

## Original Article

# Resveratrol protects rabbit ventricular myocytes against oxidative stress-induced arrhythmogenic activity and $\text{Ca}^{2+}$ overload

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**Aim:** To investigate whether resveratrol suppressed oxidative stress-induced arrhythmogenic activity and  $\text{Ca}^{2+}$  overload in ventricular myocytes and to explore the underlying mechanisms.

**Methods:** Hydrogen peroxide ( $\text{H}_2\text{O}_2$ , 200  $\mu\text{mol/L}$ ) was used to induce oxidative stress in rabbit ventricular myocytes. Cell shortening and calcium transients were simultaneously recorded to detect arrhythmogenic activity and to measure intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ).  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases II (CaMKII) activity was measured using a CaMKII kit or Western blotting analysis. Voltage-activated  $\text{Na}^+$  and  $\text{Ca}^{2+}$  currents were examined using whole-cell recording in myocytes.

**Results:**  $\text{H}_2\text{O}_2$  markedly prolonged  $\text{Ca}^{2+}$  transient duration (CaTD), and induced early afterdepolarization (EAD)-like and delayed afterdepolarization (DAD)-like arrhythmogenic activity in myocytes paced at 0.16 Hz or 0.5 Hz. Application of resveratrol (30 or 50  $\mu\text{mol/L}$ ) dose-dependently suppressed  $\text{H}_2\text{O}_2$ -induced EAD-like arrhythmogenic activity and attenuated CaTD prolongation. Co-treatment with resveratrol (50  $\mu\text{mol/L}$ ) effectively prevented both EAD-like and DAD-like arrhythmogenic activity induced by  $\text{H}_2\text{O}_2$ . In addition, resveratrol markedly blunted  $\text{H}_2\text{O}_2$ -induced diastolic  $[\text{Ca}^{2+}]_i$  accumulation and prevented the myocytes from developing hypercontracture. In whole-cell recording studies,  $\text{H}_2\text{O}_2$  significantly enhanced the late  $\text{Na}^+$  current ( $I_{\text{Na,L}}$ ) and L-type  $\text{Ca}^{2+}$  current ( $I_{\text{Ca,L}}$ ) in myocytes, which were dramatically suppressed or prevented by resveratrol. Furthermore,  $\text{H}_2\text{O}_2$ -induced ROS production and CaMKII activation were significantly prevented by resveratrol.

**Conclusion:** Resveratrol protects ventricular myocytes against oxidative stress-induced arrhythmogenic activity and  $\text{Ca}^{2+}$  overload through inhibition of  $I_{\text{Na,L}}/I_{\text{Ca,L}}$ , reduction of ROS generation, and prevention of CaMKII activation.

**Keywords:** resveratrol; cardioprotective agents; oxidative stress; cardiac arrhythmias;  $\text{Ca}^{2+}$  overload; CaMKII; late sodium current; L-type calcium current

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## Introduction

Reactive oxygen species (ROS) are increasingly generated under various pathological conditions, such as ischemia/reperfusion, myocardial infarction, and heart failure. Increased oxidative stress causes a series of detrimental effects on the heart<sup>[1]</sup>. Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), which can cause oxidative stress, is significantly elevated in hearts exposed to ischemia/reperfusion and predisposes the heart to ischemia/reperfusion injury and arrhythmias. In myocardial infarction, increasing ROS contribute to left ventricular remodeling and

arrhythmias<sup>[1, 2]</sup>. Increased oxidative stress is also associated with reduced left ventricular function in heart failure<sup>[3]</sup>. The underlying mechanisms may involve an impairment of Na and Ca homeostasis<sup>[4, 5]</sup>, which subsequently causes mechanical dysfunction, arrhythmias and even cell death<sup>[6]</sup>. Recently, Ca/calmodulin-dependent protein kinase II (CaMKII) has been suggested to be an important mediator of ROS-induced detrimental effects<sup>[6, 7]</sup>. ROS activates CaMKII via autophosphorylation- and oxidation-dependent mechanisms<sup>[8]</sup>, which in turn enhance late sodium current ( $I_{\text{Na,L}}$ ) and L-type calcium current ( $I_{\text{Ca,L}}$ ) in cardiac myocytes<sup>[6, 9]</sup>. Augmentation of  $I_{\text{Na,L}}$  and  $I_{\text{Ca,L}}$  not only causes a prolongation of action potential duration (APD) and the occurrence of early afterdepolarizations (EADs) but also leads to sodium and calcium overload,

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contractile dysfunction, the occurrence of delayed afterdepolarizations (DADs) and triggered activities<sup>[6, 7, 10]</sup>. Despite the serious detrimental effects caused by increased oxidative stress in structural heart diseases, few drugs exist that can be used for clinical treatment.

Resveratrol (*trans*-3,4',5-trihydroxystilbene) is a natural compound found in abundance in grapes, mulberries, peanuts and red wine<sup>[11]</sup>. Over the past two decades, it has been demonstrated from both *in vitro* and *in vivo* studies to have cardioprotective effects, including anti-inflammatory, antioxidative, and anti-hyperlipidemic properties as well as the prevention of platelet aggregation and cardiac hypertrophy<sup>[12-14]</sup>. These beneficial effects of resveratrol may provide explanations for the "French paradox", the finding that the consumption of red wine is associated with a decreased incidence of cardiovascular diseases<sup>[15]</sup>. Recently, studies have revealed that resveratrol can reduce ventricular arrhythmias in myocardial infarction<sup>[2, 16]</sup>, ischemia/reperfusion<sup>[17]</sup>, heart failure and other pathological conditions<sup>[18]</sup>. Accumulating evidence indicates that increased oxidative stress is an important factor predisposing the diseased heart to calcium overload and lethal arrhythmias<sup>[6, 7, 10]</sup>. However, it is unclear whether resveratrol has protective effects against oxidative stress-induced arrhythmias. Therefore, the present study aims to investigate the effects of resveratrol on exogenous H<sub>2</sub>O<sub>2</sub>-induced arrhythmogenic activity and calcium overload and explore the underlying mechanisms.

## Materials and methods

### Animals

Six-month-old New Zealand White male rabbits weighing 2.0 to 3.0 kg were used for experiments. Animal care and handling procedures were approved by the Animal Care and Use Committee, Research Institute of Medicine, Shanghai Jiao Tong University, in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH Publication No 85-23, revised 1996).

### Materials

H<sub>2</sub>O<sub>2</sub> and resveratrol were purchased from Sigma Chemical (St Louis, MO, USA). Resveratrol was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 50 mmol/L with the final concentration of DMSO at less than 0.1%. The same amount of DMSO was added to all groups to exclude the effects of DMSO on myocytes. All experiments were performed at a temperature of 25±1 °C, unless otherwise mentioned.

### Cell isolation

Ventricular myocytes were enzymatically isolated from the hearts of New Zealand White rabbits as previously described<sup>[10]</sup>.

### Measurement of cellular arrhythmias

Ca<sup>2+</sup> transients and cell shortening were simultaneously detected as previously described<sup>[19]</sup>. Freshly isolated rabbit

ventricular myocytes were incubated with a Ca<sup>2+</sup> indicator, Fura-2 AM (2 μmol/L; Molecular Probes, Carlsbad, CA, USA), at 25 °C for 10 min. Loaded cells were electrically stimulated at a pacing cycle length (PCL) of 6 s or 2 s. Cell shortening was continuously monitored and Ca<sup>2+</sup> transients were recorded every 3 min or when the cellular arrhythmias emerged. Cellular arrhythmias were induced upon addition of H<sub>2</sub>O<sub>2</sub> (200 μmol/L). It is generally accepted that after-contractions and after-transients were defined as cellular proarrhythmogenic events, in which early after-transients/contractions (EATs/EACs) may correspond to EADs and delayed after-transients/contractions (DATs/DACs) may correspond to DADs<sup>[7, 20-23]</sup>. Therefore, EATs/EACs and DATs/DACs can be used as proximal direct indices of EADs-like and DADs-like arrhythmias, respectively. The probabilities of EATs/EACs occurrence were assessed by calculating the percentage of calcium transients or cell shortenings that developed EATs/EACs within 1 min after the treatments (H<sub>2</sub>O<sub>2</sub> and resveratrol) reached steady state. The calcium transient duration (CaTD) was measured as the time from the upstroke to 80% recovery (*ie*, CaTD<sub>80</sub>).

