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BK channels regulate calcium oscillations in ventricular myocytes on different substrate stiffness

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ABSTRACT

Substrate stiffness is essential for cell functions, but the mechanisms by which cell sense mechanical cues are still unclear. Here we show that the frequency and the amplitude of spontaneous Ca^{2+} oscillations were greater in chick cardiomyocytes cultured on the stiff substrates than that on the soft substrates. The spontaneous Ca^{2+} oscillations were increased on stiff substrates. However, an eliminated dependence of the Ca^{2+} oscillations on substrate stiffness was observed after applying blocker of the large-conductance Ca^{2+}-activated K^+ (BK) channels. In addition, the activity of BK channels in cardiomyocytes cultured on the stiff substrates was decreased. These results provide compelling evidences to show that BK channels are crucial in substrate stiffness-dependent regulation of the Ca^{2+} oscillation in cardiomyocytes.

1. Introduction

Ventricular myocytes attach to the collagen-based extracellular matrix (ECM). The elasticity of ECM is altered under disease conditions. For example, the elasticity of the fibrosis myocardium matrix could be several-time stiffer than that of the normal matrix due to an excessive accumulation of collagens [1,2]. The cardiac ECM stiffness has been known to regulate multiple physiological processes including maturation, sarcomere organization, electromechanical coupling, morphology and gene expressions of neonatal cardiomyocytes [3]. The mechanism by which the ventricular myocytes sense the substrate stiffness is important to understand cardiac remodeling, especially the remodeling in pathological processes. Previous reports have suggested that cells apply traction forces from stress fibers (SFs) to substrates via focal adhesions [4] and this pathway is regulated by Ca^{2+}. It has been shown that intracellular Ca^{2+} ($[Ca^{2+}]_i$) is crucial in the dynamics of the cytoskeleton [5]. Thus, we speculated that $[Ca^{2+}]_i$ dynamics may mediate the response of ventricular myocytes to the substrate stiffness.

$[Ca^{2+}]_i$ dynamics is hypothetically modulated by certain mechanosensitive ion channels, such as BK channels. BK channels are expressed in various types of mammalian cells and involved in many important physiological processes, such as the regulation of vascular smooth muscle, endocrine cell secretion and neuronal firing [6]. Activation of BK channels hyperpolarizes the membrane and causes closure of voltage-dependent Ca^{2+} channels (VACCs). This provides a negative feedback for Ca^{2+} entry via VACCs, thereby reducing global $[Ca^{2+}]_i$. Whether BK channels modulate the Ca^{2+} oscillations and play a role in sensing substrate stiffness of ventricular myocytes is little known. As BK channels are poorly expressed in the heart, they were initially not considered as critical to cardiac function. However, the transcription of a gene encoding mouse cardiac BK channel was confirmed [7]. The expression of BK channels was also confirmed in the rat atrium and ventricle, although the expression level was limited [6]. In addition, the existence of BK channels in plasma membrane has been confirmed by our previous work using electrophysiological experiments in chick hearts (referred as SAKCaC) [8–10]. The SAKCaCs in chick heart is similar to other BK channels, which can be activated by depolarization and increased intracellular calcium ions. More importantly, it is unique that SAKCaCs can be activated by membrane stretch. All these findings imply that cardiac BK channels might regulate cellular excitability in response to calcium signaling in ventricular myocytes. Thus, we examined the effects of BK channel on the calcium oscillation in ventricular myocytes on substrate with different stiffness. Our study extends the understanding about how ventricular myocytes sense substrate stiffness.

