

Probucol prevents atrial ion channel remodeling in an alloxan-induced diabetes rabbit model

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ABSTRACT

Diabetes mellitus (DM) increases the risk of developing atrial fibrillation (AF), but the molecular mechanisms of diabetes-induced atrial remodeling processes have not been fully characterized. The aim of this study was to examine the mechanisms underlying atrial ion channel remodeling in alloxan-induced diabetes model in rabbits. A total of 40 Japanese rabbits were randomly assigned to a control group (C), alloxan-induced diabetic group (DM), probucol-treated control group (Control-P), and probucol-treated diabetic group (DM-P). Using whole-cell voltage-clamp techniques, $I_{Ca,L}$, I_{Na} and action potential durations (APDs) were measured in cardiomyocytes isolated from the left atria in the four groups, respectively. In the DM group, increased $I_{Ca,L}$ and decreased I_{Na} currents were reflected in prolonged APD_{90} and APD_{50} values. These changes were reversed in the DM-P group. In conclusion, probucol cured AF by alleviating the ion channel remodeling of atrial myocytes in the setting of diabetes and the promising therapeutic potential of anti-oxidative compounds in the treatment of AF warrants further study.

INTRODUCTION

Nowadays, atrial fibrillation (AF) is the most common arrhythmic condition encountered in clinical practice, producing significant morbidity and mortality via the development of various concurrent diseases and consequences, such as strokes and myocardial infarction [1]. Unfortunately, its prevalence and incidence continue to accelerate, placing a significant financial burden on the healthcare systems worldwide. Current available clinical treatment strategies, such as pharmacological therapy or invasive procedures such as catheter ablation have limited efficacy and are not without adverse effects, especially for patients with persistent AF. As one independent risk factor of AF, diabetes mellitus (DM) is a clinical condition that is becoming increasingly prevalent, in part due to a rising level of obesity, and has been shown to increase atrial arrhythmogenicity through structural

and electrophysiological remodeling [2-4]. The exact underlying pathophysiological mechanisms of such remodeling processes are not fully understood [5]. In an alloxan-induced diabetic rabbit model, our group had previously demonstrated that hyperglycemic conditions lead to increased interstitial fibrosis and higher likelihood of AF development [7], as well as reduction of the Na^+ current (I_{Na}) and increase of the Ca^{2+} current (I_{CaL}) in the atria [8]. In the past, it had been demonstrated that an increase in oxidative stress leads to shortened action potential durations (APDs) in the atria due to the elevated transient outward K^+ current [10] and promotes Ca^{2+} release from the intracellular store [11]. Furthermore, reduction of I_{Na} current due to reduced mRNA and protein levels was also observed [12]. Together, these changes can lead to both triggered and re-entrant arrhythmogenesis.

Probucol is an anti-hyperlipidemic medication with antioxidant effects by reducing lipid peroxidation

and increasing the activities of antioxidant enzymes. Our previous study has demonstrated that probucol exerted anti-inflammatory effects in the atria, through downregulation of a number of key genes involved in the pro-inflammatory pathways, such as NF- κ B, TGF- β , HSP70, TNF- α in DM rabbit models [9]. However, electrical remodeling under DM and the impact of probucol have not been studied and reported. In this present study, by using an integrative approach of molecular biology and electrophysiology techniques on well established animal models, we tested the hypothesis that probucol can prevent ion channel remodeling in the atria by its anti-inflammatory and anti-oxidative impact.

RESULTS

Serum oxidative stress parameters

The levels of biochemical and oxidative stress markers in the four experimental groups are illustrated in Table 1. We discovered that DM led to increased levels of cholesterol, triglyceride and LDL-c, as well as higher MPO, and lower SOD and CAT concentrations compared to control ($P < 0.05$), but not significance in renal function (BUN and creatinine values) ($P > 0.05$). The probucol treatment reversed the rise in the lipid peroxidation product, malondialdehyde (MDA) in the DM group ($P < 0.05$).