### Measurement of [Ca<sup>2+</sup>]<sub>i</sub> and cell shortening

Myocytes were paced at a frequency of 0.5 Hz (PCL=2 s). The ratio of emitted fluorescence at 340 and 380 nm was recorded as an indicator of [Ca<sup>2+</sup>]<sub>i</sub>. Cell shortening was detected using an optical edge detector and collected using a charge-coupled device camera (IonOptix; Milton, MA, USA). The data were analyzed using IonWizard 6.0 software (IonOptix; Milton, MA, USA).

### Electrophysiological study

Whole cell currents (*I*<sub>Na,L</sub>, *I*<sub>Ca,L</sub>) were recorded under voltage clamp mode with an EPC10 amplifier (Heka Electronic, Lambricht, Pfalz, Germany) as previously described<sup>[10, 24]</sup>. Current signals were filtered at 1 kHz and digitized at 10 kHz. For *I*<sub>Na,L</sub> recording, pipettes were filled with an internal solution containing (in mmol/L): 120 CsCl<sub>2</sub>, 1.0 CaCl<sub>2</sub>, 5 MgCl<sub>2</sub>, 5 Na<sub>2</sub>ATP, 10 TEACl, 11 EGTA, and 10 HEPES (pH 7.2, adjusted with CsOH). Myocytes were bathed with a modified Tyrode's solution containing (in mmol/L): 135 NaCl, 5.4 CsCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 0.3 BaCl<sub>2</sub>, 0.33 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose, 10 HEPES, and 0.001 nifedipine at pH 7.3. *I*<sub>Na,L</sub> was elicited by 300 ms depolarizing pulses from -120 to -20 mV at a PCL of 6 s. The amplitude of *I*<sub>Na,L</sub> was measured at 200 ms after the initiation of the depolarization step.

To record *I*<sub>Ca,L</sub> patch pipettes were filled with an internal solution containing (in mmol/L): 110 Cs-Aspartate, 30 CsCl, 5 NaCl, 10 HEPES, 0.1 EGTA, 5 MgATP at pH 7.2 adjusted with CsOH, and the cells were superfused with a modified Tyrode's solution in which KCl was replaced with CsCl. To inactivate the Na<sup>+</sup> current, the myocytes were stimulated by a 100 ms prepulse to -40 mV from the holding potential of -80 mV. Subsequently, *I*<sub>Ca,L</sub> was elicited by a test depolarization step to 0 mV for 300 ms. The current density was calculated by dividing the amplitude by the cell capacitance.

### Detection of intracellular ROS

The myocytes were incubated with 5  $\mu\text{mol/L}$  C-DCDHF-DA-AM (Invitrogen, Grand Island, NY, USA) for 30 min. After loading, myocytes were washed twice with Tyrode's solution. Loaded myocytes were treated with  $\text{H}_2\text{O}_2$  (200  $\mu\text{mol/L}$ ) in the absence or presence of resveratrol (50  $\mu\text{mol/L}$ ) for 20 min. Myocytes unexposed to  $\text{H}_2\text{O}_2$  were set as the control group. C-DCDHF-DA can be oxidized by ROS to dichlorofluorescein (DCF), which was used to determine ROS production. Cellular DCF fluorescence intensities were determined by fluorescence microscopy with excitation and emission spectra of 488 and 525 nm, respectively.

### Measurement of CaMKII activity

Isolated myocytes were treated with  $\text{H}_2\text{O}_2$  (200  $\mu\text{mol/L}$ ) in the absence or presence of resveratrol (50  $\mu\text{mol/L}$ ) for 10 min. Myocytes unexposed to  $\text{H}_2\text{O}_2$  were set as the control group. The CaMKII activity of myocytes was measured with the SignaTECT Calcium/Calmodulin-Dependent Protein Kinase Assay system (Promega, Madison, WI, USA) following the manufacturer's instructions. [ $\gamma$ - $^{32}\text{P}$ ]ATP was purchased from PerkinElmer, Inc (Waltham, MA, USA).

### Protein preparation and Western blotting

After intravenous anesthetization with sodium pentobarbital (30 mg/kg), rabbit hearts were immediately removed and mounted on a Langendorff apparatus and retrogradely perfused through the aorta (30 mL/min) with specific solutions depending on the condition for 10 min at 37°C: Tyrode's solution containing vehicle, Tyrode's solution containing 200  $\mu\text{mol/L}$   $\text{H}_2\text{O}_2$  and vehicle, and Tyrode's solution containing 200  $\mu\text{mol/L}$   $\text{H}_2\text{O}_2$  and 50  $\mu\text{mol/L}$  resveratrol. The left ventricular tissue was then isolated and flash frozen in liquid nitrogen. Protein preparations were performed using methods as described previously<sup>[25]</sup> and the protein contents of CaMKII (antiphospho-CaMKII, 1:1000, Abcam, Cambridge, UK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:1000, Beyotime, Haimen, China) in total protein preparations were analyzed by standard Western blotting.

### Statistical analysis

The data are expressed as the mean $\pm$ SEM. Statistical significance was assessed using Student's *t*-tests or ANOVA analysis followed by the Student-Newman-Keuls test. The frequencies of arrhythmia were compared using a chi-squared test.  $P < 0.05$  was considered statistically significant.

## Results

### Resveratrol suppressed and prevented $\text{H}_2\text{O}_2$ -induced arrhythmogenic activity in rabbit ventricular myocytes

Cell shortening and calcium transients were simultaneously recorded from isolated rabbit ventricular myocytes. To stably induce EAD-like arrhythmias, the myocytes were paced at a low frequency (0.16 Hz, PCL=6 s) as previously described<sup>[7]</sup>. After reaching steady state, the myocytes were continuously perfused with 200  $\mu\text{mol/L}$   $\text{H}_2\text{O}_2$ . As shown in Figure 1A, the

