cardiac arrhythmias in diabetes,$^4,5$ the underlying mechanisms are not clearly demonstrated. A type 2 diabetes animal model, the db/db mouse with a leptin receptor mutation, is very similar to clinical type 2 diabetes and has been proven to be a suitable model to study cardiovascular consequences of diabetes.$^6,7$ Pereira et al. reported that db/db mouse hearts have reduced L-type calcium current and ryanodine receptor Ca$^{2+}$ channel (RyR) density, which may reduce sarcoplasmic reticulum (SR) Ca$^{2+}$ load and intracellular Ca$^{2+}$ (Ca$_i$) sparks.$^8$ Belke et al. observed delayed Ca$^{2+}$ transients, lower systolic and diastolic Ca$^{2+}$ level in isolated db/db mouse cardiomyocytes.$^9$ Erickson et al. also reported that hyperglycemia-induced O-linked N-acetylglucosamine modification of calcium-calmodulin protein kinase II (CaMKII) overactivates CaMKII and results in diastolic Ca$^{2+}$ leak and ventricular premature ventricular ectopic complexes (PVCs).$^{10}$ These evidences imply that Ca$_i$ homeostasis may play an role in the arrhythmogenesis in the diabetes, although the association between altered Ca$_i$ homeostasis and
the change in electrophysiological characteristics were not yet well investigated in the type 2 diabetes model. In this study, we performed simultaneous \( \text{Ca}^{2+} \) and membrane voltage \( (V_m) \) mapping to examine the roles of abnormal \( \text{Ca}^{2+} \) dynamics and conduction disturbance on ventricular arrhythmogenesis in \( \text{db/db} \) mouse hearts.

**Methods**

This study protocol was approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital (approval No. 2013060401) and conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. In this study, 26 \( \text{db/db} \) and 28 control \( (\text{db/+}) \) mice were used.

**In vivo Electrophysiological Studies**

In vivo electrophysiology and optical mapping were performed using the same methods previously described. Mice were premedicated with xylazine (10 mg/kg intraperitoneally) and zoletil (25 mg/kg intraperitoneally), then intubated and anesthetized with isoflurane (0.25–1%) using a mouse ventilator (Model 687, Harvard Apparatus, Holliston, MA, USA). When mice were fully anesthetized and unresponsive to physical stimuli, hearts were exposed via a median thoracotomy. Electrocardiogram (ECG) was obtained with Axon Digidata (Molecular Devices, CA, USA) using three platinum electrodes, which were attached to mouse limbs. A custom-made bipolar electrode was placed on the lateral basal aspect of left ventricular epicardium for electrical stimulation. Pacing output was set at twice threshold. Effective refractory period (ERP) was measured by giving a premature stimulus after ten beats of S1S1 pacing at a pacing cycle length (PCL) of 200 ms. Both burst and extrastimulus pacing protocols were used to test VA inducibility. The burst pacing protocol is a \( \text{S}_1 \text{S}_1 \) burst pacing
for 2–3 seconds at PCLs from 200 ms down to the shortest PCL (by 10 ms each step) with 1:1 capture; the extrastimulus pacing represents a fixed S1S1 PCL of 200 ms, followed by extrastimuli from 80 ms down to the ERP (up to S4). The severity of induced VA was classified into single isolated premature ventricular complex (PVC), two consecutive PVCs (couplet), three consecutive PVCs (triplet) and ventricular tachycardia (VT, > 3 consecutive PVCs).

**Langendorff heart preparation and optical mapping**

Mice were exsanguinated by excision of hearts under deep-inhaled isoflurane anesthesia (5%). The hearts were quickly mounted on a Langendorff apparatus, retrogradely perfused with warm (37°C) oxygenated Tyrode's solution (NaCl 125, KCl 4.5, NaH₂PO₄ 1.8, NaHCO₃ 24, CaCl₂ 0.9, MgCl₂ 0.5, and glucose 5.5 mmol/L in deionized water; 0.01 mmol/L HCl used to maintain a pH of 7.40 ± 0.05) at a flow rate of 1.5–3 ml/min to keep perfusion pressure of 80 cmH₂O. Tyrode's solution was continuously bubbled with 95% O₂ - 5% CO₂ during optical mapping experiments. The hearts were immersed in a water bath chamber to maintain the temperature at 37°C. Pseudo-ECG recording was obtained utilizing three spaced electrodes located in the bath surrounding the hearts.