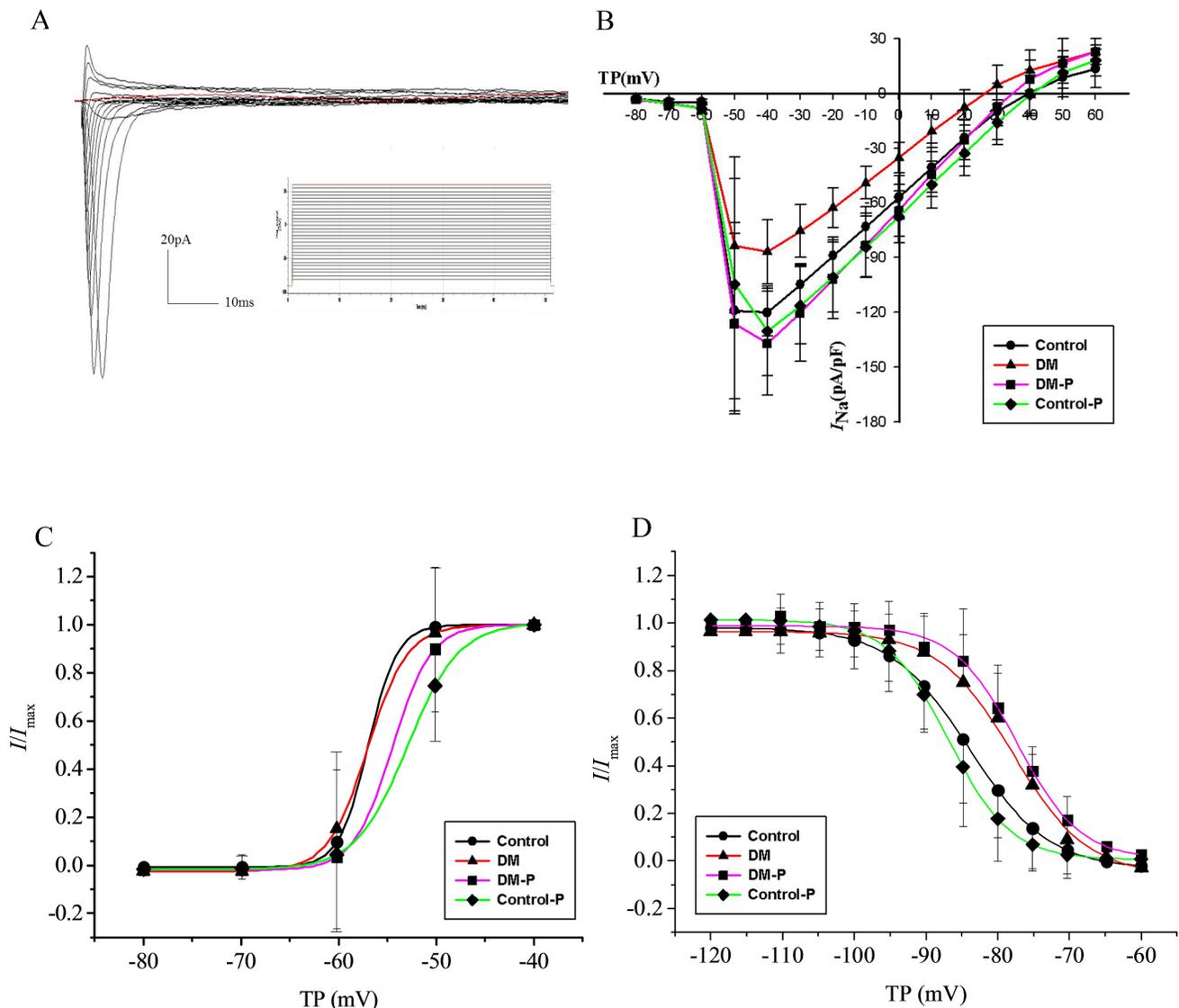


Figure 1: A. representative I_{Na} evoked by the applied protocol; B. I_{Na} densities and peak I_{Na} density as a function of test potential; C. The steady-state activation curve of I_{Na} ; D. The steady-state inactivation curve of I_{Na} , $V_{1/2inact}$, TP: Test potential.

I_{Na} densities

The average value of cell capacitance is 68.51 ± 10.52 pF ($n = 11$) in the Control group, 68.91 ± 14.23 pF ($n = 10$) in the DM group, 67.19 ± 11.17 pF ($n = 8$) in the DM-P group, and 66.20 ± 9.88 pF ($n = 9$) in the Control-P group. Figure 1(A) shows I_{Na} properties in response to the applied protocol, whereas Figure 1 (B) depicts I_{Na} densities and peak I_{Na} density as a function of test potential. Compared to the control group, the DM group did not exhibit differing morphology of the I-V curve, but demonstrated lower I_{Na} densities at all voltages (-60 mV to $+35$ mV) ($P < 0.05$), with a 72.29% reduction in I_{Na} density at -40 mV compared to the Control group. Peak I_{Na} density in the DM-P group increased by 157.92% compared with the DM group.