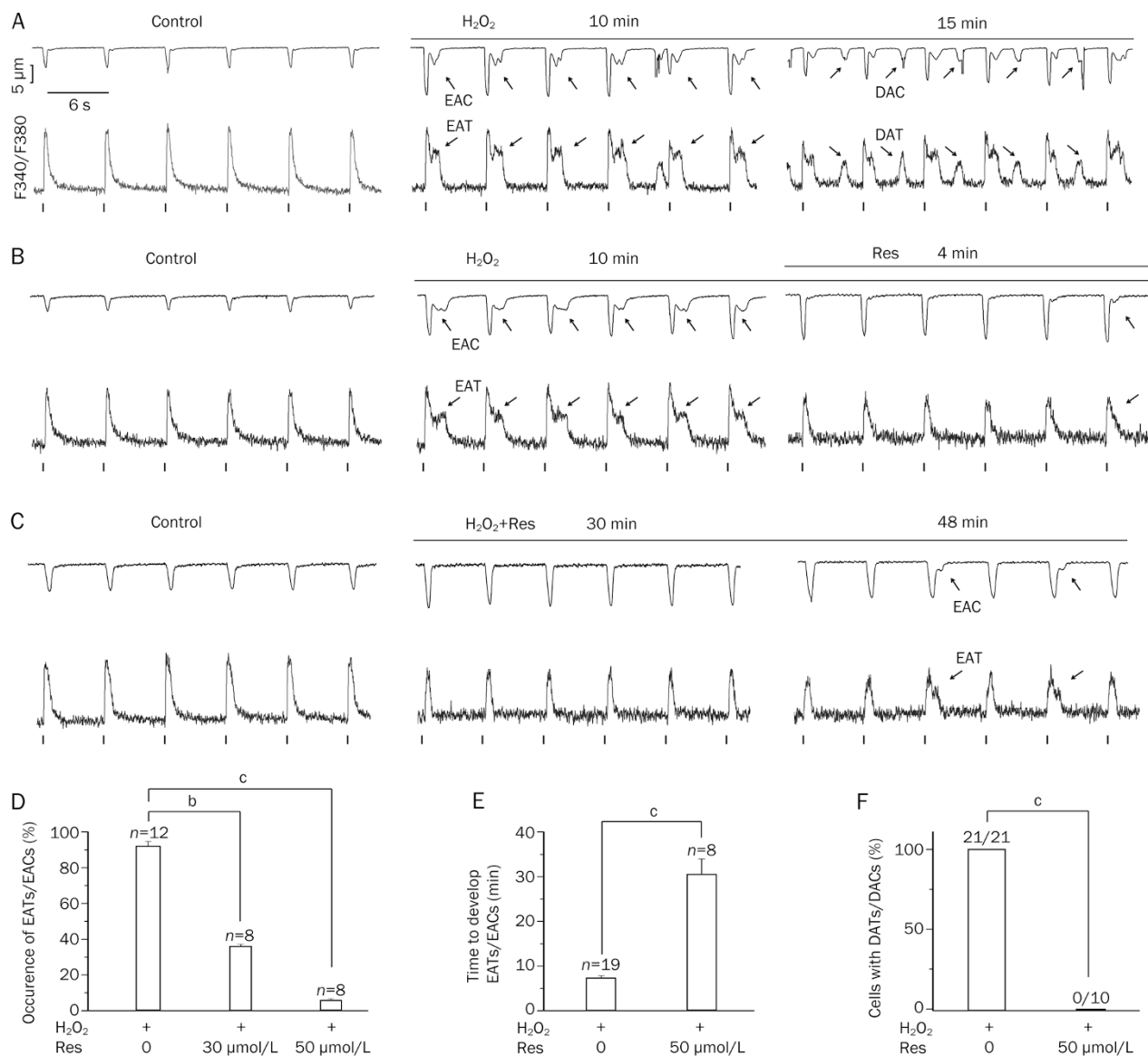
exposure of myocytes to  $\text{H}_2\text{O}_2$  led to prolongation of  $\text{CaTD}_{80}$  and induction of EATs/EACs, the EAD-like arrhythmias. Most of the cells developed EATs/EACs (90.5%, 19 out of 21 cells from 10 rabbits) after an average exposure time of  $7.4 \pm 0.5$  min with  $\text{H}_2\text{O}_2$ . Prolonged exposure to  $\text{H}_2\text{O}_2$  caused continuous EATs/EACs followed by DATs/DACs, the DAD-like arrhythmias. The other two cells (2 of 21 cells, 9.5%) did not induce EATs/EACs, but directly developed DATs/DACs.

Next, we studied the effects of resveratrol on  $\text{H}_2\text{O}_2$ -induced arrhythmogenic activity. When EATs/EACs were induced by  $\text{H}_2\text{O}_2$ , addition of resveratrol (30 or 50  $\mu\text{mol/L}$ ) resulted in a significant suppression of the EATs/EACs within 3 to 5 min (Figure 1B). As summarized in Figure 1D, the probability of EATs/EACs occurrence was significantly reduced by resveratrol at both 50 and 30  $\mu\text{mol/L}$ , in a dose-dependent manner ( $92 \pm 2.8\%$  vs  $36 \pm 1.2\%$  and  $6 \pm 0.8\%$ ,  $P < 0.05$  and  $P < 0.01$ , respectively). Accordingly, the  $\text{CaTD}_{80}$  prolongation induced by  $\text{H}_2\text{O}_2$  was also attenuated by resveratrol. The  $\text{CaTD}_{80}$  was increased by  $\text{H}_2\text{O}_2$  from  $1055 \pm 72$  to  $1892 \pm 154$  ms; resveratrol (50  $\mu\text{mol/L}$ ) decreased the  $\text{CaTD}_{80}$  to  $1340 \pm 116$  ms and attenuated by  $55 \pm 11\%$  the prolongation of CaTD caused by  $\text{H}_2\text{O}_2$  ( $P < 0.05$ ,  $n = 8$  cells from 4 rabbits).

Co-treatment of myocytes with resveratrol (50  $\mu\text{mol/L}$ ) blunted the prolongation of CaTD caused by  $\text{H}_2\text{O}_2$  and significantly prevented the induction of EATs/EACs (Figure 1C). Although resveratrol could not completely prevent the induction of  $\text{H}_2\text{O}_2$ -induced EATs/EACs, it markedly delayed the time for the development of EATs/EACs from  $7.4 \pm 0.5$  min ( $\text{H}_2\text{O}_2$  group) to  $31 \pm 3$  min ( $P < 0.01$ ,  $n = 8$  cells from 5 rabbits) (Figure 1E). In addition to preventing the induction of EATs/EACs caused by  $\text{H}_2\text{O}_2$ , resveratrol also prevented the myocytes from developing DATs/DACs (Figure 1C). All myocytes treated with  $\text{H}_2\text{O}_2$  developed DATs/DACs (100%, 21 of 21 cells from 10 rabbits), while none of the myocytes treated with resveratrol during exposure to  $\text{H}_2\text{O}_2$  developed these DAD-like arrhythmias within 40 min (0%, 0 of 10 cells from 6 rabbits,  $P < 0.01$  vs  $\text{H}_2\text{O}_2$ ) (Figure 1F). Interestingly, resveratrol completely prevented  $\text{H}_2\text{O}_2$ -induced EATs/EACs and DATs/DACs when the myocytes were paced at a higher frequency (0.5 Hz, PCL=2 s). At 0.5 Hz,  $\text{H}_2\text{O}_2$  induced EATs/EACs in 6 out of 11 myocytes (54.5%,  $P < 0.05$  vs at 0.16 Hz) at an average exposure time of  $14 \pm 2.7$  min ( $P < 0.01$  vs at 0.16 Hz) (Figure 2A). Meanwhile,  $\text{H}_2\text{O}_2$  induced DATs/DACs in 11 of 11 myocytes (100%) within 30 min. Treatment with resveratrol (50  $\mu\text{mol/L}$ ) prevented  $\text{H}_2\text{O}_2$ -induced EATs/EACs and DATs/DACs for at least 40 min ( $P < 0.05$  and  $P < 0.01$  vs  $\text{H}_2\text{O}_2$  group, respectively,  $n = 10$  cells from 6 rabbits) (Figures 2B–2D).

### Resveratrol prevented $\text{H}_2\text{O}_2$ -induced calcium overload and cell death

$\text{H}_2\text{O}_2$ -induced calcium overload is an important factor causing arrhythmias and cell death. In this series of experiments, the effects of 200  $\mu\text{mol/L}$   $\text{H}_2\text{O}_2$  on  $[\text{Ca}^{2+}]_i$  and the contractions of rabbit ventricular myocytes were determined in the absence or presence of 50  $\mu\text{mol/L}$  resveratrol (at a PCL of 2 s). As shown in Figures 3A and 3B,  $\text{H}_2\text{O}_2$  led to time-dependent increases



