Resveratrol Attenuates the Na\(^+\)-Dependent Intracellular Ca\(^{2+}\) Overload by Inhibiting H\(_2\)O\(_2\)-Induced Increase in Late Sodium Current in Ventricular Myocytes

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Abstract

**Background/Aims:** Resveratrol has been demonstrated to be protective in the cardiovascular system. The aim of this study was to assess the effects of resveratrol on hydrogen peroxide (H\(_2\)O\(_2\))-induced increase in late sodium current (I\(_{Na,L}\)) which augmented the reverse Na\(^{+}\)-Ca\(^{2+}\) exchanger current (I\(_{NCX}\)) and the diastolic intracellular Ca\(^{2+}\) concentration in ventricular myocytes.

**Methods:** The L-type Ca\(^{2+}\) current (I\(_{Ca,L}\)) and intracellular Ca\(^{2+}\) properties were determined using whole-cell patch-clamp techniques and dual-excitation fluorescence photomultiplier system (IonOptix), respectively, in rabbit ventricular myocytes.

**Results:** Resveratrol (10, 20, 40 and 80 \(\mu\)M) decreased I\(_{Na,L}\) in myocytes in the absence and presence of H\(_2\)O\(_2\) (300 \(\mu\)M) in a concentration-dependent manner. Ranolazine (3–9 \(\mu\)M) and tetrodotoxin (TTX, 4 \(\mu\)M), I\(_{Na,L}\) inhibitors, decreased I\(_{Na,L}\) in cardiomyocytes in the presence of 300 \(\mu\)M H\(_2\)O\(_2\). H\(_2\)O\(_2\) (300 \(\mu\)M) increased the reverse I\(_{NCX}\) and this increase was significantly attenuated by either 20 \(\mu\)M resveratrol or 4 \(\mu\)M ranolazine or 4 \(\mu\)M TTX. In addition, 10 \(\mu\)M resveratrol and 2 \(\mu\)M TTX significantly depressed the increase by 150 \(\mu\)M H\(_2\)O\(_2\) of the diastolic intracellular Ca\(^{2+}\) fura-2 fluorescence intensity (FFI), fura-fluorescence intensity change (\(\Delta\)FFI), maximal velocity of intracellular Ca\(^{2+}\) transient rise and decay. As expected, 2 \(\mu\)M TTX had no effect on I\(_{Ca,L}\).

**Conclusion:** Resveratrol protects the cardiomyocytes by inhibiting the H\(_2\)O\(_2\)-induced augmentation of I\(_{Na,L}\) and may contribute to the reduction of ischemia-induced lethal arrhythmias.