Activation and inactivation properties of I_{Na} channels

The steady-state I_{Na} activation curve is shown in Figure 1(C) and the different activation and inactivation parameters are summarized in Table 2. k is the slope factor of the inactivation curve. The experimental data of the voltage dependent activation were fitted by Boltzmann

equation. The membrane potential at which half-activation occurs, $V_{1/2act}$, was not significantly different between the Control and DM groups ($P > 0.05$). It was not altered by probucol in either group ($P > 0.05$). The steady-state I_{Na} inactivation curve is shown in Figure 1(D). Together, the above data indicate that neither DM nor probucol modified the voltage-dependent activation or inactivation properties of I_{Na} .

I_{CaL} changes

The mean cell capacitance was 55.22 ± 10.37 pF ($n = 12$) in the Control group, 57.63 ± 14.49 pF ($n = 10$) in the DM group, 57.79 ± 14.38 pF ($n = 10$) in the DM-P group, and 59.57 ± 9.24 pF ($n = 13$) in the Control-P group. Figure 2(A) shows I_{CaL} properties in response to the applied protocol. Figure 2(B) illustrates I_{CaL} densities and peak I_{CaL} as a function of the test potential. Compared to the Control group, the DM group did not show differing morphology in the I-V curve, but had larger I_{CaL} at all voltages steps tested between -60 mV to $+35$ mV ($P < 0.05$). Peak I_{CaL} currents in the DM-P group were reduced by 62.68% compared to the DM group. The maximal I_{CaL} amplitude was between 0 mV and 10 mV in all four experimental groups.