Dual optical mapping techniques were used to study electrophysiological characteristics of the heart as described previously.¹¹,¹² A Caᵢ indicator Rhod-2AM (5 μM, Molecular Probes, OR, USA, in 20% pluronic F-127 dissolved in dimethyl sulfoxide) and a voltage indicator RH237 (1 μM in 20 mL Tyrode’s solution, Molecular Probes, dissolved in dimethyl sulfoxide) were used for Caᵢ and Vₘ staining, respectively. Blebbistatin (15 μM, Tocris Bioscience, Minneapolis, MN, USA) was added to the Tyrode’s solution after Caᵢ and Vₘ staining to reduce motion artifacts. The hearts were illuminated with a solid-state, frequency-doubled laser light source ( Millennia, Spectra-Physics Inc., Newport Corporation, Irvine, CA, USA) of a wavelength of 532 nm. Epifluorescence was acquired.
simultaneously through two high-speed cameras (MiCAM Ultima, BrainVision, Tokyo, Japan) at 1 ms/frame through a 580 ± 20-nm bandpass filter and a 715-nm long-pass filter for Ca, and Vm images, respectively. The digital images (100×100 pixels) were gathered from a mapped field of 14×14 mm², resulting in a spatial resolution of 140×140 μm² per pixel. The average fluorescence level (F) of an individual pixel was first calculated, and the ratio of fluorescence (F-F/F) of an individual pixel was processed with both spatial (3×3 pixels Gaussian filter) and temporal (3 frames moving average) filtering to generate the maps.

A bipolar lead was used to pace the lateral wall of left ventricle (LV) at twice threshold. Arrhythmogenic alternans and conduction velocity (CV) were studied by using the same pacing protocol: from 200 ms down to the shortest PCL (by 10 ms each step) with 1:1 capture or PCLs at which VA was induced.

**Western Blot Examination**

Tissue samples from the LV of db/db and control hearts were suspended in lysis buffer (pH 8.0) containing 20 mM Tris-base (pH 8.0), 150 mM NaCl, NP-40, protease inhibitors (Roche, Basel, Switzerland). Total protein concentration of the homogenate was determined by Bradford Assay (Bio-Rad, Hercules, CA, USA). Protein samples were subjected to 4-12% SDS-PAGE and electrophoretically transferred to PVDF membranes. The membranes were then incubated in Tween-TBS with the primary antibodies: anti-protein kinase A (PKA, Millipore, Temecula, CA, USA), NaV1.5 (AVIVA, San Diego, CA, USA), calsequestrin 2 (CASQ2, Santa Cruz Biotechnology, Europe), ryanodine receptor 2 (RyR2, Santa Cruz Biotechnology, Europe), sarcoplasmic reticulum Ca²⁺-ATPase type 2a (SERCA2a, Santa Cruz Biotechnology, Europe), sodium-calcium exchanger (NCX, Thermo, Rockford, USA), Ca²⁺/calmodulin-dependent protein kinase II (CaMKII, Santa Cruz
Biotechnology, Europe), phosphorylated CaMKII at Thr287 (p-CaMKII) (Thermo, Rockford, USA), PLB (Millipore, Temecula, CA, USA), phosphorylated PLB Ser16 (p-PLB) (Millipore, Temecula, CA, USA) and β-actin antibody (Abcam, Cambridge, UK) antibodies. Then appropriate horseradish peroxidase conjugated secondary antibodies were used. Enhanced chemiluminescence films were quantitated by densitometric scanning, and protein expressions were normalized to β-actin.

Data Analyses

Action potential duration (APD_{80}) was measured from 20% depolarization to 80% repolarization at PCLs of 200, 150, 120, and 100 ms, and Ca_i transient duration (Ca_iTD_{80}) was measured using the same method as previously described.^{11, 13} APD_{80} and Ca_iTD_{80} were expressed as mean of two consecutive beats if alternans occurred. We used monoexponential fitting to compute time constant (τ) of the decay portion of the Ca_i transient between 70% of the transient peak and the diastolic baseline.^{14} Alternans was defined as differences in APD_{80} of 2 consecutive beats > 4 ms for V_m alternans and in Ca_i transient amplitude of 2 consecutive beats > 10% for Ca_i alternans. We defined phase positive for a short-long APD_{80} or a small-large Ca_i amplitude sequence (color coded by red), and negative phase represents a long-short APD_{80} or a large-small Ca_i amplitude sequence (color coded by green). Spatially discordant alternans (SDA) was evidenced in the presence of both red (positive-phase) and green (negative-phase) regions separated by a nodal line. To estimate CV, we measured distance and conduction time between the earliest activation point and two separate epicardial sites: the first distance was from the pacing site to the LV apex, and the other was along an axis parallel to the atroventricular ring at PCLs of 200, 150, 120, 100, 90, 80, and 70 ms. When CV alternans occurred, we selected the slower one for comparisons.
Statistics

