Laboratory Research

The ionic mechanisms of long QT interval in diabetic rabbits

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Objective
Abnormal QT prolongation associated with arrhythmias is considered the major cardiac electrical disorder and a significant predictor of mortality in diabetic patients. The precise ionic mechanisms for diabetic QT prolongation remained unclear. The present study was designed to analyze the changes of ventricular repolarization and the underlying ionic mechanisms in diabetic rabbit hearts.

Methods
Diabetes was induced by a single injection of alloxan (145 mg/kg, i.v.). After the development of diabetes (10 weeks), ECG was measured. Whole-cell patch-clamp technique was applied to record the action potential duration (APD50, APD90), slowly activating outward rectifying potassium current (IKs), L-type calcium current (ICa-L) and inward rectifying potassium current (IK1).

Results
The action potential duration (APD50 and APD90) of ventricular myocytes was obviously prolonged from 271.5 ± 32.3 ms and 347.8 ± 36.3 ms to 556.6 ± 72.5 ms and 647.9 ± 72.2 ms respectively (P < 0.05). Meanwhile the normalized peak current densities of IKs in ventricular myocytes investigated by whole-cell patch clamp was smaller in diabetic rabbits than that in control group at test potential of +50 mV (1.27 ± 0.20 pA/pF vs 3.08 ± 0.67 pA/pF, P < 0.05). And the density of the ICa-L was increased apparently at the test potential of 10 mV (-2.67 ± 0.41 pA/pF vs -5.40 ± 1.08 pA/pF, P < 0.05).

Conclusion
Ventricular repolarization was prolonged in diabetic rabbits, it may be partly due to the increased L-type calcium current and reduced slow delayed rectifier K+ current.

Key words diabetes; QT prolongation; slowly activating outward rectifying potassium current; inward rectifying potassium current; L-type calcium current; patch clamp

Introduction
The most prominent cardiac electrical disturbance in diabetic mellitus (DM) is the abnormality of QT interval. A prolongation of QT interval has been associated with an increased risk of sudden cardiac death in patients with diabetes due to occurrence of lethal ventricular arrhythmias known as torsades de pointes or long QT syndrome (LQTS). Indeed, QT prolongation has been suggested as a predictor of mortality in both insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). The precise ionic mechanisms for diabetic QT prolongation remained unclear. In diabetic rabbits model, studies suggested that the decreased amplitude of the transient outward K+ current (Ito) and the reduced expression of Kv4.3 and Kv4.2 channel proteins were responsible for the elongation of repolarization in diabetic rats. In a recent study, a significant reduction in the amplitude of Ito and the slow component of the delayed rectifier potassium current (IKs) was observed in diabetic dogs, while other ion currents such as inward calcium current (ICa-L), inward rectifier and rapid delayed rectifier potassium currents (IK1 and IKr) remained unaltered. In other recently published works, a reduction in IKr was found in diabetic rabbits; however, no information was presented on the other ion currents. Our study was designed to establish diabetic rabbit model to dissect the relative contribution of these ion currents/channels in diabetic QT/APD prolongation and to shed light on the potential underlying mechanisms.

Methods
Preparation of rabbit model of Type 1 IDDM
Male New Zealand white rabbits weighing 2-2.5 kg were used. The animals were randomly assigned to control and IDDM groups. To establish diabetes, a single injection of pre-warmed (37°C) alloxan monohydrate (140 mg/kg, Sigma-Aldrich), freshly dissolved in saline at a concentration of 100 mg/ml, was administrated via marginal ear vein under local anesthesia. To prevent fatal hypoglycemia from massive insulin release, 10% glucose solution (100 mg/kg, s.c.) was administered 4 and 6 hours after alloxan treatment. The
blood glucose was measured using a glucometer and monitored weekly till 10 weeks, then the animals were sacrificed for further experiments. Only those animals with serum glucose concentrations ≥ 15 mmol/L were considered diabetic and were used for further studies.