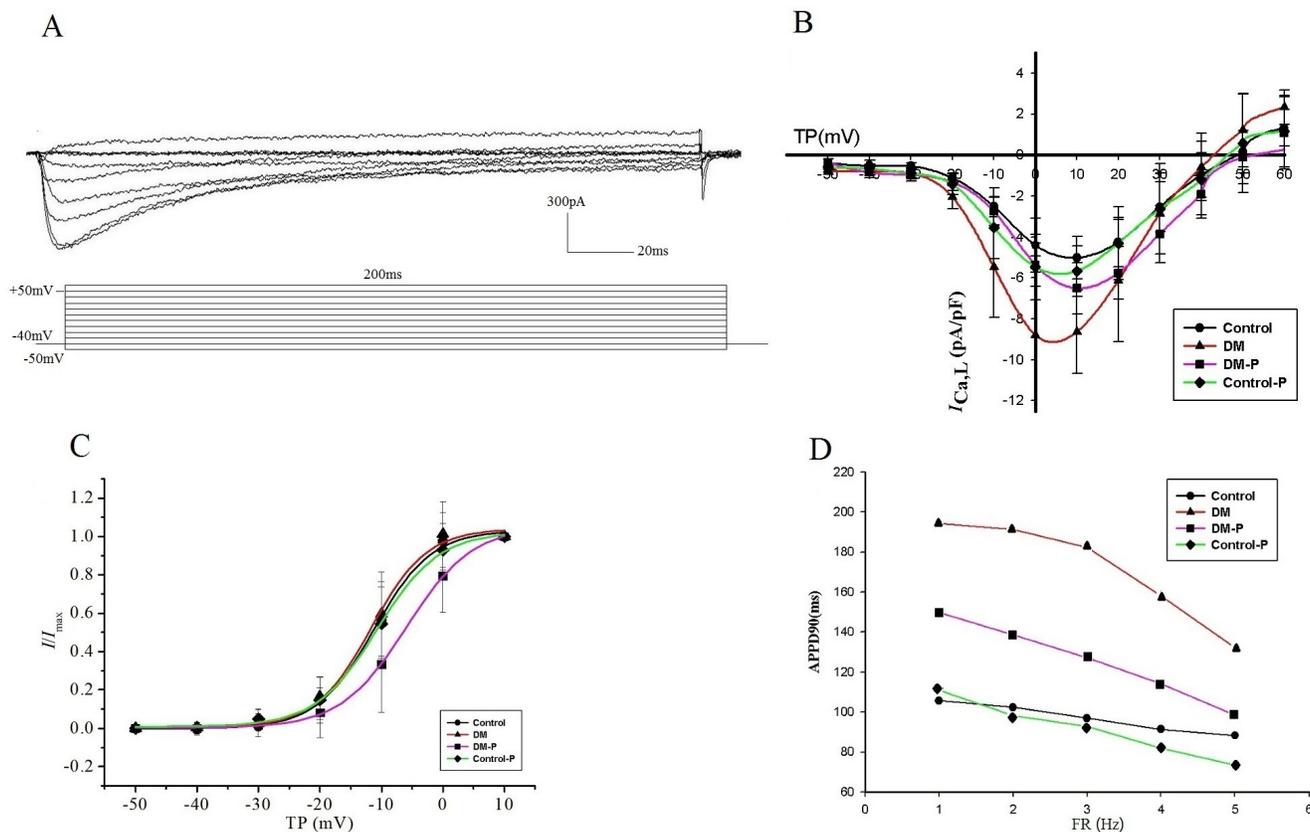


Figure 2: A. representative I_{CaL} evoked by the applied protocol; B. I_{CaL} densities and peak I_{CaL} density as a function of test potential, TP: Test potential; C. The steady-state activation curve of I_{CaL} . TP: Test potential; D. Rate-dependent APD90 changes.

Activation characteristics of I_{CaL} in isolated atrial myocytes

The steady-state I_{CaL} activation curve is shown in Figure 2 (C), in which the data were fitted by Boltzmann equation. $V_{1/2act}$ was not significantly different between the Control and DM groups ($P > 0.05$). Probucol increased $V_{1/2act}$ compared to the Control or DM groups (Control- 11.21 ± 0.51 mV, DM- 11.63 ± 0.49 mV, DM-P- 5.91 ± 0.71 mV, Control-P- 10.69 ± 0.56 mV, $P < 0.05$). A right shift in the activation curve was observed in the DM-P group, i.e. a given value of V_m the degree of current activation was smaller in the DM-P group. k in four groups are Control 4.67 ± 0.49 , DM 4.50 ± 0.46 , DM-P 5.37 ± 0.57 , Control-P 5.00 ± 0.54 , respectively, $P > 0.05$.

Properties of the cellular action potential

The resting membrane potential was not altered in DM group with an average of -68.41 ± 6.04 mV ($n = 12$ cells) in control rabbits compared with -67.70 ± 6.20 mV ($n = 12$), -64.26 ± 6.94 mV ($n = 13$), and -64.14 ± 5.28 mV (n

= 10) in DM, DM-P, and Control-P rabbits, respectively ($P > 0.05$). APD_{90} was shortened with increasing stimulation frequencies in all four experimental groups (Figure 2D)). Prolonged APD_{90} and APD_{50} values in the DM group were observed compared to the Control group; these changes were abolished by probucol (Figure 3, Table 3). Mean APD, AP amplitude and maximum upstroke velocity are displayed in DM did not significantly affect rate-dependent APD properties.

DISCUSSION

In this study, we demonstrated: 1) diabetes reduced I_{Na} densities in atrial tissue, while leaving $V_{1/2act}$ or k of I_{Na} intact; 2) Whilst diabetes did not alter the morphology of the I_{CaL} I-V curve, it increased I_{CaL} densities. Probucol prevented the reduction of I_{Na} densities and the increase in I_{CaL} densities, increased $V_{1/2act}$ and produced a right shift in the activation curve, and I_{CaL} was smaller at any given value of V_m compare with the DM group; 3) DM prolonged APD_{90} and APD_{50} , and these changes were abolished by probucol.