Introduction

Despite intensive research has been conducted in recent years, cardiac arrhythmias remain a serious problem. Late sodium current (I\(_{Na,L}\)) has been recognized as an important factor contributing to the abnormal repolarization in ischemic and failed hearts [1]. I\(_{Na,L}\) plays an important role in determining the action potential duration (APD) [2] and the alteration of the intracellular Na\(^{+}\) concentration ([Na\(^{+}\)]\(_i\)) [3,4]. It has also been reported that hypoxia increased I\(_{Na,L}\) in rat ventricular myocytes [4], and the increase in Na\(^{+}\) inflow during hypoxia increased [Na\(^{+}\)]\(_i\), which in turn rose the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) via the Na\(^{+}\)-Ca\(^{2+}\) exchanger (NCX) resulting in a Na\(^{+}\)-dependent intracellular Ca\(^{2+}\) overload induced by I\(_{Na,L}\) [5,6,7]. An increase in [Ca\(^{2+}\)]\(_i\) caused cardiac arrhythmias and irreversible cell damage [8]. Furthermore, increased I\(_{Na,L}\) caused arrhythmic activity and contractile dysfunction [9,10]. Therefore, inhibition of I\(_{Na,L}\) is considered to be a new potential target for therapeutic intervention in patients with myocardial ischaemia and heart failure [10–14]. Resveratrol (trans-3, 4’, 5-trihydroxystilbene), a polyphenol in various vegetables and fruits, is abundant in grapes. The root extracts of Polygonum cuspidatum, a constituent of Chinese and Japanese folk medicine, is also a good source of resveratrol [15]. Sufficient clinical and epidemiological evidence showed that the consumption of red wine reduced the incidence of mortality and morbidity in patients with coronary heart disease [16]. Among all the evidence, the well-known one is now popularly termed as the “French paradox” [16,17]. Resveratrol has been considered to be responsible for the cardiovascular benefits after moderate wine consumption [18]. It is speculated that resveratrol may act as an antioxidant, which modulates the vascular cell functions [19], inhibits platelet aggregation [20], and reduces lipoprotein oxidation [21], to serve as a cardioprotective agent. H\(_2\)O\(_2\), a reactive oxygen species, is a by-product of oxidative metabolism in which energy activation and electron reduction are involved, and was...
enhanced during ischemia-reperfusion of the heart [22]. Excessive amount of H$_2$O$_2$ augmented $I_{Na,L}$ in ventricular myocytes [10,23], but the reducing agents, e.g., dithiothreitol (DTT) and glutathione (GSH), reversed these changes induced by either H$_2$O$_2$ or hypoxia [24,25]. Since resveratrol acts as an antioxidant [26], we presumed that it might inhibit the increase in $I_{Na,L}$ induced by H$_2$O$_2$.

To further clarify the pharmacological mechanisms and the scope of application of the agent, it is critical to determine the effect of resveratrol on $I_{Na,L}$. Previous investigation showed that 50 µM of resveratrol reduced $I_{Na,L}$ in a recombinant expression system with the R1623Q LQT3 mutation [27]. To our knowledge, the effect of resveratrol on $I_{Na,L}$ in ventricular myocytes with increased H$_2$O$_2$ has not been reported. Therefore, this study was designed to address the impact of resveratrol on the Na$^+$-dependent Ca$^{2+}$ overload induced by H$_2$O$_2$-induced increase in $I_{Na,L}$, in ventricular myocytes, with the intention to shed some light on its potential clinical application in the future.

Materials and Methods

Isolation of Ventricular Myocytes

Adult New Zealand white rabbits (body weight 1.7–2 kg) of either sex were heparinized (2000 U) and anesthetized with ketamine (30 mg kg$^{-1}$ i.v.) and xylazine (7.5 mg kg$^{-1}$ i.m.). Hearts were excised rapidly and perfused retrogradely on a Langendorff apparatus for 5 min with a Ca$^{2+}$-free Tyrode’s solution containing (in mM): NaCl 135, KCl 5.4, MgCl$_2$ 1, NaH$_2$PO$_4$ 0.33, HEPES 10 and glucose 10 (pH 7.4, adjusted with NaOH), and then a Tyrode’s solution containing enzyme (collagenase type I, 0.1 g/l) and bovine serum albumin (BSA, 0.5 g/l) for 40–50 min. The perfusate was finally switched to KB solution containing (in mM): KOH 70, taurine 20, glutamic acid 50, KCl 40, KH$_2$PO$_4$ 20, MgCl$_2$ 3, EGTA 0.5, HEPES 10, and glucose 10 (pH 7.4), for 5 min. All perfusates were bubbled with 100% O$_2$ and maintained at 37°C. The left ventricles were then cut into small chunks and gently agitated in KB solution. The cells were filtered through nylon mesh and stored in KB solution at 25°C. The use of animals in this investigation was approved by the Institutional Animal Care and Use Committee of Wuhan University of Science and Technology and conformed to the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH publication no. 85-23, revised 1996) and the Guide for the Care and Use of Laboratory Animals of Hubei Province, China.