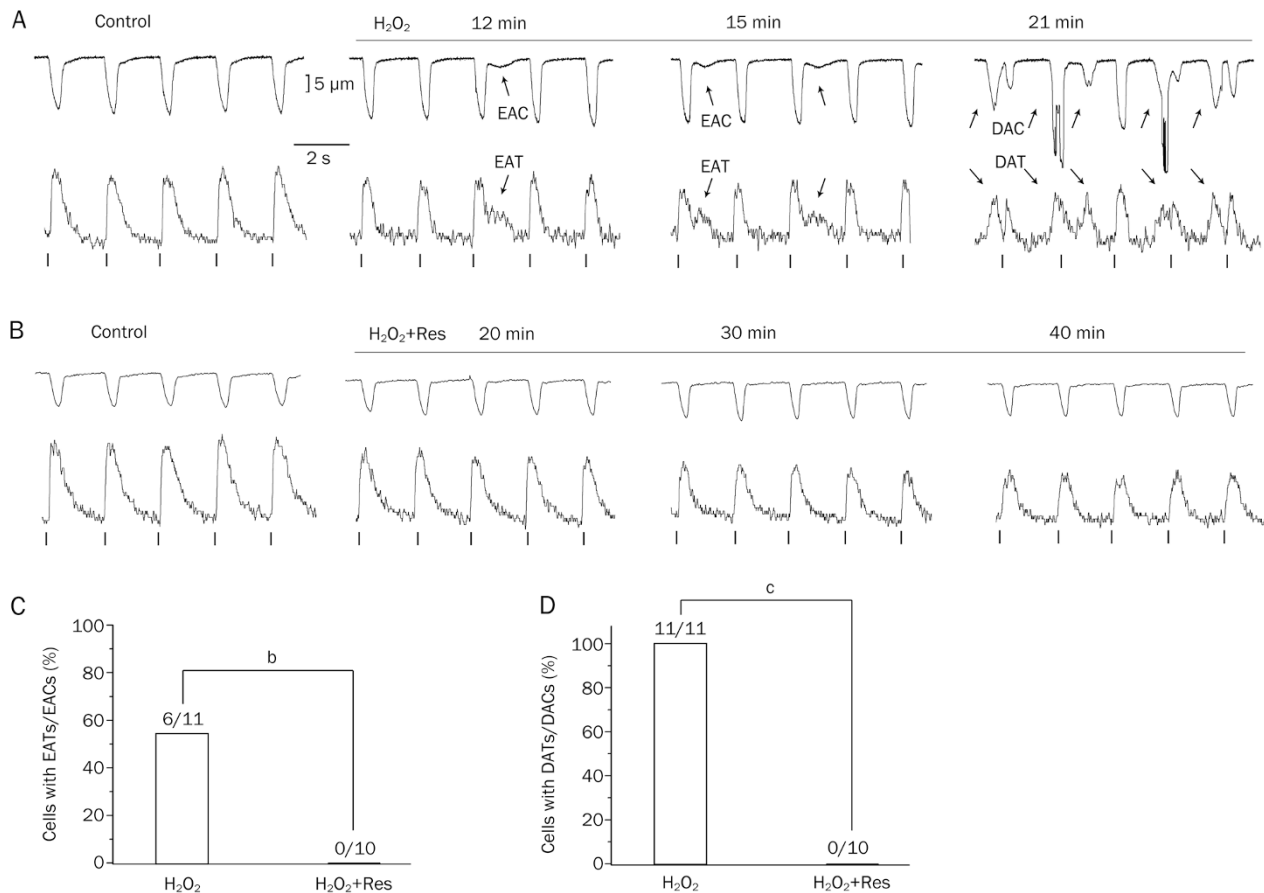
**Figure 1.** Effects of resveratrol on H<sub>2</sub>O<sub>2</sub>-induced arrhythmogenic activity at 0.16 Hz. In each panel, the top recordings are cell shortening and the bottom ones are calcium transients (PCL=6 s, 0.16 Hz). (A) Arrhythmogenic activity induced by H<sub>2</sub>O<sub>2</sub> (200 μmol/L) in a rabbit ventricular myocyte. Continuous EATs/EACs developed after 10 min of H<sub>2</sub>O<sub>2</sub> perfusion. At 15 min, continuous DATs/DACs developed. (B) After EATs/EACs were induced by 200 μmol/L H<sub>2</sub>O<sub>2</sub>, addition of resveratrol (Res, 50 μmol/L) suppressed EATs/EACs after approximately 4 min. (C) In the presence of resveratrol (Res, 50 μmol/L), a myocyte exposed to H<sub>2</sub>O<sub>2</sub> (200 μmol/L) did not develop EATs/EACs until 48 min and no DATs/DACs were induced within 48 min. (D) Summarized histogram showing dose-dependent inhibitory effects of resveratrol (Res, 30 and 50 μmol/L) on the incidence of EATs/EACs induced by H<sub>2</sub>O<sub>2</sub>. (E) Summarized histogram shows the time to develop EATs/EACs in the presence of H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>+Res (50 μmol/L). (F) Summarized histogram shows the proportion of cells displaying DATs/DACs in the presence of H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>+Res (50 μmol/L). Mean±SEM. <sup>b</sup>P<0.05, <sup>c</sup>P<0.01 vs H<sub>2</sub>O<sub>2</sub>.

of diastolic [Ca<sup>2+</sup>]<sub>i</sub>. Treatment with resveratrol significantly blunted the time-dependent increases of diastolic [Ca<sup>2+</sup>]<sub>i</sub> during exposure to H<sub>2</sub>O<sub>2</sub>. After a 21 min incubation of myocytes with H<sub>2</sub>O<sub>2</sub>, diastolic [Ca<sup>2+</sup>]<sub>i</sub> was increased to 143%±2% and 114%±3% of the control in the absence and presence of resveratrol, respectively (P<0.01; Figure 3B).

Consistent with this finding, resveratrol prevented hypercontracture caused by H<sub>2</sub>O<sub>2</sub>. As shown in Figure 3C, H<sub>2</sub>O<sub>2</sub>-induced calcium overload led to the development of hyper-

contracture, which is a permanent reduction (<50%) of the longitudinal cell length attributable to a persistent activation of myofilaments<sup>[6]</sup>. H<sub>2</sub>O<sub>2</sub> caused the development of hypercontracture within 21±1.3 min (n=43 cells from 10 rabbits). However, in the presence of resveratrol, myocytes (n=22 cells from 8 rabbits) exposed to H<sub>2</sub>O<sub>2</sub> never developed hypercontracture within 40 min. A Kaplan-Meier survival analysis (Figure 3D) revealed that resveratrol effectively prevented ROS-induced hypercontracture and cell death.





**Figure 2.** Effects of resveratrol on H<sub>2</sub>O<sub>2</sub>-induced arrhythmogenic activity at 0.5 Hz. In each panel, the top recordings are cell shortening and the bottom ones are calcium transients (PCL=2 s, 0.5 Hz). (A) Arrhythmogenic activity induced by H<sub>2</sub>O<sub>2</sub> (200 μmol/L) in rabbit ventricular myocytes. (B) In the presence of resveratrol (Res, 50 μmol/L), myocytes exposed to H<sub>2</sub>O<sub>2</sub> (200 μmol/L) did not develop EATs/EACs and DATs/DACs for at least 40 min. (C) Summarized histogram shows proportion of cells displaying EATs/EACs in the presence of H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>+Res (50 μmol/L). (D) Summarized histogram shows proportion of cells displaying DATs/DACs in the presence of H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>+Res (50 μmol/L). Mean±SEM. <sup>b</sup>P<0.05, <sup>c</sup>P<0.01 vs H<sub>2</sub>O<sub>2</sub>.

### Resveratrol inhibited ROS-induced $I_{Na,L}$ augmentation

It has been reported previously<sup>[6, 7, 10]</sup> that augmentation of  $I_{Na,L}$  plays a key role in ROS-induced cellular Na<sup>+</sup> and Ca<sup>2+</sup> overload and arrhythmias. Therefore, we first examined whether resveratrol could inhibit ROS-induced  $I_{Na,L}$  augmentation. When myocytes were exposed to H<sub>2</sub>O<sub>2</sub>,  $I_{Na,L}$  gradually increased in a time-dependent manner and reached a steady state at approximately 10–14 min after H<sub>2</sub>O<sub>2</sub> treatment. Treatment with resveratrol markedly prevented the time-dependent increase of  $I_{Na,L}$  induced by H<sub>2</sub>O<sub>2</sub> (Figure 4A). At the point of 12 min after H<sub>2</sub>O<sub>2</sub> exposure,  $I_{Na,L}$  increased from  $-0.31\pm 0.014$  pA/pF to  $-1.06\pm 0.037$  pA/pF in the H<sub>2</sub>O<sub>2</sub> group, increased by  $245\pm 23\%$  ( $n=6$  cells from 4 rabbits). While in the resveratrol treatment group,  $I_{Na,L}$  increased from  $-0.31\pm 0.032$  pA/pF to  $-0.53\pm 0.028$  pA/pF, only a  $75\pm 11\%$  increase of  $I_{Na,L}$  ( $n=6$  cells from 4 rabbits,  $P<0.01$ , Figure 4B). Resveratrol also reversed the H<sub>2</sub>O<sub>2</sub>-induced  $I_{Na,L}$  augmentation (Figure 4C).  $I_{Na,L}$  increased from  $-0.31\pm 0.013$  pA/pF to  $-1.07\pm 0.045$  pA/pF after the incubation of myocytes with H<sub>2</sub>O<sub>2</sub> ( $P<0.05$ ,  $n=6$  cells from 5 rabbits). The addition of resveratrol reduced the current to  $-0.53\pm 0.031$  pA/pF in the presence of H<sub>2</sub>O<sub>2</sub> ( $P<0.05$ ; Figure 4D),