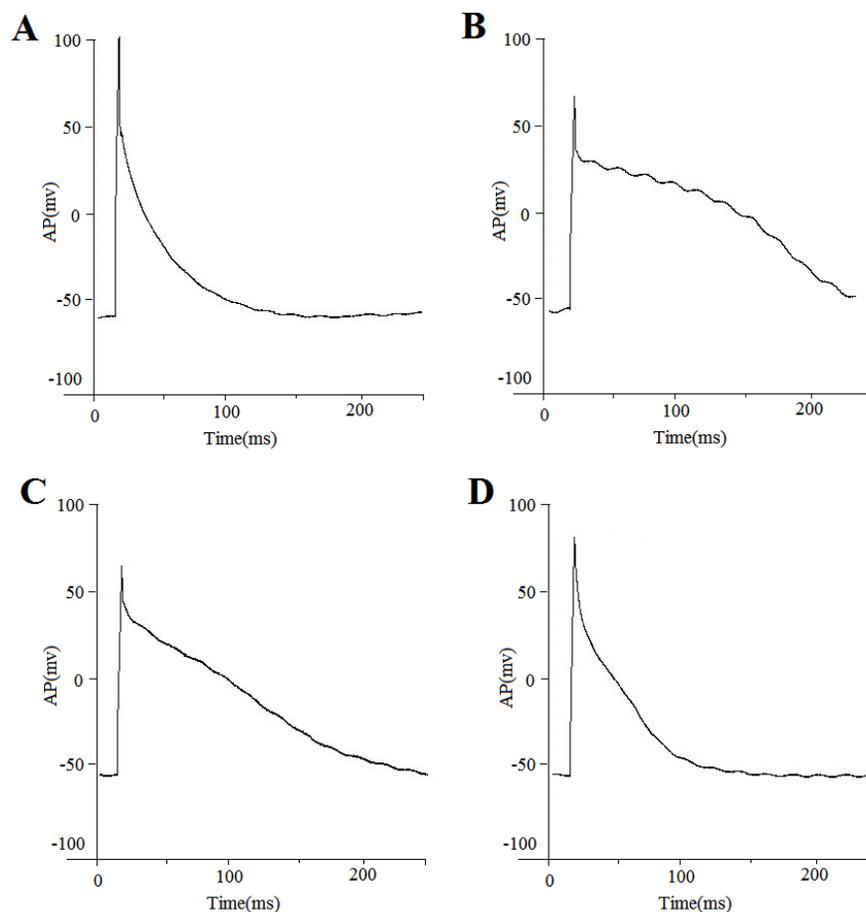


Figure 3: Action potential recordings from representative cells obtained from a A. control group; B. DM group; C. DM-P group and D. Control-P group. Action potentials were recorded in current-clamp mode at the frequencies indicated.

Table 1: Biochemical and oxidative stress parameters

	Control group (n = 10)	DM group (n = 10)	DM-P group (n = 10)	control-P group (n = 10)
Weight, Kg	2.19±0.17	2.29±0.47	2.29±0.20	2.34±0.08
Glu level at 8W,mmol/L	5.58± 0.75	20.86±4.37*	19.28±8.82*	6.54±0.52#
BUN, mmol/L	5.65±2.00	6.87±2.23	5.08±1.01	4.49±1.40
Cr, umol/L	98.5±16.31	103.7±29.22	95.13±27.18	89.66±9.48
TG, mmol/L	1.19±0.31	1.52±1.23	1.30±0.29	1.25±0.38
TC, mmol/L	1.14±0.57	1.25±0.39	0.87±0.35	0.92±0.36
HDL-c, mmol/L	0.56±0.16	0.49±0.20	0.43±0.14	0.47±0.17
LDL-c, mmol/L	0.36±0.16	0.38±0.16	0.31±0.15	0.24±0.10
INS, uIU/ml	16.24±7.87	5.71±3.01*	7.33±1.56*	13.89±5.11#
Serum CAT, U/ml	6.40±2.46	4.79±2.06	4.80±0.96	5.35±1.74
Serum SOD, U/ml	588.08±82.09	556.29±47.7	589.18±63.44	640.12±82.02
Serum MPO, U/L	63.66±29.44	76.41±27.02	56.66±20.58	64.30±14.35
Serum MDA, nmol/ml	17.69±3.59	22.17±4.15*	18.32±1.63#	18.14±2.62#
CAT in atrium, U/mg prot	4.30±1.29	3.82±1.87	4.00±1.13	4.78±1.33
SOD in atrium, U/mg prot	98.41±12.07	72.11± 11.08*	88.48±8.81#	93.03±20.04#
MPO in atrium, U/mg prot	0.51±0.13	1.00±0.38*	0.78±0.08*	0.55±0.91#
MDA in atrium, mol/mg prot	0.78±0.12	1.10±0.24*	0.84±0.32#	0.64±0.17#