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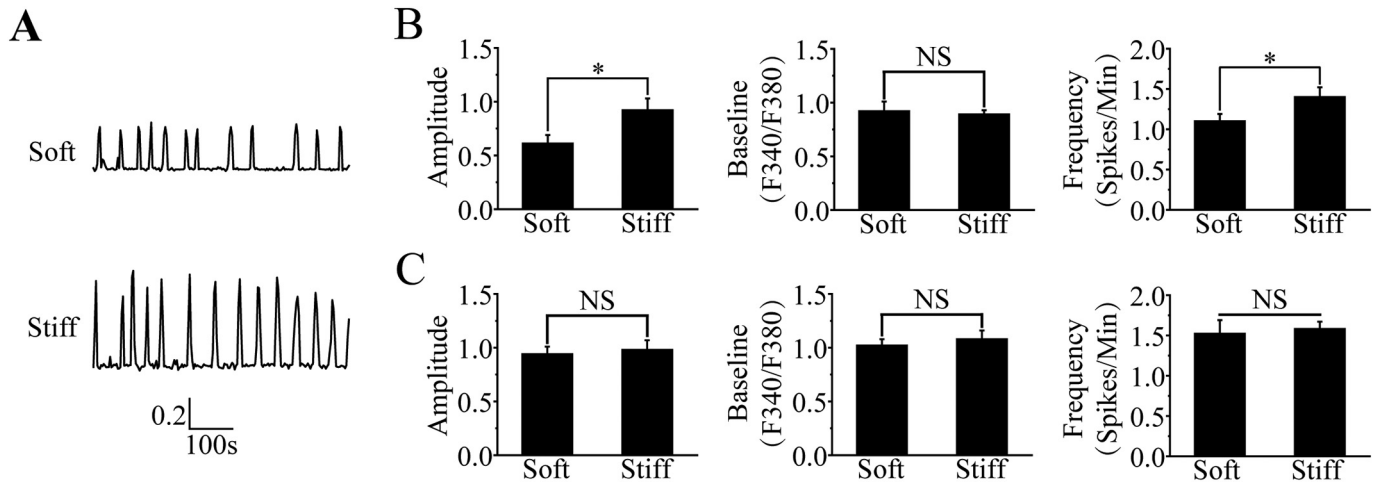

Fig. 1. Effects of substrate stiffness on calcium oscillations in CCVMs. (A) Representative time elapse recordings of Ca^{2+} oscillations from CCVMs on soft and stiff substrates. (B) Summary of the magnitude (left), the basal cytosolic Ca^{2+} concentrations (middle) and the frequency (right) of Ca^{2+} oscillations in 62 CCVMs on soft and stiff substrates from three preparations. (C) Summary of the magnitude (left), the basal cytosolic Ca^{2+} concentrations (middle) and frequency (right) of Ca^{2+} oscillations in 60 CCVMs on soft and stiff substrates in presence of IBTX (100 nM) for 10 min from three preparations. $^{*}P < 0.05$, and NS, no significant difference.

2. Materials and methods

2.1. PDMS fabrication

The Polydimethylsiloxane (PDMS) was prepared using sylgard 184 (Corning, NY, USA). Different stiffness of PDMS was achieved by different mass ratios of curing agent (35 mm × 10 mm, Corning) and oligomeric base (1:5 and 1:45). The mixture was poured into a single-well petri dish and solidified at 60 °C for 3 h. Then the sterilization step was carried out by irradiation under UV for 2 h. The Young's moduli were 1.014 ± 0.056 and 0.046 ± 0.011 MPa in 1:5 and 1:45, referred as "soft" and "stiff" substrates. PDMS was measured by the spherical indentation method [11].

2.2. Cell culture

The white leghorn chicken embryos came from China Agricultural University, Beijing, China. The animal materials and experimental methods used in this study conformed to ethical principles and was approved by Tsinghua University. Ventricles were isolated from chicken embryos aged 10–12 days. HS-DMEM contains DMEM (Corning) that was supplemented with 1% penicillin/streptomycin and 10% heat-inactivated horse serum, which inhibited proliferation of low-ratio cardiac fibroblasts coexisted in isolated cardiomyocytes. The cardiomyocytes were cultured at 5% CO_2, with 95% air humidity at 37 °C.

2.3. Cell seeding

The UV-sterilized PDMS substrate was coated by 5 pg/mL poly-D-lysine (MW 220,000: Sigma, MO, USA) at 37 °C for 40 min. The isolated cardiomyocytes were seeded at a concentration of 5×10^5 cells per plate on the PDL-coated PDMS substrates.

2.4. Electrophysiology

Single-channel and whole-cell patch-clamp recordings were performed as previously described [8–10]. The chick ventricular myocytes were cultured for 3–4 days on stiff and soft substrates, respectively. In single-channel recording (inside-out model), the pipette solution includes (in mM): 145 K-gluconate, 1 EGTA, 10 HEPES and 5 glucose with pH adjusted to 7.4. Bath solution includes (in mM): same as the pipette solution except various Ca^{2+} concentrations [8]. The channel open probability (P_o) was calculated by using pClamp software. For whole-cell recording, pipette solution includes (in mM): 145 KCl, 10 EGTA, 10 HEPES (pH 7.3 with Tris) and 1 $CaCl_2$. Bath solution includes (in mM): 1.3 $CaCl_2$, 0.8 $MgSO_4$, 5.4 KCl, 0.4 KH_2PO_4, 136.9 NaCl, 0.3 Na_2HPO_4, 10 D-glucose and 4.2 $NaHCO_3$.