Protocol of Experiments

Isolated cells were perfused with Tyrode’s solution saturated oxygenated with 100% O$_2$ (control) and were then exposed to Tyrode’s solution containing 300 µM H$_2$O$_2$ for 7 min. Next, isolated cells were perfused with Tyrode’s solution containing both 300 µM H$_2$O$_2$ and one of the following, resveratrol (10, 20, 40 or...
80 μM) for 10 min, 4 μM tetrodotoxin (TTX) for 10 min, or 4 μM ranolazine for 5 min.

Solutions and Drugs
To record \( I_{\text{Na,L}} \), the intracellular pipette solution contained (mM): CsCl 120, CaCl\(_2\) 1, MgCl\(_2\) 5, Na\(_2\)ATP 5, TEACl 10, EGTA 11, HEPES 10 (pH 7.3). The extracellular solution contained (mM): NaCl 135, CsCl 70, CaCl\(_2\) 1, MgCl\(_2\) 1, CdCl\(_2\) 0.05, glucose 5, HEPES 5 (pH 7.4). In addition, 1 μM nicardipine was used to block the L-type Ca\(_{\text{2+}}\) channels.

To record Na\(^{+}\)-Ca\(_{\text{2+}}\) exchanger current (\( I_{\text{NCX}} \)), the intracellular pipette solution contained (mM): NaCl 20, CaCl\(_2\) 10, aspartic acid 50, MgCl\(_2\) 3, MgATP 5 and CsOH 120 (pH 7.3). The bath solution contained (mM): NaCl 140, CaCl\(_2\) 2, MgCl\(_2\) 2, HEPES 5, and glucose 10 (pH 7.4). In addition, 1 μM nicardipine was used to block the L-type Ca\(_{\text{2+}}\) channels.

Krebs-Henseleit bicarbonate (KHB) buffer for intracellular Ca\(_{\text{2+}}\) fluorescence measurement, the bath solution contained (mM): NaCl 131, KCl 4, CaCl\(_2\) 1, MgCl\(_2\) 1, glucose 10, and HEPES 10 (pH 7.4).

To record L-type calcium current (\( I_{\text{Ca,L}} \)), the intracellular pipette solution contained (mM): CsCl 80, CsOH 60, aspartic acid 40, CaCl\(_2\) 0.65, HEPES 5, EGTA 10, MgATP 5 and disodium creatine phosphate 5 (pH 7.2). The bath solution contained (mM): NaCl 135, KCl 5.4, MgCl\(_2\) 0.5, CaCl\(_2\) 1.8, NaH\(_2\)PO\(_4\) 0.33, HEPES 10, glucose 10 (pH 7.4).

H\(_2\)O\(_2\) was a product of Wuhan Zhongnan Chemical Reagent Co. (Wuhan, China). All other chemicals were purchased from Sigma. Stock solutions of drugs were prepared in water. Each of the stocks was diluted to the required concentrations in the external recording solution immediately before use.

Electrical Recordings
Experiments were performed at room temperature (22–24°C). Rabbit ventricular myocytes were placed into a recording chamber that was bathed with normal extracellular solution, in the absence and presence of drug(s), at a rate of 2 ml min\(^{-1}\). \( I_{\text{Na,L}}, I_{\text{NCX}} \) and \( I_{\text{Ca,L}} \) were recorded in voltage-clamp mode by using whole-cell patch-clamp techniques in rabbit ventricular myocytes. Patch electrodes were pulled with a two-stage puller (PP-830, Narishige Group, Tokyo, Japan). Their resistances were in the range of 1–1.5 MΩ. Capacitance and series resistances were adjusted to obtain minimal contribution of the capacitative transients. A 60% to 80% compensation of the series resistance was usually achieved without ringing. Currents were obtained with an EPC 9 amplifier (Heka Electronic, Lambrecht, Pfalz, Germany) and a Multiclamp 700B amplifier (Axon Instruments, Inc. USA), filtered at 2 kHz, digitized at 10 kHz, and stored on a computer hard disk for further analysis.
Intracellular Ca$^{2+}$ Fluorescence Measurement