Continuous variables were expressed in mean ± standard deviation values, and categorical variables were presented as absolute numbers (percentages). Unpaired Student’s t-test was performed for comparison between two groups. Cochran-Mantel-Haenszel test was used for in vivo VA inducibility and severity comparisons, and Fisher’s exact test was used for in vitro VA inducibility and SDA inducibility comparisons. A P value < 0.05 was considered statistically significant.

Results

The mean body weight of db/db mice was significantly heavier than control mice (56 ± 9g, n = 26 vs. 27 ± 4g, n = 28, P < 0.001), and the mean heart weight of db/db mice was also heavier (0.21 ± 0.05g, vs. 0.17 ± 0.04g, P = 0.01). As a result, db/db mice had a significantly lower heart weight/body weight ratio than the control mice (0.38% vs. 0.63%, P < 0.001). The mean ages of db/db and db/+ mice were 22.3 ± 5.3 and 22.0 ± 5.4 weeks old (P = 0.852), respectively. The numbers of female gender in the db/db and db/+ mice were 26 (100%) and 24 (86%).

Electrophysiological studies

In the electrophysiological studies, we acquired data from 10 db/db mice and 12 control mice. The db/db mice had a longer mean ERP than the control mice (62 ± 10 ms vs. 52 ± 6 ms, P = 0.018).

Figure 1 summarized the inducible VA severity classification. Figure 1A and 1B show representative ECG tracings of VAs induced by S1-S4 pacing and burst pacing in db/db mice. The db/db mice were more susceptible to VA induction than the control ones. With S1-S4 pacing protocol, 2, 2, 1, 0 out of 12 control mice and 7, 7, 4, 3 out of 10 db/db mice developed isolated PVC, couplet, triplet and VT, respectively (P = 0.017, Figure 1C); and there were 5 isolated PVCs, 14 couplets, 4 triplets, 0 VT
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group). The representative tracings in Figure 3B reveal that the Ca decay was 34 ms and 31 ms in these particular db/db and control mice, respectively.

Conduction velocity

The mean CVs measured from the pacing site to LV apex and along the atrioventricular ring were significantly slower in the db/db group than the control mice group (data were summarized in Figure 4A). Figure 4B shows a representative example: the CVs from the pacing site to LV apex were 114, 114, 103, 103, 94, 81, 71 cm/s in this particular control mouse and 63, 63, 54, 52, 48, 45, 45 cm/s in this particular db/db mouse (P < 0.001) at PCLs of 200, 150, 120, 100, 90, 80, 70 ms, respectively; and the CVs from the pacing site along atrioventricular ring were 99, 99, 83, 83, 72, 72, 68 cm/s in this particular control mouse and 81, 69, 65, 61, 57, 54, 49 cm/s in this particular db/db mouse at PCLs of 200, 150, 120, 100, 90, 80, 70 ms, respectively.

Thresholds of spatially concordant alternans and spatially discordant alternans

Although spatially concordant V_m and Ca_i alternans could be provoked in all control and db/db mice by dynamic pacing, but there was a trend for a longer PCLs to provoke alternans in the db/db group than the control group: the longest PCLs to provoke concordant alternans were 92 ± 11 ms vs. 82 ± 11 ms in the db/db and the control groups, respectively (P = 0.064, n = 10 in each group). Furthermore, the db/db mice hearts were more apt to SDA provocation than the control ones: SDA was able to be provoked in all 10 (100%) db/db mice and only in 5 of 10 (50%) control mice (P = 0.033). Figure 5A shows an example of SDA development in a db/db mouse heart: burst pacing at a PCL of 70 ms provoked SDA. Spatially concordant alternans could be provoked at a PCL of 80 ms (left subpanel). When PCL was further shortened to 70 ms, SDA was provoked and the mapping field was separated.
by a nodal line (black arrows), which moved toward the pacing site at PCL of 60 ms. Note that \( V_m \) and Ca\(_i\) transients were out-of-phase (long-short and large-small vs. short-long and small large) in different regions of the heart during SDA.