2.5. Cytosolic Ca^{2+} measurement

After ventricular myocytes were cultured for 6 days, Ca^{2+} concentrations in cytoplasm were determined by Fura-2 atiometric calcium imaging. In brief, cultured chick ventricular myocytes (CCVMs) were incubated at 37 °C with 2 mM Fura-2/acetoxymethyl ester (Life Technologies) in DMEM for 30 min, and then washed with phosphate buffer saline for three times. PBS was replaced with MEM containing 2% FBS. The Ca^{2+} oscillations were imaged with Olympus IX71 (Till Photonics, Germany). The excitation is at 340 nm and 380 nm and the emission light is at 510 nm. The images were analyzed by TILLvisION4.0 program. The specific calculation method was shown in the previous study [12].

2.6. Statistical analysis

Calcium imaging and electrophysiological curve were made and fitted with the Origin 8.0 software. The open probability and $[Ca^{2+}]_i$ relationship was fitted with Hill equation: $P_o = [Ca^{2+}]_i^n / (K_d^n + [Ca^{2+}]_i)$, where n is the Hill coefficient and K_d is the dissociation constant. The P_o-voltage relationship was fitted with the Boltzmann equation: $P_o = 1/[1 + \exp. (V_{1/2} - V)/k]$, where $V_{1/2}$ and k are half-maximal channel activation voltage and slope, respectively. All data were presented as mean ± SD. Significant differences between two groups were performed using one-way ANOVA. Statistical evaluation of the significance was defined as p-value < 0.05.

3. Results

3.1. Stiff substrate enhances spontaneous Ca^{2+} oscillations in CCVMs

Ca^{2+} is a second messenger in pathways of various intracellular signal transduction [5]. To assay the effects of substrate stiffness on Ca^{2+} homeostasis, we firstly measured spontaneous Ca^{2+} oscillations in CCVMs. The spontaneous intracellular Ca^{2+} oscillations were observed in the most of CCVMs. Interestingly, both magnitude and frequency of Ca^{2+} oscillations were greater in CCVMs on stiff substrates

than these on the soft substrates (Fig. 1A and B). In contract, we found that the basal $[Ca^{2+}]_i$ slightly decreased in cells on the stiff substrates. These results indicate that, the extracellular substrate stiffness can affect Ca^{2+} oscillations in CCVMs and the frequency and magnitude of Ca^{2+} oscillations are more sensitive to substrate stiffness than the basal $[Ca^{2+}]_i$. Because the BK channel is involved in modulating the intracellular Ca^{2+} concentration [6], we then examined the roles of BK channels on regulating spontaneous Ca^{2+} oscillations in CCVMs. Interestingly, when we applied 100 nM iberiotoxin (IBTX), a specific BK channel inhibitor, we found that both the frequency and the magnitude of Ca^{2+} oscillations were similar in CCVMs on both soft and stiff substrates, suggesting IBTX abolished their dependency on substrate stiffness in CCVMs (Fig. 1C). All these results indicate that BK channels are critical in substrate stiffness-dependent regulation of the Ca^{2+} oscillations in cardiomyocytes.

3.2. Soft substrate increases the whole cell currents of BK channels in CCVMs

To further investigate the effects of substrate stiffness on the function of BK channels, we measured the BK channel currents by whole-cell recording. We recorded robust outward K^+ currents and these outward currents were strongly suppressed by 100 nM IBTX, indicating that the currents were mostly carried by the BK channel in CCVMs (Fig. 2A). In addition, the BK channel current density was significantly higher in myocytes on the soft substrates. The current density was 119.7 ± 8.47 pA/pF at +100 mV on the soft substrates versus 91.6 ± 6.12 pA/pF on the stiff substrates ($p < 0.05$) (Fig. 2B). All together, these results indicate that soft substrates increase the function of BK channel in CCVMs.