Myocytes were loaded with fura-2-AM (0.5 mM) for 10 min at 25°C, and fluorescence measurements were recorded with a dual excitation fluorescence photomultiplier system (Ionoptix). Myocytes were imaged through an Olympus IX-70 Fluor 40 x 60 oil objective. The cells were field stimulated with a suprathreshold (150%) voltage and at a frequency of 0.5 Hz, 3-ms duration, using a pair of platinum wires placed on opposite sides of the chamber connected to a HFC stimulator (Brunswick, NE, USA). The polarity of the stimulatory electrodes was reversed frequently to avoid possible build up of electrolyte by-products. Cells were exposed to light emitted by a 75-W lamp and passed through either a 340- or 380-nm filter (bandwidths were ±15 nm) while being stimulated to contract at 0.5 Hz. Fluorescence emissions were detected between 480 and 520 nm by a photomultiplier tube after first illuminating the cells at 340 nm for 0.5 s then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 excitation scan was repeated at the end of the protocol, and qualitative changes in intracellular Ca$^{2+}$ level were inferred from the ratio of the fura-fluorescence intensity (FFI) at both wavelengths. Intracellular Ca$^{2+}$ fluorescence measurements were assessed using the following indices: diastolic intracellular Ca$^{2+}$ level (diastolic FFI) (340/380 ratio), electrically stimulated rise in intracellular Ca$^{2+}$ (ΔFFI) (340/380 ratio), maximal velocity of Ca$^{2+}$ rise and Ca$^{2+}$ decay (340/380 ratio).

Data Analysis

Whole-cell recordings were analyzed using clampfit 9.0 (Axon Instruments, Inc., USA) and PulseFit (v8.74, HEKA). Figures were plotted by Origin (v7.0, OriginLab Co., MA, USA). All amplitudes of $I_{Na,L}$ were tested at 200 ms in depolarization testing pulse to eliminate the influence of transient sodium current ($I_{Na,T}$).

Results

Effects of Resveratrol and TTX on $I_{Na,L}$ Under Normal Condition

To identify $I_{Na,L}$, the current was recorded first in the absence and then in the presence of 4 μM TTX with 300 ms voltage steps from a holding potential (HP) of −120 to −20 mV. The values of current recorded before (control condition) and after application of TTX were $-0.0400±0.0050$ and $-0.154±0.030$ pA/pF (n = 6, P<0.05 versus control), respectively, indicating that this TTX-sensitive current recorded was $I_{Na,L}$. Statistical significance between two groups and multiple groups were evaluated by Student’s t-test and one-way analysis of variance (ANOVA), respectively. All values were expressed as mean ± SD, and the number of cells (n) in each group was given. P<0.05 was considered to be statistically significant.

Figure 3. Ranolazine (RAN) inhibited the increase in $I_{Na,L}$ caused by 300 μM H$_2$O$_2$. A. 3, 6 and 9 μM ranolazine decreased the amplitudes of the increased $I_{Na,L}$ induced by 300 μM H$_2$O$_2$ in a concentration dependent manner. B. The I-V relationships of $I_{Na,L}$ after the sequential application of H$_2$O$_2$ plus 3, 6 and 9 μM ranolazine. C. The inhibition amounts of 3, 6 and 9 μM ranolazine on $I_{Na,L}$ induced by 300 μM H$_2$O$_2$. Values are expressed as mean ± SD, n = 8 cells/group. *P<0.01 versus H$_2$O$_2$ group; #P<0.01, ##P<0.05 versus 3 μM ranolazine group; ^P<0.01, ^P<0.05 versus 6 μM ranolazine group.

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in a concentration dependent manner (Figure 1A, 1B). Figure 1B showed the I-V relationship of $I_{Na.L}$ after the administration of 10, 20, 40 and 80 mM resveratrol, without a shift of the voltage at which the $I_{Na.L}$ amplitude was maximal (Figure 1B). Figure 1C shows the inhibition amounts of 10, 20, 40 and 80 mM resveratrol on the $I_{Na.L}$ with an IC50 of 34.442 mM.