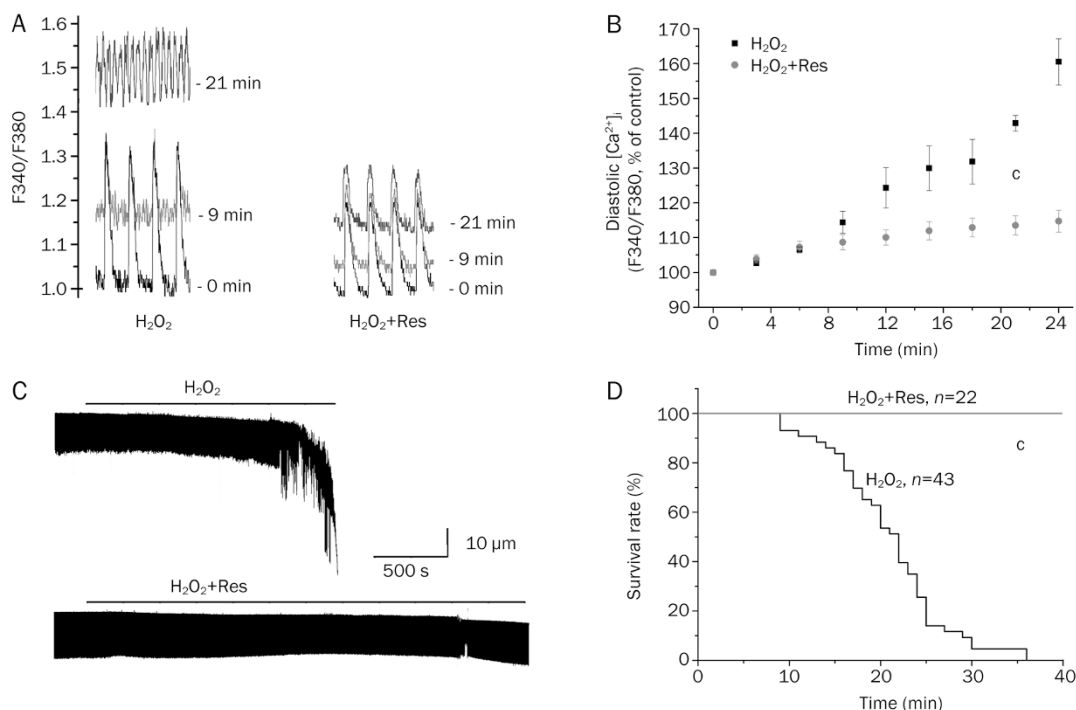
a  $178\pm 17\%$  decrease of the H<sub>2</sub>O<sub>2</sub>-induced  $I_{Na,L}$ .

### Resveratrol inhibited ROS-induced $I_{Ca,L}$ augmentation

Because reactivation of  $I_{Ca,L}$  contributes to the H<sub>2</sub>O<sub>2</sub>-induced EAD in rabbit ventricular myocytes<sup>[7]</sup>, we next investigated the potential involvement of  $I_{Ca,L}$  in the inhibitory effect of resveratrol on H<sub>2</sub>O<sub>2</sub>-induced EATs/EACs. As shown in Figures 5A and 5B, H<sub>2</sub>O<sub>2</sub> increased  $I_{Ca,L}$  from  $-8.2\pm 0.8$  pA/pF to  $-12.3\pm 1.4$  pA/pF ( $P<0.05$ ,  $n=6$  cells from 4 rabbits) and resveratrol reduced the current to  $-9.1\pm 0.8$  pA/pF ( $P<0.05$ ), indicating that resveratrol markedly inhibited H<sub>2</sub>O<sub>2</sub>-induced  $I_{Ca,L}$  augmentation.

### Resveratrol reduced H<sub>2</sub>O<sub>2</sub>-induced ROS production

To determine whether resveratrol inhibits the arrhythmogenic activity and calcium overload via decreasing intracellular ROS, the effect of resveratrol on intracellular ROS levels was measured. ROS production was monitored by detecting the fluorescence from the reaction of intracellular ROS with C-DCFDA using fluorescence microscopy. As shown in Figures 6A and 6B, DCF fluorescence intensity was signifi-



**Figure 3.** Effects of resveratrol on H<sub>2</sub>O<sub>2</sub>-induced calcium overload and hypercontracture. In this part, cells were paced at 0.5 Hz (PCL=2 s). (A) Original traces of Ca<sup>2+</sup> transient in the absence and presence of 50 μmol/L resveratrol (Res) before (0 min) and after 9 and 21 min exposures to 200 μmol/L H<sub>2</sub>O<sub>2</sub>. (B) Time course of changes in diastolic [Ca<sup>2+</sup>]<sub>i</sub>, which was normalized as a percentage of the control (before application of drug). Each point represents data collected from 5 to 10 cells (n=5–10). (C) Representative cell shortening curve of myocytes exposed to H<sub>2</sub>O<sub>2</sub> in the absence (top) or presence (bottom) of resveratrol (Res, 50 μmol/L). (D) Kaplan-Meier analysis of hypercontracture development. \*P<0.01 vs H<sub>2</sub>O<sub>2</sub> group.

cantly increased after a 20 min exposure with H<sub>2</sub>O<sub>2</sub>. Treatment with resveratrol significantly decreased the generation of ROS (P<0.05 vs H<sub>2</sub>O<sub>2</sub> group).

#### Resveratrol prevented H<sub>2</sub>O<sub>2</sub>-induced CaMKII activation

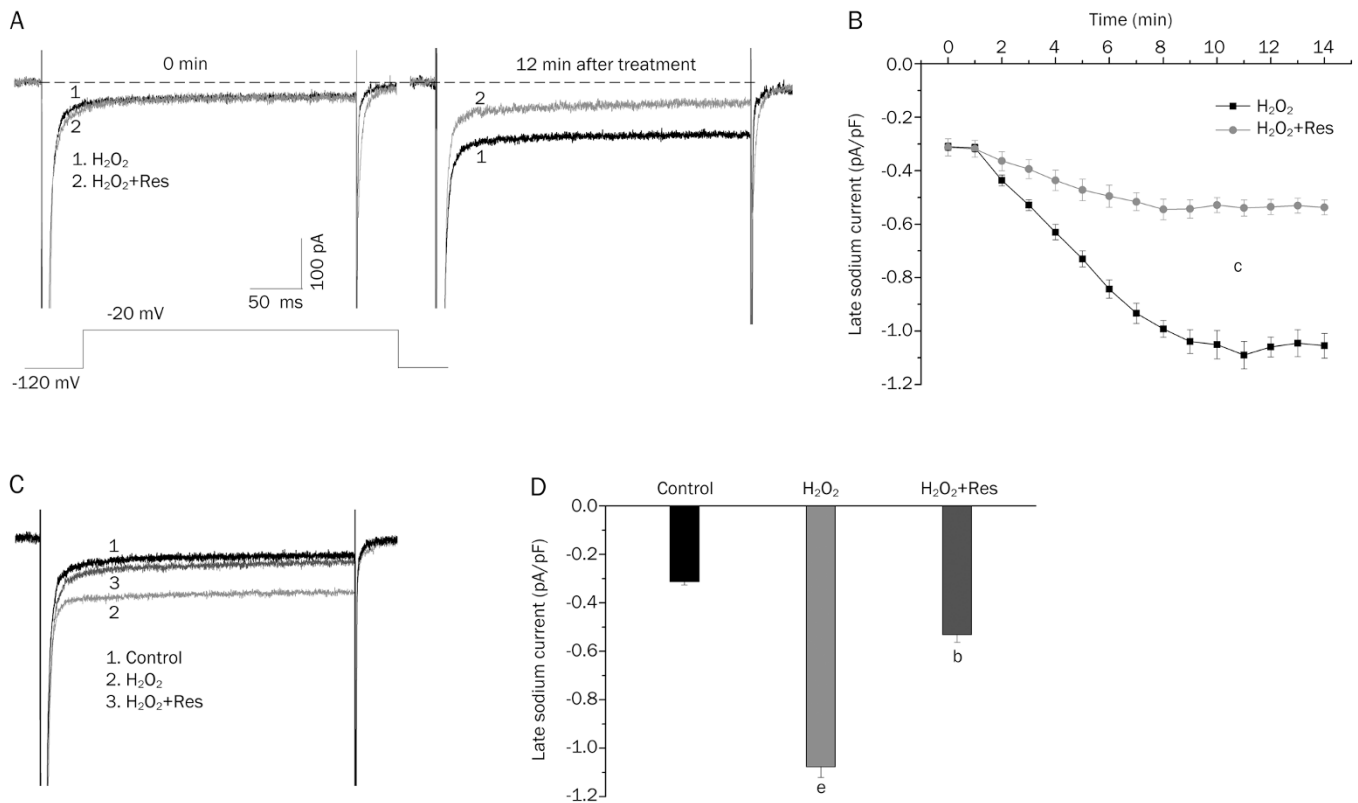
It has been reported that ROS can markedly activate CaMKII<sup>[6]</sup>. Having confirmed that resveratrol reduced ROS production, we next tested whether ROS-induced CaMKII activation could be prevented by resveratrol. Using a CaMKII assay kit, we found that H<sub>2</sub>O<sub>2</sub> (200 μmol/L) effectively increased CaMKII activity and resveratrol (50 μmol/L) significantly prevented this activation in isolated myocytes (P<0.05 vs H<sub>2</sub>O<sub>2</sub> group; Figure 7A). With a Western blotting analysis using the antibody against p-CaMKII, we further confirmed that resveratrol prevented H<sub>2</sub>O<sub>2</sub>-induced CaMKII activation. As shown in Figures 7B and 7C, CaMKII phosphorylation was significantly increased in hearts perfused with 200 μmol/L H<sub>2</sub>O<sub>2</sub> for 10 min (P<0.05 vs control), and resveratrol decreased the activation of CaMKII (P<0.05 vs H<sub>2</sub>O<sub>2</sub> group).