Electrocardiography
Conventional ECG recordings were taken for each rabbit under anaesthesia at the beginning and end of the experiment. Standard lead II ECG was sampled by a signal processing system under the control of a personal computer. ECG parameters (RR, QT and QTc intervals) were determined manually using cursors. QTc intervals were derived according to Carlsson’s formula \[QTc = QT - 0.175(RR)^{-0.5} - 300\].

Isolation of rabbit ventricular myocytes
Myocytes were isolated from rabbit left ventricular endocardium via enzymatic digestion with the procedures similar to previously described. Rabbits were anesthetized by sodium pentobarbital (60 mg/kg, i.v.). Hearts were rapidly excised and mounted on a Langendorff apparatus and perfused retrogradely with the following three solutions in a sequential order: 1 mmol/L Ca²⁺ Tyrode (2 min), Ca²⁺-free Tyrode (3-5 min), Ca²⁺-free Tyrode containing collagenase (Worthington type II) for 25-35 min. The rabbit left ventricle was divided into epicardial, M, and endocardial regions during collecting the isolated myocytes. The freshly isolated myocytes were gently centrifuged and resuspended in the KB solution for patch-clamp studies. Only ventricular myocytes from endocardium were used for electrophysiological studies. Tyrodes solution (mmol/L): NaCl 126, KCl 5.4, MgCl₂ 1.0, CaCl₂ 1.8, NaH₂PO₄ 0.33, glucose 10, HEPES 10, pH 7.4 (adjusted by NaOH). The KB solution (mmol/L): Glutamic acid 70, Taurine 15, KH₂PO₄ 10, MgCl₂ 15, EGTA 15, HEPES 10, Glucose 10, 1% albumin, pH 7.4.

Whole-cell patch-clamp recording
Patch-clamp techniques have been described in detail elsewhere. Currents were recorded in the whole cell voltage-clamp mode and action potentials (APs) were recorded in the current-clamp mode, with an Axopatch-200B amplifier (Axon Instruments). These electrodes had resistances between 2 and 4 MΩ when filled with pipette solution containing (mmol/L): K-aspartate 100, KCl 25, ATP 3, MgCl₂ 1, EGTA 10 and HEPES 5, or KCl 110, KOH 40, EGTA 10, HEPES 10, TEACl 20, MgATP 5, GTP 0.25, when measuring potassium or calcium currents respectively. The pH of the pipette solution was adjusted to 7.2 by KOH. When potassium currents were measured, the inward L-type calcium current (ICa-L) was blocked by 1 μmol/L nisoldipine to the external solution. TTX (1 μmol/L) was used to prevent INa. 4-Aminopyridine (1 mmol/L) was used to inhibit Ito for recording other currents and glyburide (10 μmol/L), plus Mg-ATP (5 mmol/L) in the pipette solution to prevent ATP sensitive K⁺ current. Dofetilide (1 μmol/L) was used to block IKr. L-type inward calcium current (ICa-L) was recorded in the presence of 3 mmol/L 4-aminopyridine added to the external solution to block Ito. The extracellular solution (in mmol/L: Tris-Cl 136, CsCl 5.4, CaCl₂ 2, MgCl₂ 2.6, H₂O 1, HEPES 10, and Glucose 5, pH 7.4 with Tris-OH) and pipette solution (in mmol/L: CsCl 20, MgCl₂ 2.6, H₂O 1, MgATP 5, EGTA 10, and CsCl: H₂O 110, Aspartate 110, pH 7.2 with CsOH) were used to record the ICa-L. Series resistance and capacitance was compensated and leak currents were subtracted.

Statistical analysis
Group data were expressed as means ± S.E. Statistical comparisons (ANOVA followed by Dunnett’s method) were carried out using SPSS. A two-tailed \(P < 0.05\) was taken to indicate a statistically significant difference. Nonlinear least square curve fitting was performed with GraphPad Prism.

Results
Blood glucose increment and diabetic QT prolongation
Rabbits developed typical characteristics of type I diabetes 12-24 hours after single injection of alloxan. Serum glucose level was elevated significantly (18.4±2.04 mmol/L) as compared with the normal value (5.91±0.69 mmol/L) in control animals (\(P < 0.05\), Fig.1).