Values are mean ± SD; Abbreviations: LA-left atrial; LV-left ventricular; *:Compared with Control group $p < 0.05$; #: Compared with DM group $p < 0.05$

Effects of diabetes mellitus and probucol on I_{Na}

Many isoforms of the voltage-gated Na^+ channels have been described in many current literatures and the predominant isoform was found in cardiac tissue. It is known that Nav1.5 is encoded by SCN5A and I_{Na} activation contributes to the upstroke of cardiac AP [15]. Our previous experiments have shown that increased atrial interstitial fibrosis and higher vulnerability to AF was evident under hyperglycemic conditions in a DM rabbit model induced by alloxan [8]. These pathological changes were associated with higher expression levels of several proteins involved in inflammation, including transforming growth factor- β (TGF- β), TNF- α and NF- κ B [7-9]. Moreover, prolonged APD₉₀ and APD₅₀ values in isolated left atrial cardiomyocytes from diabetic rabbit hearts were demonstrated, and these changes were associated with I_{Na} downregulation and I_{CaL} upregulation [8]. We argue the ion channel remodeling can be explained by altered cellular signaling, such as protein kinase C (PKC) activation [16-17]. The activity of the cardiac Na^+ channel can be regulated by post-translational modification such as phosphorylation events, through increased protein kinase A (PKA) and PKC [18]. This reduction in I_{Na} can be prevented by the antioxidant enzyme SOD or a PKC inhibitor. PKC α -mediated phosphorylation of the Na^+ channel has been reported to result in immediate reduction of Nav1.5 channels [19]. Also, previous studies suggest that PKC decreases I_{Na} , but this was not due to altered channel gating mechanisms [20]. Instead, this

may involve alterations in transcriptional activity of the channel. In supporting of this notion, the promoter region of the SCN5a gene has a consensus NF- κ B binding site [21]. Indeed, angiotensin II, which is elevated in cardiometabolic disorders, can lower Na^+ channel expression mediated by higher levels of oxidative stress from H_2O_2 . The latter can promote NF- κ B binding to the Na^+ channel promoter in turn leading to a decrease in Na^+ channel expression in the plasma membrane [22]. Therefore, multiple interacting molecular mechanisms can alter the activity and expression of Na^+ channels to mediate adverse electrophysiological remodeling.

Probucol is a potent antioxidant compound that has promising therapeutic effects in patients with DM in randomized clinical trials. It is an anti-hyperlipidemic agent that can reduce redox imbalances by reducing myocardial lipid peroxidation and increasing the antioxidant system [23, 24] and by downregulating a number of key pro-inflammatory mediators such as NF- κ B, TGF- β , HSP70 and TNF- α in a diabetes rabbit model produced by alloxan [9]. Therefore, it is likely that probucol exerts multiple effects on Na^+ channel during pathophysiological conditions through reduction in NF- κ B- and PKC-mediated oxidative stress.

Effects of diabetes mellitus and probucol on I_{Ca} and action potential

Voltage-gated Ca^{2+} channels (VGCCs) modulated by cytosolic Ca^{2+} determine myocardial contractility and

Table 2: The shifts in activation and inactivation variables of I_{Na} ($x \pm s$)

Group	Control	DM	DM-P	Control-P
k	1.49±0.37	2.03±0.69	1.98±0.28	2.63±0.34
$V_{1/2}$ (mV)	-56.97±0.78	-57.01±1.12	-54.48±0.74	-52.93±0.48
$V_{1/2inact}$ (mV)	5.53±0.52	5.20±0.59	4.38±0.42	4.33±0.31
k_{inact}	-84.00±0.57	-77.91±0.70	-77.32±0.49	-86.86±0.35

$V_{1/2act}$ (membrane voltage [Vm] at which half-activation occurs; $V_{1/2inact}$: Vm at which half-inactivation occurs; k : the activation slope factor; k_{inact} : inactivation slope factor

Table 3: Mean values of APD90 and APD50($x \pm s$)