#### Discussion

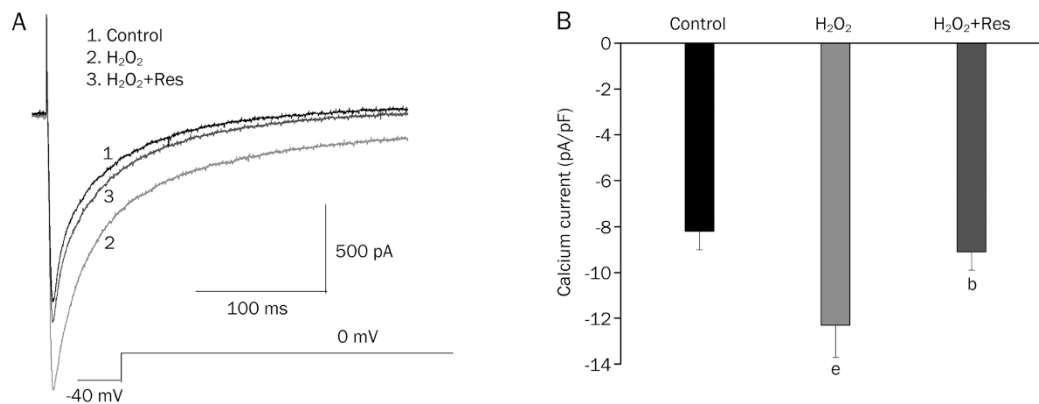
The cardioprotective effects of resveratrol are complex and are not completely understood. In the present study, we provide the first evidence showing that resveratrol prevents and suppresses oxidative stress-induced arrhythmogenic activity. It also prevents oxidative stress-induced calcium overload and

cell death. Furthermore, our study suggests that the underlying mechanisms involve: (1) inhibition of I<sub>Na,L</sub>/I<sub>Ca,L</sub>; (2) reduction of ROS generation; and (3) prevention of CaMKII activation.

EAD-mediated triggered activity plays an important role in ROS-induced arrhythmias<sup>[26]</sup>. In this study, we observed that resveratrol significantly suppressed and prevented H<sub>2</sub>O<sub>2</sub>-induced EATs/EACs, *ie*, EAD-like arrhythmias. At 0.16 Hz (PCL 6 s), resveratrol markedly delayed the time to develop EATs/EACs. It also reversed H<sub>2</sub>O<sub>2</sub>-induced EATs/EACs when added after EATs/EACs were induced. Recent studies<sup>[7,10]</sup> have implicated that ROS-activated CaMKII mediates EAD formation. Downstream targets of ROS include CaMKII, I<sub>Ca,L</sub>, and I<sub>Na,L</sub>. ROS-activated CaMKII causes I<sub>Ca,L</sub> and I<sub>Na,L</sub> augmentation through the regulation of calcium and sodium channel phosphorylation sites<sup>[27]</sup>. Xie *et al*<sup>[7,10]</sup> have demonstrated that both I<sub>Ca,L</sub> and I<sub>Na,L</sub> are required for ROS-induced EAD formation: activation of late I<sub>Na</sub> to reduce the repolarization reserve (*ie*, to prolong APD) and the modification of I<sub>Ca,L</sub> to enhance its reactivation properties to generate the EAD upstroke. In the present study, we found that resveratrol effectively reduced H<sub>2</sub>O<sub>2</sub>-induced ROS generation. With a CaMKII assay kit, we showed that resveratrol significantly prevented H<sub>2</sub>O<sub>2</sub>-induced CaMKII activation in isolated myocytes. Using CaMKII phosphorylation at threonine-287 as a marker of CaMKII activity, we further confirmed that resvera-



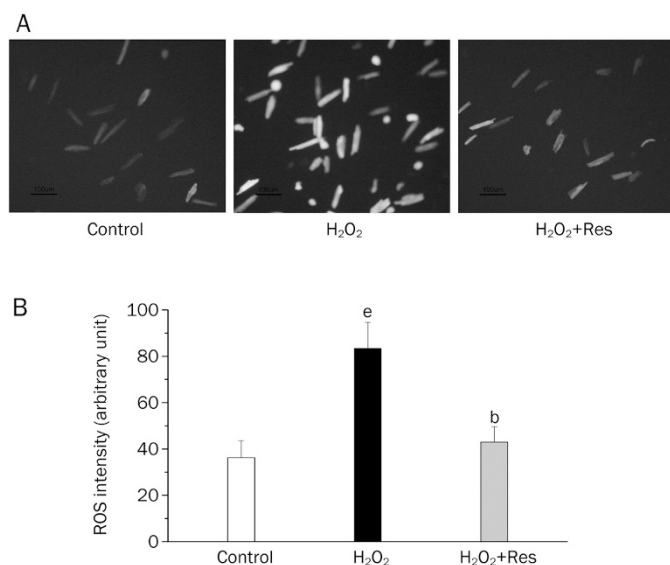
**Figure 4.** Effects of resveratrol on H<sub>2</sub>O<sub>2</sub>-induced  $I_{Na,L}$  augmentation.  $I_{Na,L}$  was measured at a PCL of 6 s (protocol in inset) in isolated rabbit myocytes. Original traces (A) and mean data (B) showed that H<sub>2</sub>O<sub>2</sub> significantly increased  $I_{Na,L}$  in myocytes. This increase was significantly prevented by resveratrol (Res, 50  $\mu$ mol/L). Original traces (C) and mean data (D) showed that resveratrol (Res, 50  $\mu$ mol/L) reversed the H<sub>2</sub>O<sub>2</sub>-induced  $I_{Na,L}$  augmentation. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  vs H<sub>2</sub>O<sub>2</sub> group. <sup>e</sup> $P < 0.05$  vs control. Mean  $\pm$  SEM.  $n = 6$  for each group.