Remarkably, heart rate-corrected QT interval (QTc interval) was consistently prolonged in rabbits after treatment with alloxan (177.0±2.9ms) for 10 weeks compared with the baseline values obtained before treatment (156.3±2.4ms, \(P < 0.05\), \(n = 7\)). These data indicated a 13.2% prolongation of QTc interval in the IDDM rabbits (Fig.2).

To delineate the cellular mechanism underlying the QTc prolongation, rabbit ventricular myocytes were isolated from rabbit left ventricular endocardium via enzymatic digestion. Only ventricular myocytes from endocardium were used for electrophysiological studies. Tyrodes solution (mmol/L): NaCl 126, KCl 5.4, MgCl₂ 1.0, CaCl₂ 1.8, NaH₂PO₄ 0.33, glucose 10, HEPES 10, pH 7.4 (adjusted by NaOH). The KB solution (mmol/L): Glutamic acid 70, Taurine 15, KH₂PO₄ 10, MgCl₂ 15, EGTA 15, HEPES 10, Glucose 10, 1% albumin, pH 7.4.

Fig. 1 Blood glucose concentration in control and IDDM. Ctrl: control; IDDM: insulin-dependent diabetes mellitus. \(n = 7\). \(\*P < 0.05\) vs ctrl.

Fig. 2 QTc interval in insulin-dependent diabetes mellitus. Ctrl: control. IDDM: insulin-dependent diabetes mellitus. \(n = 7\). \(\*P < 0.05\) vs ctrl.
prolongation in our IDDM model, single cell action potentials (APs) were recorded in enzymatically dispersed myocytes from left ventricular endocardium. As illustrated in Fig.3, 50% and 90% APD (APD50 and APD90) measured in diabetic rabbit cardiac myocytes (556.6±72.5ms and 647.9±72.2ms) are longer than those in control group (271.5±32.3 ms and 347.8±36.3 ms, P < 0.05).

Diabetes-induced changes in ionic currents

To unravel the changes of ion currents that may account for the QTc/APD prolongation and the associated arrhythmias in our IDDM animals, we performed whole cell patch clamp studies of ion currents under physiological conditions in ventricular myocytes, including IK1, IKs, ICa-L. Our results revealed increment of multiple ion currents (IK1 and ICaL) and reduction of IKs in cells isolated from IDDM rabbits, compared with healthy rabbits. The IK1 current was evoked by depolarizing test pulses to voltages between -120 and +50 mV, arising from the holding potential of -40 mV. IK1 current density was found larger in IDDM myocytes at potential between -40 mV and +10 mV. At -40mV, the current amplitude was (1.76±0.27) pA/pF in controls and (3.10±0.38) pA/pF in diabetic rabbits. At -120mV, the current amplitude was (-12.72±0.94) pAPf in control and (-26.19±2.77) pAPf in diabetic rabbits (P < 0.05, n = 16, Fig.4).

The calcium current was evoked by 300-ms-long depolarizing test pulses to voltages between -40 and +50 mV, arising from the holding potential of -40 mV. Peak values of ICa-L were plotted against the respective test potentials. The density of ICa-L was significantly increased ranging from 0mV to +50 mV in IDDM hearts. At +10 mV, the current amplitude was (-2.67±0.41) pA/pF in controls (n=14) and (-5.40±1.08) pA/pF in diabetic rabbits (n=13, P < 0.05, Fig.5).

As displayed in Fig.6, the amplitude of IKs was significantly less in diabetic rabbits (n=16) than in non-diabetic rabbits (n=17) at +50 mV [ (1.27±0.20) pA/pF vs (3.08±0.67) pA/pF, P < 0.05]. The change of the amplitude in IKs tail was the same to the IKs step current. At the test potential of +70 mV, the current amplitude was (2.78±0.1) pA/pF in control.

Fig.3 Prolongation of action potential duration (APD) in IDDM rabbits. A: The action potentials were elicited by a train of 10-consecutive stimuli of 2-ms duration and twice threshold strengths at a frequency of 10kHz. B: APD50 and APD90 are obtained from data analysis. Mean data are from 10 cells (IDDM) and 11 cells (Ctl). Ctl: control. IDDM: insulin-dependent diabetes mellitus. *P<0.05 vs Ctl.