	Control (n = 12)	DM(n = 12)	DM-P (n = 13)	Control-P(n = 10)
RP(mv)	-78.41±6.04	-77.70±6.20	-74.26±6.94	-74.14±5.28
APD90-1Hz	105.69±29.77	194.34±45.47*	149.75±52.24#	110.93±23.37#
APD90-2Hz	102.34±26.39	191.19±42.04*	138.62±48.76#	98.08±30.55#
APD90-3Hz	96.92±25.01	182.40±40.93*	127.25±43.13#	92.95±30.41#
APD90-4Hz	91.38±23.33	158.28±16.09*	114.11±35.16#	81.95±26.03#
APD90-5Hz	88.27±22.2	132.21±11.62*	98.51±25.32#	73.62±25.11#
APD50-1Hz	35.26±20.34	69.65±24.02*	70.22±35.90*	44.50±11.53
APD50-2Hz	29.80±13.57	64.10±20.15*	57.71±25.89*	35.21±13.17
APD50-3Hz	27.97±8.14	182.40±40.93*	46.32±12.04#	30.26±10.76#
APD50-4Hz	24.50±6.23	63.05±28.62*	44.63±12.39	24.27±11.23
APD50-5Hz	20.51±5.15	55.83±28.40*	37.62±9.59#	22.06±10.68
AP-amplitude(mv)	204.96±20.14	134.54±24.58*	134.69±14.01*	188.85±14.79#
APA (mV)	204.96±20.14	134.54±24.58*	134.69±14.01*	188.85±14.79#
Vmax (V/s)	81.44±7.42	42.37±10.64*	52.23±9.39*#	82.31±7.74#

Abbreviations: RP: resting potential ;APA: action potential amplitude; Vmax: maximum rate of rise of the action potential; APD50, APD90: action potential duration at 50 and 90% repolarization; * Compared with Control group $P < 0.05$; # Compared with DM group $P < 0.05$

APDs in cardiac tissue. Normally, VGCCs are closed during electrical diastole and activated by membrane depolarization, and it contributes a significant amount of inward current during the plateau of the APs. This inward Ca^{2+} influx is responsible for inducing further release from the intracellular store, the sarcoplasmic reticulum, leading to contractile activation [25].

Previous reports found reduced expression of VGCCs and SERCA2 in AF patients [26-30]. However, the role of VGCCs in postoperative AF is less clear. Van Wagoner et al [31] observed that higher $I_{Ca,L}$ in cardiomyocytes isolated from the right atria in postoperative AF patients compared to those without AF, but a different study found no significant difference in $I_{Ca,L}$ or APD in these two groups [32]. We believe that these conflicting results are likely due to different baseline characteristics of the subjects involved and different methods of detecting AF [33]. In our study, prolongations in both APD₉₀ and APD₅₀ values were observed in left atrial cardiomyocytes isolated from diabetic rabbit hearts. These changes were linked to higher $I_{Ca,L}$ densities. These findings are consistent with those by Van Wagoner et al [31]. Prophylactic treatment using calcium channel antagonists can reduce or prevent alterations in ion channel expression in the diabetic atria [34, 35]. Since

calcium overload is an important mechanism in mediating electrical instability [36], we hypothesized that higher I_{Ca} in DM is responsible for this observation. AF can be caused by triggered activity or by re-entry [37-39]. EADs develop in the context of APD prolongation, when inward currents ($I_{Ca,L}$ or late Na^+ current (I_{NaL})) are increased, or when outward currents (I_{K^+}) decreased [46, 47]. The causal relationship between abnormal Ca^{2+} handling and AF development is bidirectional [32]. Thus, hydrogen peroxide (H_2O_2), a reactive oxygen species, can alter molecular signaling mechanisms that ultimately lead to altered cellular calcium homeostasis [47]. The data generated from our group demonstrate that through a reduction in oxidative stress, as shown using the antioxidant compound probucol, can prevent or reverse electrophysiological remodeling in the atria [9].

MATERIALS AND METHODS

This study was approved by the Experimental Animal Administration Committee of Tianjin Medical University and Tianjin Municipal Commission for Experimental Animal Control, which follow the guidelines established by the U.S. National Institutes of Health.

Experimental animals

Forty Japanese rabbits, weighted ~1.5–2.0 kg at the beginning of the study (Beijing Medical Animals Research Institute, Beijing, China), were randomly assigned to control group (Control, $n = 10$), alloxan-induced diabetic group (DM, $n = 10$), probucol-treated group (Control-P, $n = 10$), and probucol-treated diabetic group (DM-P, $n = 10$). In the DM and DM-P groups, alloxan monohydrate (Sigma ldrich Chemical, Saint Louis, MO, USA) was dissolved in sterile sodium chloride to achieve a concentration of 5% (W/V) and 120 mg/kg, and then was immediately administered into the marginal ear vein. The diabetic state was confirmed 48 hours later by measuring blood glucose levels of ≥ 14 mmol/L. Rabbits in the DM-P and Control-P groups received probucol (Lunan Pharmaceutical Company, Shandong, China) orally (1,000 mg/day) for 8 weeks. The animals in the four groups were housed in cages on a standard laboratory pellet diet during the 8 week experimental period.