**VA induction**

The \( db/db \) mice were more susceptible to VA induction than the control ones. Figure 5 illustrates VA induction following development of SDA in a \( db/db \) mouse: panel B illustrates the mapping field, and panel C shows the pseudo-ECG (top tracing) and the \( V_m \) (bottom tracings) signals recorded at sites “a” (LV base) and “b” (on a nodal line) during extra-stimulus with VT induction. The VT was induced by \( S_1-S_4 \) extrastimulus pacing (\( S_1-S_2-S_3-S_4 \): 200, 90, 60 and 50 ms). When the \( S_3-S_4 \) coupling pacing interval was shortened to 50 ms, dispersion of refractoriness occurred and the wavefront was blocked at a nodal line and propagated laterally along the nodal line (frame 611 ms, Figure 5E), waiting for the refractory region to repolarize and then re-entered the blocked region to initiate reentrant wavefronts (supplementary online video). During the initiation of VT, the core of the reentrant wavefront was anchoring at site “b”, where fragmented \( V_m \) transients were shown (red arrow, Figure 5C) and phase singularity point was located (Figure 5D).

**Protein Expression**

To elucidate the role of Ca\(^{2+}\) handling proteins in the arrhythmogenesis in \( db/db \) mice, we further performed Western blot of the associated molecules. Western blot analyses showed that expression levels of CaMKII and PLB did not differ between the groups, but p-CaMKII (\( P = 0.01 \)) and p-PLB (\( P = 0.01 \)) was strongly increased in the \( db/db \) group compared to the control group (\( n = 6 \) in each group). In addition, RyR2 expression was reduced in \( db/db \) mice (\( P < 0.001 \)). Other Ca\(^{2+}\) handling
protein, (SERCA2a, CASQ2 and NCX), NaV1.5, and PKA protein expressions showed no significant differences between groups (Figure 6).

Discussion

In the present study, we examined electrophysiological characteristics in vivo and in Langendorff-perfused hearts, and protein expressions of LV tissue from db/db and control mice. Our data showed that db/db mice had increased incidence and severity of inducible VA by programmed electrical stimulation. Optical mapping analyses also revealed prolonged Ca, decay, reduced CV, and enhanced SDA induction in db/db mice. The increased p-CaMKII, p-PLB expressions and reduced RYR2 may underlie, at least partly, the molecular mechanisms of altered electrophysiological characteristics and increased arrhythmogenicity in the db/db mouse model.

Interaction of Abnormal Ca, Handling and Arrhythmogenic Alternans

CaMKII phosphorylation plays roles in arrhythmogenicity of db/db mouse hearts: phosphorylated CaMKII can further phosphorylate RyR2 to enhance diastolic SR Ca\(^{2+}\) leak,\(^{15}\) phosphorylate Na\(^{+}\) channels to slow open-state inactivation of \(I_{Na}\),\(^{16}\) and phosphorylate L-type Ca\(^{2+}\) channels to increase L-type Ca\(^{2+}\) current amplitude and to slow L-type Ca\(^{2+}\) inactivation.\(^{17,18}\) NCX plays also an important role in the cardiac Ca\(^{2+}\) homeostasis. However, the total protein of NCX was not different between the db/db and control groups. Ca\(^{2+}\) decay prolongation might be associated with increased phosphorylated-CaMKII, which slowed open-state inactivation of L-type Ca\(^{2+}\) and resulted in increased late \(I_{Na}\). The combined effects enhance NCX activity in the reverse mode and lead to Ca\(^{2+}\) overload. The interactions are implicated in the initiation of early (EAD) and delayed afterdepolarizations (DAD).\(^{19}\) The effects of CaMKII prolong APD and Ca\(^{2+}\) transient duration.
through slow inactivation of $I_{\text{Na}}$, increase amplitude and slow inactivation of L-type Ca\(^{2+}\) currents, and further enhanced arrhythmogenic afterdepolarization. The mechanisms are compatible with our observations, including prolonged action potential duration, prolonged Ca\(_i\)TD, and prolonged Ca\(_i\) decay.