3.3. Substrate stiffness regulates the BK channel gating properties in CCVMs

To understand the mechanism by which BK channels were altered by substrate stiffness, we examined the channel open probability Po and the conductance of the BK channel in CCVMs by single channel recordings. We found that the Po was decreased in the cells on the stiffer substrate (Fig. 3A and B), but the conductance of BK channels was similar in CCVMs on both soft and stiff substrates (Fig. 3E). Next, we studied the effects of substrate stiffness on the open and closed time of BK channels. We examined the single channel activities in the presence of 1 μM Ca^{2+} under a membrane potential of +40 mV. We recorded both open and closed times of BK channels in CCVMs. The opening times were 10.3 ± 1.01 ms for BK channels in cells on soft substrates and 7.2 ± 0.48 ms for the stiff ones. The closed times were 11.3 ± 0.8 ms for soft groups and 15.6 ± 1.7 ms for stiff groups (Fig. 3C–D). These results show that softer substrate reduces the closed time and increases open time of the BK channels in CCVMs.

BK channels are consisted of α and β subunits. The α subunit forms the K-selective pore, while β subunits modulate the voltage and Ca^{2+}-sensitivity of BK channels [6]. To understand the role of β-subunit of BK channel in respond to the substrate stiffness, we treated CCVMs with tamoxifen (a β1 subunit-specific BK channel activator), which is known to activate BK channels only when it binds to the β1-subunit [13]. In the presence of 100 nmol/L $[Ca^{2+}]$i, application of tamoxifen (1 μmol/L) evoked a 2.4-fold increase in the Po of BK channels in CCVMs on soft substrates, whereas, on stiff substrates, tamoxifen only evoked a 1.6-fold increase (Fig. 4). These results indicate that the functional expression of the BK β1 accessory subunit may result in the differences in the Po of BK channels between soft and stiff substrates. Taken together, in CCVMs, substrate stiffness regulates the BK channel biophysical properties, such as the open probability, the open time and closed time of these channels, and this regulation is likely due to the expression of BK β1 subunit in response to different substrates stiffness.

4. Discussion

Myocardial fibrosis is one of the important pathophysiological processes involving cardiac remodeling [1,14]. It can stiffen the myocardium, decrease compliance and cause dysfunction of systole and diastole, resulting in heart failure and arrhythmias [1–3]. The stiffened myocardium decreases the matrix elasticity far beyond the normal ventricular ones. Reducing elasticity is one physical signal around the cardiac cells and is likely an important factor to impair the normal functions of cells and leading to dysfunction in cardiac disease [2]. However, the mechanism underlying cells sense substrate stiffness is unclear. Cells respond to the substrate stiffness by the traction forces generated by actomyosin (Myosin-II) motors in SFs[4]. $[Ca^{2+}]$i is a crucial regulator of traction forces in cells. Ca^{2+} oscillations regulate cell division, apoptosis, fertilization, migration and many other cellular