Serum biochemical and oxidative stress parameters

Serum cholesterol, triglyceride, low density lipoprotein cholesterol (LDL-c), high density lipoprotein cholesterol (HDL-c), fasting insulin blood urea nitrogen (BUN), and creatinine (Cr) levels were measured using automated techniques. Antioxidant enzyme activities levels in serum and left atrial tissue, including superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO) and malondialdehyde (MDA), were detected using antioxidant enzyme activities kits (Nanjing Jianchen Bioengineering Institute, Jiangsu province, China).

Single-cell electrophysiology

Cell isolation

The left atrium (LA) was used for isolation of single cardiomyocytes. The procedure for isolating cells was followed from a previously developed method [13]. A rabbit was anesthetized with 3% Pelltobarbitalum Natricum (30 mg/kg), and then the rabbit's heart was quickly removed from the torso and was placed in cold perfusion fluid (4 °C). The aorta was cannulated and connected to a Langendorff perfusion system filled with warmed (37 ± 0.5 °C) Ca^{2+} -free Tyrode's solution. Next, the heart was perfused at 30 ml/min for 20 minutes, followed by a 20-minute perfusion containing collagenase (0.075%, CLS II, Worthington Biochemical, Lakewood, CO, USA) and 0.2% bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO, USA). Cardiac tissue became opaque and mottled in appearance. Tissue from

a well-perfused region of the LA free wall was removed using forceps, gently triturated, and maintained at room temperature in a high- K^+ storage solution (KB solution). A small aliquot of the solution containing isolated cells was placed in a 2-mL chamber mounted on the stage of an inverted microscope (IX-50, Olympus Co., Tokyo, Japan). Five minutes following cell adhesion to the bottom of the chamber, cells were superfused with Tyrode's solution (3 mL/min) for 5 minutes, followed by extracellular solution for the recording of I_{Na} or I_{CaL} at the same velocity and time of flow. Recordings of I_{Na} and I_{CaL} were obtained 5 minutes following membrane rupture.

Data acquisition

Membrane currents were measured at room temperature (22 ± 1 °C) using the whole-cell configuration of the patch-clamp technique [14]. Briefly, whole-cell configuration was made in Tyrode's solution. Pipette resistances were 2–4 mV. After achieving a gigaseal, the test-pulse current was nulled by adjusting the pipette capacitance compensator with both fast and slow components. After break-in, the whole-cell charging transient was nulled by adjusting whole cell capacitance and series resistance. Voltage control protocols were generated with Axopatch 200B amplifier/Digidata 1200B acquisition system using pCLAMP-10 software (Molecular Devices/Axon, Sunnyvale, CA). Whole-cell recording was analyzed using Clampfit 10.2. I_{Na} , I_{CaL} and action potentials (APs) were recorded and filtered at 0.5 kHz using a low pass filter. The sampling frequency was set at 0.5 Hz for recording I_{Na} and 0.2 Hz for I_{CaL} . The current-voltage relation was determined in the extracellular solution over a voltage range of -80 to +60 mV increased in 10-mV steps from a hold potential (HP) of -90 mV for I_{Na} and -40 to +60 mV increased in 10-mV steps from a HP of -50 mV for I_{CaL} . The APs were recorded at 60, 120, 180, 240 and 300 bpm. APD was stabilized within 5 action potentials at each cycle length, and was measured at 50% (APD_{50}) and 90% (APD_{90}) of full repolarization.

Statistical analysis

All results are presented as mean \pm standard deviation. Comparisons between the groups were analyzed for statistical significance using the one-way analysis of variance (ANOVA) and Fisher's exact test, respectively. Differences with $P < 0.05$ were considered statistically significant. In order to ensure data validity from voltage clamp studies, similar numbers of cells from different regions of each heart were studied utilizing the same protocol (i.e., cells were distributed evenly across rabbits).

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CONFLICTS OF INTEREST

All authors declare no potential conflicts of interest.

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