Chronic overactivity of sympathetic nervous system is very common in diabetic patients and may contribute to increased p-PLB level without increased protein level of PKA.\(^{20}\) The activated PKA further enhance phosphorylation of CaMKII. Activated PKA and CaMKII phosphorylate PLB to remove inhibition on SERCA. Increased p-PLB expression is a result of increased p-CaMKII and can releases its inhibition on SERCA activity.\(^{21}\) However, the modest upregulation of p-PLB may be an adaptive mechanism in this diseased state and is not adequate for full compensation in p-CaMKII induced slow Ca\(^{2+}\) inactivation. The increased p-CaMKII still plays the pivotal role in the arrhythmogensis in this db/db mice model. Reduced RYR2 expression may further cause impaired Ca\(^{2+}\)-induced SR Ca\(^{2+}\) release. Thus, the impaired SR Ca\(^{2+}\) release and reuptake enhance the susceptibility to alternans because myocytes cannot restore cytosolic Ca\(^{2+}\) during each beat at faster heart rates.\(^{22}\) In agreement with the altered Ca\(^{2+}\) handling proteins expression, the Ca\(_i\) transients in \textit{db/db} mice hearts (Figure 3) showed longer decay time constant and longer Ca\(_i\)TD\(_{80}\), implying a slower Ca\(^{2+}\) reuptake compared with the control ones. In addition, Ca\(_i\) transient alternans was induced at a longer PCL during dynamic pacing. The longer APD\(_{80}\) in \textit{db/db} mice hearts (Figure 2) may also reflect increased late $I_{\text{Na}}$ that was probably associated with increased p-CaMKII expression.

The interactions among CaMKII, PKA, RyR2, CASQ2 and other associated Ca\(^{2+}\) handling protein are complicated and some concepts remain controversial. CaMKII-mediated RyR2 phosphorylation and oxidation is associated with altered Ca\(^{2+}\) spark frequency,\(^{23}\) leading to increased frequency of afterdepolarization. A previous report showed that elevated reactive oxygen species...
(ROS) activities triggered CaMKII activation, phosphorylated RyR2, and contributes spontaneous SR Ca\(^{2+}\) release in a diabetes mouse model.\(^{24}\)

**Mechanisms of Conduction Disturbance**

Although limited data showed slow conduction velocity in \(db/db\) mice, reduced cardiac conduction reserve has been reported previously in streptozocin-induced type 1 diabetic rats\(^{25}\) and Zucker Diabetic Fatty (ZDF) type 2 diabetic rats.\(^{26}\) In accordance with these reports, our mapping data showed slower CV at all PCLs in \(db/db\) mice hearts compared with the control ones (Figure 4).

Propagation of the action potentials through the working myocardium depends on \(I_{Na}\) activation followed by gap junction conduction. The conduction disturbance in the diabetic rats has been explained by increased lateralization of connexin-43, which is associated with non-functional gap junctions.\(^{25,26}\) Meantime, calmodulin/CaMKII has been proposed not only directly regulating connexin-43 gating properties but also indirectly modifying their expression and subcellular localization in the intercalated disc.\(^{27}\) On the other hand, decreased Na\(^{+}\) current could reduce cardiomyocytes excitability and thereby lead to slow CV. Wagner et al. reported that CaMKII\(\delta\)c overexpression may shift the voltage dependence of Na\(^{+}\) channel availability to more negative membrane potentials, enhance intermediate inactivation, and slow recovery from inactivation, resulting in a loss of \(I_{Na}\) function.\(^{16}\) Other factors, such as lipotoxicity, could decrease \(I_{Na}\) and thereby slow CV in type 2 diabetes hearts,\(^{26}\) provoking arrhythmogenesis.

**Arrhythmogenetic SDA in \(db/db\) Mice Hearts**

Abnormal Ca\(_i\) dynamics facilitates induction of triggered activity and concordant alternans; and conduction disturbance perpetuates inducibility of SDA.\(^{28}\) SDA causes wavebreaks most likely
because they are sites of abrupt dispersion of refractoriness, which increases the susceptibility to functional conduction block. Recently, Takanari et al. reported that calmodulin/CaMKII inhibition by W7, a calmodulin inhibitor, improves conduction characteristics and enhances localization of connexin-43 in the intercalated disc. It implies that calmodulin/CaMKII could be served as a potential therapeutic target to reduce arrhythmogenic susceptibility in diabetic patients.