**Figure 5.** Effects of resveratrol on H<sub>2</sub>O<sub>2</sub>-induced  $I_{Ca,L}$  augmentation. (A) Representative  $I_{Ca,L}$  traces under control conditions, in the presence of H<sub>2</sub>O<sub>2</sub>, and H<sub>2</sub>O<sub>2</sub>+resveratrol (Res, 50  $\mu$ mol/L). (B) A bar graph summarizing the H<sub>2</sub>O<sub>2</sub>-induced increase of  $I_{Ca,L}$ , which is significantly suppressed by resveratrol (Res, 50  $\mu$ mol/L). <sup>b</sup> $P < 0.05$  vs H<sub>2</sub>O<sub>2</sub>. <sup>e</sup> $P < 0.05$  vs control. Mean  $\pm$  SEM.  $n = 6$ .

tol significantly prevented H<sub>2</sub>O<sub>2</sub>-induced CaMKII activation in rabbit hearts. By reduction of ROS generation and CaMKII activation, resveratrol may indirectly inhibit  $I_{Na,L}$  and  $I_{Ca,L}$  and subsequently prevent and suppress H<sub>2</sub>O<sub>2</sub>-induced EATs/EACs. Previous studies<sup>[16, 28]</sup> have reported that resveratrol can directly modulate ion channels. For instance, resveratrol can

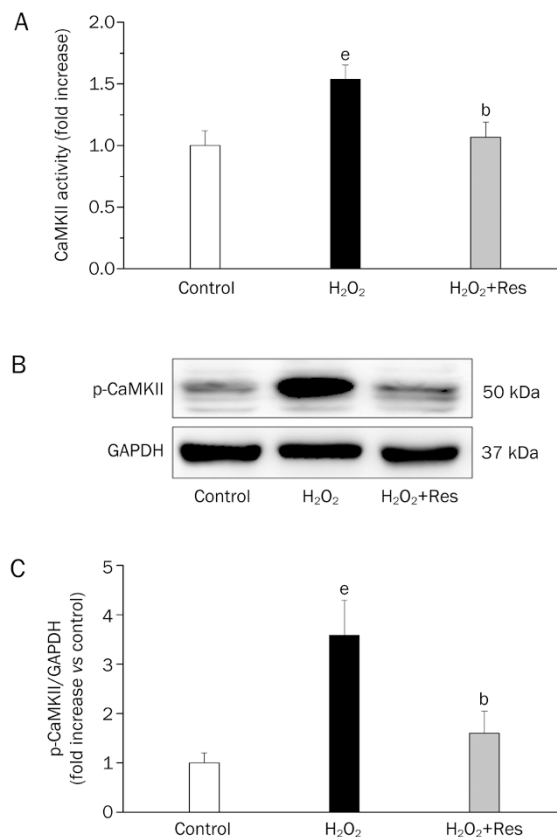
inhibit  $I_{Ca,L}$  and enhance the ATP-sensitive potassium current ( $I_{K,ATP}$ ) in isolated ventricular myocytes<sup>[16]</sup>. It can also inhibit the sodium current ( $I_{Na}$ ), transient ( $I_{to}$ ) and sustain ( $I_{ss}$ ) outward potassium currents<sup>[17]</sup>. Therefore, we cannot rule out the possibility that a direct inhibition of  $I_{Na,L}$  and  $I_{Ca,L}$  by resveratrol contributes to its inhibition of EATs/EACs.



**Figure 6.** Effects of resveratrol on H<sub>2</sub>O<sub>2</sub>-induced ROS production. (A) DCF fluorescence images of myocytes exposed to H<sub>2</sub>O<sub>2</sub> (200 μmol/L) in the absence and presence of resveratrol (Res, 50 μmol/L). (B) DCF fluorescence intensity measured as the image optical density (IOD) per unit area. <sup>b</sup>*P*<0.05 vs H<sub>2</sub>O<sub>2</sub>. <sup>e</sup>*P*<0.05 vs control. Mean±SEM. *n*=3.

Interestingly, when myocytes were paced at a higher frequency (0.5 Hz, at a PCL of 2 s), resveratrol completely prevented the H<sub>2</sub>O<sub>2</sub>-induced EATs/EACs for at least 40 min, indicating that resveratrol has enhanced anti-arrhythmias properties at a higher frequency. This may be due to a larger *I*<sub>Na,L</sub> at lower frequencies<sup>[24, 29]</sup>, which makes myocytes more prone to EATs/EACs. In sinus rhythm, *I*<sub>Na,L</sub> is much smaller than it is at 0.16 Hz. Thus, resveratrol may be expected to be more effective in preventing H<sub>2</sub>O<sub>2</sub>-induced EATs/EACs under sinus rhythm.

Calcium overload is an important factor causing DAD-induced arrhythmias and cell death<sup>[6]</sup>. In this study, we showed that resveratrol significantly blunted time-dependent [Ca<sup>2+</sup>]<sub>i</sub> increase induced by H<sub>2</sub>O<sub>2</sub>. As a result, resveratrol prevented H<sub>2</sub>O<sub>2</sub>-induced hypercontracture and cell death. Accordingly, resveratrol completely prevented the development of DATs/DACs, the DAD-like arrhythmias. Recent studies indicate that CaMKII activation and *I*<sub>Na,L</sub> augmentation play important roles in the ROS-induced Ca<sup>2+</sup> overload and arrhythmias<sup>[4]</sup>. Under various cardiac pathological conditions, there exists a lethal cycle between CaMKII and *I*<sub>Na,L</sub><sup>[30, 31]</sup>. ROS dramatically enhances *I*<sub>Na,L</sub> via CaMKII activation; the enhanced *I*<sub>Na,L</sub> further activates CaMKII, leading to CaMKII over-activation<sup>[6, 30]</sup>. Consequently, on the one hand, the ROS-induced over-activation of CaMKII phosphorylates RyR2 and causes sarcoplasmic reticulum (SR) calcium leak<sup>[32]</sup>; on the other hand, the CaMKII activation-enhanced *I*<sub>Na,L</sub> leads to sodium overload and subsequently calcium overload via the NCX<sup>[4]</sup>. Calcium overload and SR calcium leak always induce arrhythmogenic calcium wave and cause DAD-like arrhythmias<sup>[22]</sup>. Additionally, calcium overload leads to the



**Figure 7.** Effects of resveratrol on H<sub>2</sub>O<sub>2</sub>-induced CaMKII activation. (A) Histograms illustrating the CaMKII activity in response to a 10 min exposure to 200 μmol/L H<sub>2</sub>O<sub>2</sub> in the absence or presence of resveratrol (Res, 50 μmol/L). <sup>b</sup>*P*<0.05 vs H<sub>2</sub>O<sub>2</sub>. <sup>e</sup>*P*<0.05 vs control. *n*=4. (B) Western blotting analysis showing the levels of phosphorylated CaMKII in response to a 10 min exposure to 200 μmol/L H<sub>2</sub>O<sub>2</sub> in the absence or presence of resveratrol (Res, 50 μmol/L). (C) Mean data of CaMKII phosphorylation normalized to the total amount of GAPDH from 3 experiments. <sup>b</sup>*P*<0.05 vs H<sub>2</sub>O<sub>2</sub>. <sup>e</sup>*P*<0.05 vs control. Mean±SEM. *n*=3.

development of hypercontracture and cell death<sup>[6]</sup>. Therefore, elevated ROS leads to the development of a lethal cycle between CaMKII and *I*<sub>Na,L</sub> in diseased hearts. In the present study, we showed that resveratrol prevented H<sub>2</sub>O<sub>2</sub>-induced CaMKII activation and augmentation of *I*<sub>Na,L</sub> and effectively blocked the lethal cycle between them. Thus, resveratrol prevented the H<sub>2</sub>O<sub>2</sub>-induced calcium overload and DAD-like arrhythmias. In addition, ROS can directly oxidize RyR2<sup>[33]</sup>, leading to an increased calcium spark, DAD-like arrhythmias and calcium overload. By reducing ROS generation, resveratrol may reduce RyR2 oxidation and also suppress DAD-like arrhythmias and calcium overload.

A limitation of the present study was that we did not investigate the anti-arrhythmias properties of resveratrol at the organ/whole heart level. This requires further study in the future.

In conclusion, resveratrol significantly suppressed and prevented oxidative stress-induced arrhythmogenic activity and calcium overload by inhibition of *I*<sub>Na,L</sub>/*I*<sub>Ca,L</sub>, reduction of ROS



generation, and prevention of CaMKII activation. This study provides a new way to treat arrhythmias and calcium overload induced by ROS. It also provides a novel drug to prevent CaMKII over-activation, a hallmark of pathological conditions, such as heart failure.