**Effects of Resveratrol, Ranolazine and TTX on the Increased $I_{Na.L}$ by H2O2**

Currents were recorded using depolarizing pulses with a duration of 300 ms at a rate of 0.25 Hz from a HP of −120 mV, in 10 mV increments between −70 and −20 mV. Administration of resveratrol at concentrations of 10, 20, 40 and 80 mM resulted in a decrease in the amplitudes of $I_{Na.L}$ in a concentration dependent manner in myocytes exposed to H2O2 (Figure 2). H2O2 (300 mM) increased the amplitudes of $I_{Na.L}$ but 10, 20, 40 and 80 mM resveratrol decreased the amplitudes of $I_{Na.L}$ in the continued presence of H2O2 (Figure 2A). Shown in figure 2B are the I-V relationships of $I_{Na.L}$ after the sequential application of 300 mM H2O2, 10, 20, 40 and 80 mM resveratrol respectively, without a shift of the voltage at which the $I_{Na.L}$ amplitude was maximal. Figure 2C shows the inhibition amounts of 10, 20, 40 and 80 mM.
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resveratrol on the $\Delta I_{\text{Na,L}}$ ($\text{H}_2\text{O}_2$ induced increase in $I_{\text{Na,L}}$) induced by 300 $\mu$M $\text{H}_2\text{O}_2$ with an IC$\text{so}_{50}$ of 26.192 $\mu$M. Ranolazine (3, 6 and 9 $\mu$M) attenuated the increased $I_{\text{Na,L}}$ caused by 300 $\mu$M $\text{H}_2\text{O}_2$. The values of $I_{\text{Na,L}}$ under the control conditions, after application of 300 $\mu$M $\text{H}_2\text{O}_2$ and 4 $\mu$M TTX were $-0.323 \pm 0.087$, $-0.878 \pm 0.071$ (n = 6, P < 0.05 versus control) and $-0.258 \pm 0.045$ pA pF$^{-1}$ (n = 6, P < 0.05 versus $\text{H}_2\text{O}_2$), respectively.

**Effects of Resveratrol, Ranolazine and TTX on Increased Electrogenic $I_{\text{NCX}}$ by $\text{H}_2\text{O}_2$**

Electrogenic $I_{\text{NCX}}$ was measured to determine whether the reverse NCX was activated by the increase of $I_{\text{Na,L}}$. Membrane currents were elicited using ramp voltage-clamp pulses from a HP of −40 mV to +60 mV for 100 ms and then ramped to −120 mV over a period of 2 seconds (i.e., at 90 mV s$^{-1}$) before returning to −40 mV. The current-time relationship was constructed from the declining slope of the ramp pulse (Figure 4A, 4B, 4D, 4E, 4G, 4H). Figure 4B, 4E, 4H shows the Ni$^{2+}$-sensitive (NCX) current obtained by subtracting the data in the trace d, h or l from the data in the trace a, c, i, b, f, j, c, g or k in the panel 4A, 4D or 4G. Figure 4C, 4F, 4I are $I_{\text{NCX}}$ measured at voltage levels of +50 mV and −100 mV, respectively, as the Ni$^{2+}$-sensitive current by subtracting the current recorded in the presence from that in the absence of 5 mM NiCl$_2$.

$I_{\text{NCX}}$ was recorded after 7 minutes of exposure of $\text{H}_2\text{O}_2$. The mean current density of the inward $I_{\text{NCX}}$ had little change, while the reverse $I_{\text{NCX}}$ increased significantly (n = 7, Figure 4B, 4C, 4E, 4F, 4H, 4I). 20 $\mu$M resveratrol, 4 $\mu$M ranolazine or 4 $\mu$M TTX diminished the increase of $I_{\text{NCX}}$ (n = 7, Figure 4B, 4C, 4E, 4F, 4H, 4I).