Fig.4 Current-voltage (I-V) relationship of the inward rectifier potassium current (IK1) in rabbit ventricular myocytes isolated from healthy and diabetic animals. A: Representative IK1 families. B: Average results presented as I-V curves. Symbols and bars are means ± SEM. n denotes the number of cells tested. Ctl: control. IDDM: insulin-dependent diabetes mellitus. *P<0.05 vs Ctl.
and (1.56±0.08) pA/pF in diabetic rabbits (P<0.05, respectively).

**Discussion**

This study firstly described the diabetes-induced changes in the main transmembrane cardiac ion currents underlying the repolarization in the rabbit, a species having ventricular repolarization relatively similar to that of the human. The major finding of this study was that experimentally induced diabetes mellitus caused a statistically significant lengthening of the QTc interval in the rabbit heart, which was associated with a marked reduction in the density of IKs and an outstanding increase in the density of ICa-L.

IKs acts as repolarization reserve to limit excessive slowing of repolarization. Previous study revealed that in diabetic rabbit model the repolarization lengthened due to the blockage of IKr. The subsequent increase in action potential duration would facilitate the activation of IKs and provide a negative feedback mechanism to limit further lengthening. Here, Our study demonstrated that IKs was inhibited in diabetic rabbit. Without the accumulation of IKs, the inhibition of other repolarizing current such as IKr could lead to more pronounced APD/QT prolongation. It is likely that the decreased IKs in diabetes mellitus would attenuate the repolarization reserve, and as such, it would increase the proarrhythmic risk especially when another repolarizing potassium current is also diminished (e.g. due to a genetic defect of a K+ channel, or in case of acquired long QT syndrome). Consequently, diabetic patients may carry an increased proarrhythmic risk due to their compromised repolarization reserve capacity even if their QTc interval is close to normal. This must be borne in mind when designing pharmacotherapy for diabetic patients.

ICa-L also contributes to maintain the AP plateau. Some studies have shown that Ca2+ entrying through the voltage dependent L-type Ca2+ channel was reduced in cardiac myocytes from streptozotocin-induced diabetic rats and from obese db/db mice, a well-known model of type II diabetes. A recent study showed that, in a type I diabetic
mice model L-type, Ca\(^{2+}\) current (ICa-L) decreased density in myocytes compared with non-diabetic control littermates.\(^{25}\) However, the rat and mice are different from other species such as human, canines, rabbits, whose repolarization includes plateau phase. Our data suggested that the function of ICa-L was facilitated in cardiac myocytes after diabetes mellitus with QT prolongation. The increased calcium channel density could induce the prolongation of APD and consequently trigger malignant arrhythmias. Increasing ICa-L induced the disturbance of intracellular calcium and the restoration of SR free Ca\(^{2+}\). ICa-L may also contribute to the differences in APD restitution curves and was also an important determinant of dynamic wave instability during ventricular tachyarrhythmias.\(^{26}\)

Inward rectifying potassium current (IK1) is responsible for maintaining membrane resting potential of cardiac myocytes and phase 3 (rapid repolarization phase) of action potential. It is well known that the magnitude of the outward component of IK1 plays an important role in controlling the frequency of VT/VF. From our present data, both the inward and outward currents of IK1 were increased markedly in the cardiomyocytes from diabetic rabbits. In theory, any increase in net outward current may act to increase frequency and stability of reentry in the heart. It should accelerate terminal ventricular repolarization which tends to accelerate and stabilize rotors.\(^{20}\)

The major contribution of this study was the description of the ionic changes in diabetes' rabbits and the further illustration of mechanism of QT prolongation. On account of IKs decreases in its current density in diabetic cardiac myocytes, the repolarization reserve attenuated and the ventricular repolarization was prolonged significantly. Meanwhile, the increment of ICa-L current density accelerates the QT prolongation potentiality. Of course, the further molecular mechanisms essential to elucidate arrhythmias caused by diabetes is needed to be explored.

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