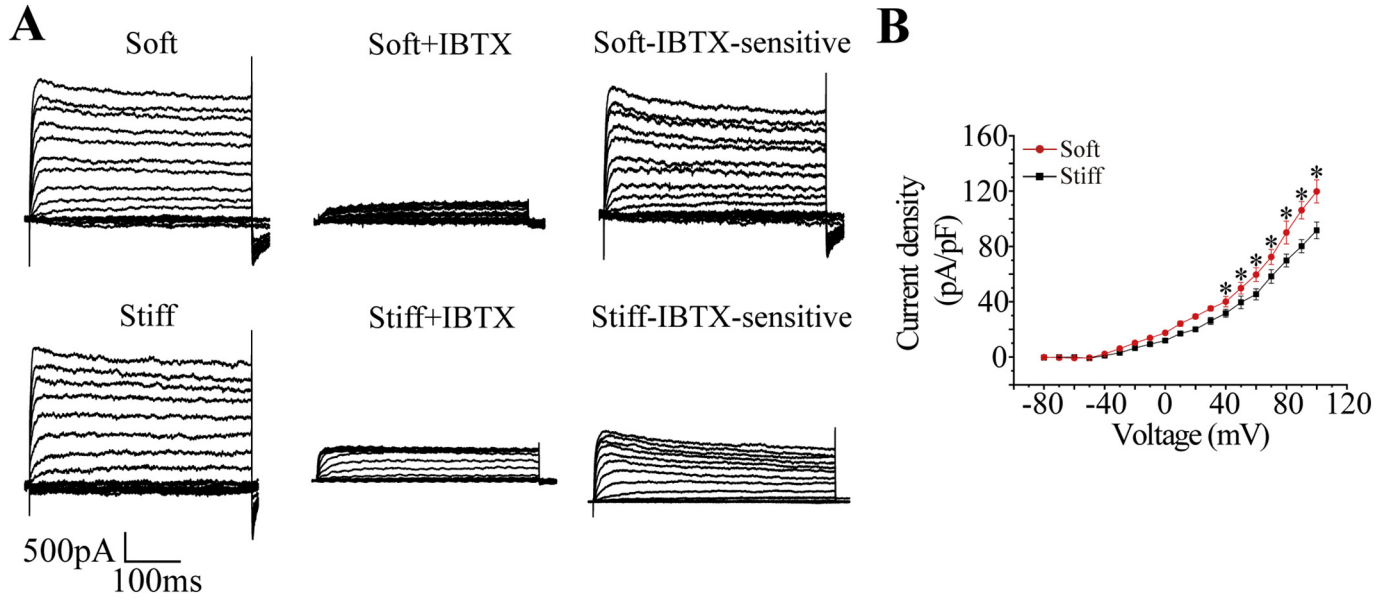

Fig. 2. Whole-cell BK channel currents in CCVMs on soft and stiff substrates. (A) Representative whole cell recordings of outward K^+ currents from cells on the soft and stiff substrates before (left panel), and 10 min after application of 100 nM IBTX (middle panel) and IBTX-sensitive currents (right panel). (B) The mean IBTX sensitive current density versus voltage in 9 CCVMs on soft and stiff substrates from three preparations. $^*P < 0.05$.

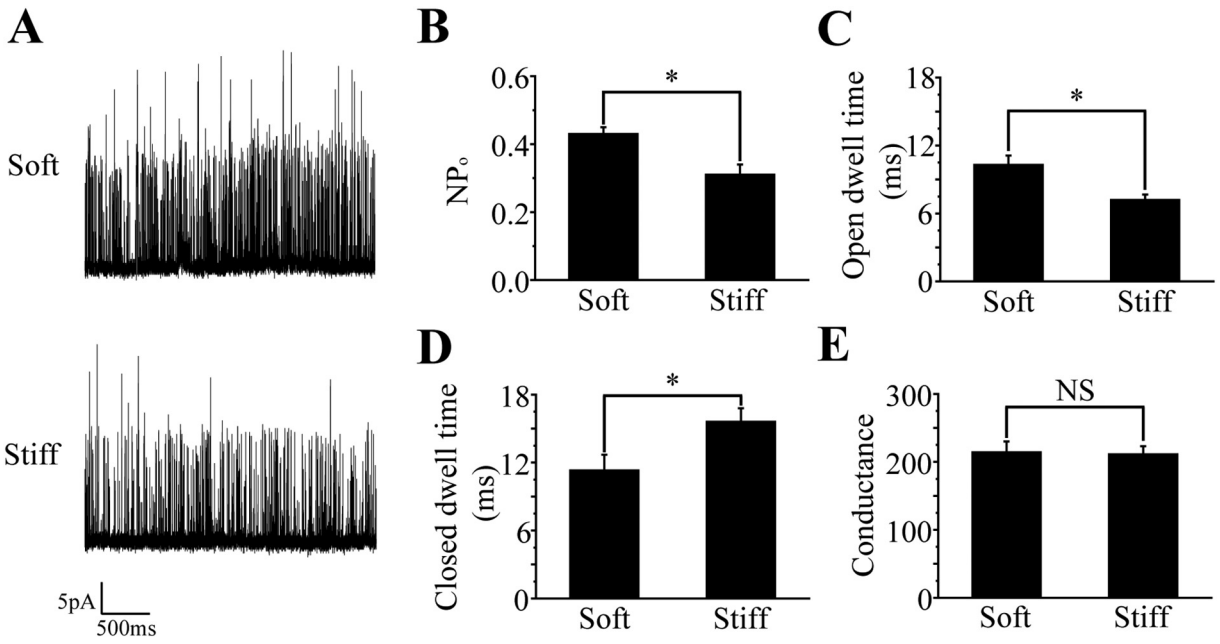

Fig. 3. Effects of substrate stiffness on BK channel biophysical activity and gating properties in CCVMs. (A) Representative trace of single-channel currents at a membrane voltage of +40 mV in CCVMs on soft and stiff substrates (1 μM $[Ca^{2+}]$ in bath solution) respectively. (B, C, D and E) Summary of the BK channel normalized *N*Po (B), open dwell time (C) and closed dwell time (D), and conductance (E) in 10–11 CCVMs on soft and stiff substrates from three preparations. $^{*}P < 0.05$, and NS, no significant difference.