**Effects of Resveratrol and TTX on Increased Intracellular Ca$^{2+}$ Transient by $\text{H}_2\text{O}_2$**

As shown earlier, resveratrol could reduce the increase in $I_{\text{Na,L}}$ and $I_{\text{NCX}}$ by $\text{H}_2\text{O}_2$, theoretically it should also decrease the increase in intracellular Ca$^{2+}$ transients by $\text{H}_2\text{O}_2$. To minimize the cell contracture by 300 $\mu$M $\text{H}_2\text{O}_2$ due to the increase in the amplitude of calcium transients and diastolic calcium concentration, the concentration of $\text{H}_2\text{O}_2$ used was 150 $\mu$M. Cells were perfused with KHB solution for 5 min and then with KHB containing 150 $\mu$M $\text{H}_2\text{O}_2$ for 10 min. The diastolic intracellular Ca$^{2+}$ fura-2 fluorescence intensity (FFI) fura-fluorescence intensity change (ΔFFI), maximal velocity of Ca$^{2+}$ rise and Ca$^{2+}$ decay were all enhanced by 150 $\mu$M $\text{H}_2\text{O}_2$ (Figure 5). However, 10 $\mu$M resveratrol reversed all these enhancements, as shown in Figure 5A, 5C, 5D, 5E, 5F. TTX (2 $\mu$M) also depressed these enhancements of the abovementioned parameters induced by 150 $\mu$M $\text{H}_2\text{O}_2$ (Figure 5B, 5G, 5H, 5I, 5J). These results indicated that both resveratrol (10 $\mu$M) and TTX (2 $\mu$M) could attenuate the $\text{H}_2\text{O}_2$-induced augmentations in diastolic Ca$^{2+}$ concentration and calcium transients amplitude.

**Effects of TTX on $I_{\text{Ca,L}}$**

Recent evidence suggests a potential for TTX to inhibit L-type Ca$^{2+}$ channels [28]. The results in this study showed that 2 $\mu$M TTX inhibited $\text{H}_2\text{O}_2$-induced augmentations in diastolic Ca$^{2+}$ concentration and amplitude of calcium transients. To identify the effect of 2 $\mu$M TTX on intracellular Ca$^{2+}$ was from its blocking of $I_{\text{Na,L}}$ but not the inhibition of L-type Ca$^{2+}$ channels, $I_{\text{Ca,L}}$ was measured. The results indicated that at a low concentration (2 $\mu$M) TTX is relatively a selective $I_{\text{Na,L}}$ blocker. Using depolarizing pulses with a duration of 300 ms applied at 0.5 Hz from a HP of −80 mV, in 10 mV increments between −40 and +40 mV, $I_{\text{Ca,L}}$ was recorded in the absence (Figure 6A) and presence (Figure 6B) of 2 $\mu$M TTX. Figure 6C showed the effect of TTX (2 $\mu$M) application on the current-voltage relationship of

![Figure 6. Effects of 2 $\mu$M TTX on $I_{\text{Ca,L}}$ under control conditions in rabbit ventricular myocytes. A. $I_{\text{Ca,L}}$ Under control conditions. B. $I_{\text{Ca,L}}$ after the application of 2 $\mu$M TTX. C. Effects of TTX (2 $\mu$M) on current-voltage relationship of $I_{\text{Ca,L}}$. Values are expressed as mean ± SD, n = 6 cells/group. P > 0.05 versus control group. doi:10.1371/journal.pone.0051358.g006](image-url)
Treated cells in a concentration dependent manner (Figure 1, 2). Induced augmentations in diastolic Ca$^{2+}$ have been suggested to inhibit the cardiac ryanodine receptor (IC50 = 10 μM) and ultimately causing cell damage [10,23,38,39]. Reducing agent, e.g., dithiothreitol (DTT) and reduced glutathione (GSH), could reverse the increased INa,L in rabbit ventricular myocytes [5,24,25]. Resveratrol, a natural antioxidant, has beneficial effects against coronary heart disease. Previous studies have shown that resveratrol effectively suppressed ischemia/reperfusion-induced arrhythmia [40,41] and reduced peak INa and INa,L in the R1623Q LQT3 mutation in a recombinant expression system [27]. The effect of resveratrol on the increased INa,L and reverse INa,CX under proarrhythmic conditions (H2O2) in rabbit ventricular myocytes has not been investigated yet. The data from this study addressed the impact of resveratrol on the Na$^{+}$-dependent Ca$^{2+}$ overload.