**Study Limitations**

There are still several limitations in this study. Although the phenotypic presentations are similar to clinical diabetes type 2, the manifestations of cardiac arrhythmias in this diabetic animal model might not be completely the same as patients. Because mice and human have different expression of calcium handling proteins and ion channels, leading to different action potential morphology and cardiac electrophysiology. Blebbistatin, an excitation-contraction uncoupler, is required to suppress motion artifacts in optical mapping studies, but also has significant electrophysiological effects, including APD prolongation, ERP increase and restitution curve alteration. The electrophysiological presentations in optical mapping studies might be different from in-vivo condition. Since no spontaneous VA was mapped during optical mapping studies, we could not demonstrate if diastolic SR Ca\(^{2+}\) leak (Ca\(_{\text{i}}\) rise preceding to V\(_{\text{m}}\) rise) underlies the mechanism of VPC. The development of alternans is associated with the interaction of calcium homeostasis and action potential dynamics. In a previous report, ex-vivo hearts experiments were conducted at low temperature (21 °C), and the longest PCL to induce alternans was 250 ms. Since our mapping setting is different, especially the chemical concentrations, dyes and temperature during the experiments, the PCL threshold of alternans induction may not be the same. For example, hypothermia may induce APD prolongation, delay L-type calcium current inactivation and reduce SR calcium release. Our previous study in a mouse
model of myotonic dystrophy also revealed that the longest CL to induce alternans was 94 ms in the normal control group. Most of the mice in this study were female gender because limited availability of male mice, and ion channels expression might be different between male and female mice.

Conclusions
The db/db mice show abnormal Ca\textsubscript{i} handling and slow cardiac conduction reserve. The combined effects enhance spatially discordant alternans induction and the susceptibility to VA. Abnormal Ca\textsubscript{i} protein expressions may underlie the molecular mechanisms of arrhythmogenicity in type 2 diabetes.

Author contributions

References


impulse propagation in the heart and is antiarrhythmic under conditions when fibrosis is absent. Cardiovasc Res 2016; 111: 410-421.


Figure 1. Electrophysiological studies to test VA inducibility in vivo. A. Representative ECG traces of S1-S4 pacing. B. Representative ECG traces of burst pacing. C. Summary of inducibility of VA by S1-S4 pacing. D. Summary of inducibility of VA by burst pacing.
Figure 2. Optical mapping and action potential recording. A. Mean $\text{APD}_{80}$ at pacing cycle lengths (PCL) of 200, 150, 120 and 100 ms, respectively ($n = 10$ in each group). B. Representative action potential traces and $\text{APD}_{80}$ maps at PCLs of 200, 150, 120 and 100 ms in two groups. $V_m$ alternans occurred at PCL of 100 ms in the $db/db$ mouse heart.
Figure 3. Optical mapping and Ca\textsubscript{i} transient recording. A. Mean Ca\textsubscript{i}TD\textsubscript{80} at PCLs of 200, 150, 120 and 100 ms, respectively (n = 10 in each group). B. Representative Ca\textsubscript{i} traces and Ca\textsubscript{i}TD\textsubscript{80} maps at PCLs of 200, 150, 120 and 100 ms in two groups. Ca\textsubscript{i} alternans occurred at PCL of 100 ms in the db/db mouse heart.
Figure 4. Optical mapping and conduction velocity (CV). A. Mean CV at PCLs of 200, 150, 120, 100, 90, 80 and 70 ms, respectively (n = 10 in each group). B. Representative isochronal maps at PCLs of 200, 150, 120, 100, 90, 80 and 70 ms in two groups. Black arrows indicate the directions of CV measurement; square waves indicate the pacing site. AV, atrioventricular; LV, left ventricle.
Figure 5. Spatially discordant alternans (SDA) and ventricular arrhythmia (VA) induction in a db/db mouse heart. **A.** $V_m$ and $Ca_i$ alternans maps. Concordant alternans was induced by burst pacing at PCL of 80 ms, preceded to SDA during burst pacing at a shorter PCL (70 ms). Black arrows indicate the nodal lines, which moved towards the pacing sites during burst pacing at PCL of 60 ms. **B.** Mapping field. Red and orange dot lines indicate the corresponding sites of nodal lines induced by PCLs of 70 and 60 ms, respectively. **C.** Pseudo-ECG (upper subpanel) and the corresponding $V_m$ traces (bottom subpanel) show the initiation of ventricular tachycardia (VT) by S1-S4 pacing. Red square indicates the corresponding period in $V_m$ traces; red arrow indicates fragmented $V_m$ transient during rotor anchoring at site “b”. **D.** Phase singularity map. A phase singularity was formed at site “b” during VT. **E.** Isochronal maps. The number below each frame is the time (ms) with the onset of data acquisition as time zero. White arrows indicate the directions of wave fronts propagation. AV, atrioventricular; LV, left ventricle; RV, right ventricle.
Figure 6. Western blot analyses of Ca\textsuperscript{2+} handling and ion channel proteins in two groups. A. Representative Western blot autoradiograms. B. Summary of protein expression (n = 6 in each group).

The protein expression was normalized to actin. *, P < 0.05.
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