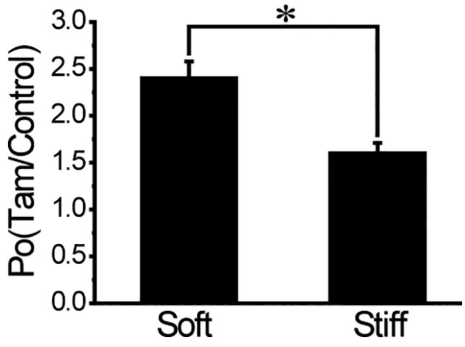

Fig. 4. Effects of tamoxifen (1 μmol/L) on BK channel activity. Bar plot summarizes the mean ± SD fold change in the Po of BK channels after the application of tamoxifen for 10 min in 10–11 CCVMs on soft and stiff substrates from three preparations. $[Ca^{2+}]$ in the bath solution: 100 nmol/L, holding potential: +40 mV. $^{*}P < 0.05$.

processes [15–19]. Spontaneous calcium oscillations occur in several cell types, including cardiomyocytes, oocytes and fibroblasts [15,16]. Here, we have observed the spontaneous calcium oscillation in majority of CCVMs, consistent with previous studies [20]. Our results have shown that spontaneous $[Ca^{2+}]$i oscillation is different in CCVMs on substrates with varying stiffness. Both the frequency and the magnitude of Ca^{2+} oscillations are obvious higher in cells on the stiff substrate, which is likely to be controlled by the BK channels (Fig. 1B). Blocking the BK channels significantly abolishes the difference of both the frequency and magnitude of Ca^{2+} oscillations in CCVMs on soft and stiff substrates (Fig. 1C). These results lead to the idea that the BK channel plays a very critical role in substrate stiffness-regulated Ca^{2+} oscillations in CCVMs.

Whole-cell recordings show that BK channel current significantly decreased in the CCVMs cultured on stiff substrates (Fig. 2). Stiff substrate shortens the opening time and lengthens the closing time (Fig. 3C–D). To date, this is the first evidence suggesting that shorter open times could be partly responsible for the reduction of BK channel current on stiff substrates. The BK β1-subunits are formed from small two transmembrane-segment peptides that affect the channel properties. For example, it increases the Ca^{2+} concentration and membrane potential sensitivity and slows the activation of the BK channel [21]. In consequence, we speculate that the BK β1-subunits play an important role in increasing the Po of BK channels and lengthen the opening time of BK channels in cells on soft substrates. Moreover, the open dwell times of BK channels in the CCVMs cultured on soft substrate were higher than those from stiff substrate. This is consistent with increased function of the BK channel β1-subunit in cells on soft substrate (Fig. 4). Considering the large conductance and the high input resistance of ventricular myocyte membrane, small changes in the Po of channel could make a difference in the membrane potential and cellular excitability. When activated, the BK channel shifts the membrane voltage towards the equilibrium potential of K^{+}, resulting in membrane hyperpolarization. Therefore, the reduced activation of BK channels in cells on stiff substrate may weaken the negative feedback of $[Ca^{2+}]$i to increase membrane depolarization and cell constriction.

In summary, this study has manifested that BK channels in cardiomyocytes play a key role in substrate stiffness-regulated Ca^{2+} oscillations. This finding helps to understand the effects of the mechanical properties of extracellular matrix on cardiac function.

Author contributions

H.Z. and X.G. conceived the research, designed the experiments, and prepared and revised the article; S.L., Y.Y., Z.Y. and S.Z. performed the experiments and analyzed the data; X.G. and X.W. revised the article; and all authors participated in discussion and commented on the article.

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Declaration of competing interest

The authors declare no conflicts of interest.

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