In this study, INa,L was increased by H2O2 (Figure 2, 3). Ranolazine attenuated the increased INa,L by H2O2 in a concentration dependent manner (Figure 3) and 4 μM TTX attenuated the increased INa,L increased by H2O2 as well. These data are consistent with other reports and our previous studies that the INa,L inhibitors ranolazine and TTX significantly inhibited late INa,L at clinical relevant concentrations [42,43]. H2O2-induced intracellular Na$^{+}$ and Ca$^{2+}$ overload was associated with an enhanced INa,L and therefore was attenuated by the INa,L inhibitors ranolazine and TTX [10]. The INa,L blocking agents may be effective in preventing arrhythmias by reducing [Na$^{+}$]i load and subsequently the [Ca$^{2+}$]i load. However, ranolazine has been suggested to inhibit the cardiac ryanodine receptor (IC50 = 10 μM) [44], which could also modulate intracellular Ca$^{2+}$ levels. Ranolazine is currently approved as an antianginal agent that reduces the Na$^{+}$-dependent Ca$^{2+}$ overload via inhibition of the INa,L and thus improves diastolic tone and oxygen handling during myocardial ischemia [7]. INa,L is an important contributing factor to intracellular Ca$^{2+}$ overload in the pathogenesis of myocardial ischemia and infarction. In rabbit ventricular myocytes, low concentrations of TTX (1.5–4.0 μM) did not alter the L-type Ca$^{2+}$ current (Figure 6) and INa,T [6,25,45], but obviously inhibited the INa,L. Accordingly TTX was used to confirm the process of Na$^{+}$-dependent Ca$^{2+}$ overload induced by H2O2. The effect of resveratrol on INa,L is similar to ranolazine and TTX. Resveratrol inhibited INa,L in both normal and H2O2-treated cells in a concentration dependent manner (Figure 1, 2). This result is consistent with our previous studies that DTT and reduced glutathione could reverse the increase in INa,L induced by either H2O2 or hypoxia [24,25], indicating resveratrol may act as an antioxidant to eliminate the detrimental effects of H2O2 on INa,L. Changes in redox potential or surface charge may account for some ionic current block [46], therefore it is possible that the antioxidant properties of resveratrol may contribute to the INa,L inhibition observed in this study.

Recently, it has been reported that reverse INa,CX was increased along with the increased INa,L during hypoxia and was decreased along with the INa,L inhibition by TTX in hypoxic ventricular myocytes, suggesting that the increased INa,L contributed to the increase in the reverse INa,CX [6]. In this study, 300 μM H2O2 increased the reverse INa,CX while the inward INa,CX was not affected obviously, whereas ranolazine or TTX attenuated the increase in the reverse INa,CX significantly (Figure 4). Different from INa,L, INa,L can be blocked by a low concentration of ranolazine and TTX, and the consequent reduction of Na$^{+}$ loading via the decrease of the INa,L can prevent the increase in the reverse INa,CX-induced intracellular Ca$^{2+}$ accumulation [47]. Ranolazine (4 μM) and TTX (4 μM) decreased the reverse INa,CX through the inhibition of INa,L. Similarly, resveratrol (20 μM) attenuated the increase in the reverse INa,CX by H2O2. Thus, we concluded that the effect of resveratrol to inhibit the increased reverse INa,CX caused by H2O2 was from its inhibition of INa,L.

In this study, 150 μM H2O2 significantly increased the amplitude of calcium transients and diastolic calcium concentration in the ventricular cell which could be reversed by TTX (2 μM). The intracellular Ca$^{2+}$ overload caused by ROS was due to an increase in [Na$^{+}$]i, followed with an increase in Ca$^{2+}$ influx via the reverse mode of the NCX [48]. Then the large entry of Ca$^{2+}$ into the cell will cause intracellular Ca$^{2+}$ overload [49,50]. TTX also inhibited L-type Ca$^{2+}$ channel with an IC50 value of 55.2 μM [20]. In this study in rabbit ventricular myocytes, 2 μM TTX inhibited INa,L and restrained Ca$^{2+}$ overload induced by H2O2 but not affected L-type Ca$^{2+}$ channels (Figure 6), supporting that INa,L played an important role in the genesis of Ca$^{2+}$ overload induced by H2O2. TTX also reversed the increase in calcium transients amplitude and diastolic calcium concentration through inhibiting the increased INa,L by H2O2. Resveratrol (10 μM) also restrained the increased calcium transients amplitude and the diastolic calcium concentration induced by H2O2 (150 μM). Therefore the effects of resveratrol on the Na$^{+}$-dependent Ca$^{2+}$ overload induced by enhanced INa,L were similar to 2 μM TTX, suggesting that the reduction of Ca$^{2+}$ overload by resveratrol may have similar mechanism to TTX, i.e., inhibition of INa,L. Indeed, resveratrol has also been suggested to inhibit the ryanodine receptor-induced intracellular Ca$^{2+}$ increase [51] which may account for the reduction of [Ca$^{2+}$]i. The results in this study indicated that resveratrol reduced both INa,L and reverse INa,CX which was responsible for the reversal of intracellular Ca$^{2+}$ overload in the presence of H2O2. Resveratrol may inhibit both the ryanodine receptor-induced intracellular Ca$^{2+}$ overload and INa,L-induced increase in reverse INa,CX to attenuate the intracellular Ca$^{2+}$ overload. Further research will be needed to clarify the contribution of the two pathways by resveratrol in the absence and presence of H2O2.

Conclusions
INa,L is an important target for resveratrol to prevent or treat ventricular arrhythmias. INa,L, increased by H2O2 induces intracellular Ca$^{2+}$ overload (the increased diastolic calcium concentration) through the increase in the reverse INa,CX. The inhibitive effect of resveratrol on H2O2-induced INa,L may reduce the concentration of [Na$^{+}$]i, lower [Ca$^{2+}$]i, by attenuating reverse NCX.

Discussion
The mechanisms underlying the genesis of ischemia- and reperfusion-induced arrhythmias are notoriously complex and controversial. There has been an interest in the concept that oxygen free radicals play a role in the pathogenesis of myocardial ischemia and infarction. It has been reported that a burst of H2O2, an important reactive oxygen species, is generated in the myocardium during ischemia and reperfusion [29–33] and causes Ca$^{2+}$ overload through many ways [34,35,36,37]. For example, the activation of ryanodine receptors with H2O2 could also account for the increased cytosolic Ca$^{2+}$ levels found with ROS production, which could account for the Ca$^{2+}$ overload in cells [34]. Furthermore, the excessive amount of H2O2 could increase INa,L in cardiomyocytes, subsequently leading to intracellular Ca$^{2+}$ overload through reverse NCX (the Na$^{+}$-dependent Ca$^{2+}$ overload induced by INa,L) and accounting for that the effects of 2 μM TTX to inhibit H2O2-induced augmentations in diastolic Ca$^{2+}$ concentration and amplitude of calcium transients were from its inhibition on INa,L and subsequently the reverse INa,CX.

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to eliminate Ca\(^{2+}\) overload, and ultimately inhibit the electrical abnormalities.

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