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### Author contribution

Yi-gang LI and Yue-peng WANG designed the research; Wei LI, Ling GAO, Peng-pai ZHANG, Qing ZHOU, and Quan-fu XU performed the research; Zhi-wen ZHOU, Kai GUO, and Ren-hua CHEN analyzed data; Wei LI wrote the paper; Yi-gang LI, Yue-peng WANG, and Huang-tian YANG revised the manuscript.

### References

- Kinugawa S, Tsutsui H, Hayashidani S, Ide T, Suematsu N, Satoh S, et al. Treatment with dimethylthiourea prevents left ventricular remodeling and failure after experimental myocardial infarction in mice: role of oxidative stress. *Circ Res* 2000; 87: 392–8.
- Xin P, Pan Y, Zhu W, Huang S, Wei M, Chen C. Favorable effects of resveratrol on sympathetic neural remodeling in rats following myocardial infarction. *Eur J Pharmacol* 2010; 649: 293–300.
- Mallat Z, Philip I, Lebreton M, Chatel D, Maclouf J, Tedgui A. Elevated levels of 8-iso-prostaglandin F2alpha in pericardial fluid of patients with heart failure: a potential role for *in vivo* oxidant stress in ventricular dilatation and progression to heart failure. *Circulation* 1998; 97: 1536–9.
- Song Y, Shryock JC, Wagner S, Maier LS, Belardinelli L. Blocking late sodium current reduces hydrogen peroxide-induced arrhythmogenic activity and contractile dysfunction. *J Pharmacol Exp Ther* 2006; 318: 214–22.
- Wagner S, Seidler T, Picht E, Maier LS, Kazanski V, Teucher N, et al. Na<sup>+</sup>-Ca<sup>2+</sup> exchanger overexpression predisposes to reactive oxygen species-induced injury. *Cardiovasc Res* 2003; 60: 404–12.
- Wagner S, Ruff HM, Weber SL, Bellmann S, Sowa T, Schulte T, et al. Reactive oxygen species-activated Ca/calmodulin kinase II is required for late I<sub>Na</sub> augmentation leading to cellular Na and Ca overload. *Circ Res* 2011; 108: 555–65.
- Xie LH, Chen F, Karagueuzian HS, Weiss JN. Oxidative-stress-induced afterdepolarizations and calmodulin kinase II signaling. *Circ Res* 2009; 104: 79–86.
- Erickson JR, Joiner ML, Guan X, Kutschke W, Yang J, Oddis CV, et al. A dynamic pathway for calcium-independent activation of CaMKII by methionine oxidation. *Cell* 2008; 133: 462–74.
- Song YH, Choi E, Park SH, Lee SH, Cho H, Ho WK, et al. Sustained CaMKII activity mediates transient oxidative stress-induced long-term facilitation of L-type Ca<sup>2+</sup> current in cardiomyocytes. *Free Radic Biol Med* 2011; 51: 1708–16.
- Zhao Z, Fefelova N, Shanmugam M, Bishara P, Babu GJ, Xie LH. Angiotensin II induces afterdepolarizations via reactive oxygen species and calmodulin kinase II signaling. *J Mol Cell Cardiol* 2011; 50: 128–36.
- Husken A, Baumert A, Milkowski C, Becker HC, Strack D, Mollers C. Resveratrol glucoside (Piceid) synthesis in seeds of transgenic oilseed rape (*Brassica napus* L). *Theor Appl Genet* 2005; 111: 1553–62.
- Bhat KPL, Kosmeder JW 2nd, Pezzuto JM. Biological effects of resveratrol. *Antioxid Redox Signal* 2001; 3: 1041–64.
- Movahed A, Yu L, Thandapilly SJ, Louis XL, Netticadan T. Resveratrol protects adult cardiomyocytes against oxidative stress mediated cell injury. *Arch Biochem Biophys* 2012; 527: 74–80.
- Thandapilly SJ, Wojciechowski P, Behbahani J, Louis XL, Yu L, Juric D, et al. Resveratrol prevents the development of pathological cardiac hypertrophy and contractile dysfunction in the SHR without lowering blood pressure. *Am J Hypertens* 2010; 23: 192–6.
- Renaud S, de Lorgeril M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* 1992; 339: 1523–6.
- Chen YR, Yi FF, Li XY, Wang CY, Chen L, Yang XC, et al. Resveratrol attenuates ventricular arrhythmias and improves the long-term survival in rats with myocardial infarction. *Cardiovasc Drugs Ther* 2008; 22: 479–85.
- Chen WP, Su MJ, Hung LM. *In vitro* electrophysiological mechanisms for antiarrhythmic efficacy of resveratrol, a red wine antioxidant. *Eur J Pharmacol* 2007; 554: 196–204.
- Lakatta EG, Sollott SJ. The "heartbreak" of older age. *Mol Interv* 2002; 2: 431–46.
- Zhang CM, Gao L, Zheng YJ, Yang HT. Berberine increases myocardial contractility via a Ca<sup>2+</sup>-independent mechanism. *J Cardiovasc Pharmacol* 2011; 58: 40–8.
- Janiak R, Lewartowski B. Early after-depolarisations induced by noradrenaline may be initiated by calcium released from sarcoplasmic reticulum. *Mol Cell Biochem* 1996; 163-164: 125–30.
- Sag CM, Wadsack DP, Khabbazzadeh S, Abesser M, Greff C, Neumann K, et al. Calcium/calmodulin-dependent protein kinase II contributes to cardiac arrhythmogenesis in heart failure. *Circ Heart Fail* 2009; 2: 664–75.
- Gonano LA, Sepulveda M, Rico Y, Kaetzel M, Valverde CA, Dedman J, et al. Calcium-calmodulin kinase II mediates digitalis-induced arrhythmias. *Circ Arrhythm Electrophysiol* 2011; 4: 947–57.
- Dallas ML, Yang Z, Boyle JP, Boycott HE, Scragg JL, Milligan CJ, et al. Carbon monoxide induces cardiac arrhythmia via induction of the late Na<sup>+</sup> current. *Am J Respir Crit Care Med* 2012; 186: 648–56.
- Wu L, Ma J, Li H, Wang C, Grandi E, Zhang P, et al. Late sodium current contributes to the reverse rate-dependent effect of I<sub>Kr</sub> inhibition on ventricular repolarization. *Circulation* 2011; 123: 1713–20.
- Qi X, Yeh YH, Chartier D, Xiao L, Tsuji Y, Brundel BJ, et al. The calcium/calmodulin/kinase system and arrhythmogenic afterdepolarizations in bradycardia-related acquired long-QT syndrome. *Circ Arrhythm Electrophysiol* 2009; 2: 295–304.
- Morita N, Lee JH, Xie Y, Sovari A, Qu Z, Weiss JN, et al. Suppression of re-entrant and multifocal ventricular fibrillation by the late sodium current blocker ranolazine. *J Am Coll Cardiol* 2011; 57: 366–75.
- Ashpole NM, Herren AW, Ginsburg KS, Brogan JD, Johnson DE, Cummins TR, et al. Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) regulates cardiac sodium channel Nav1.5 gating by multiple phosphorylation sites. *J Biol Chem* 2012; 287: 19856–69.
- Zhang Y, Liu Y, Wang T, Li B, Li H, Wang Z, et al. Resveratrol, a natural ingredient of grape skin: antiarrhythmic efficacy and ionic mechanisms. *Biochem Biophys Res Commun* 2006; 340: 1192–9.
- Guo D, Lian J, Liu T, Cox R, Margulies KB, Kowey PR, et al. Contribution of late sodium current (I<sub>NaL</sub>) to rate adaptation of ventricular repolarization and reverse use-dependence of QT-prolonging agents. *Heart Rhythm* 2011; 8: 762–9.
- Yao L, Fan P, Jiang Z, Viatchenko-Karpinski S, Wu Y, Kornyejev D, et al. Nav1.5-dependent persistent Na<sup>+</sup> influx activates CaMKII in rat ventricular myocytes and N1325S mice. *Am J Physiol Cell Physiol*

- 2011; 301: C577-86.
- 31 Coppini R, Ferrantini C, Yao L, Fan P, Del Lungo M, Stillitano F, *et al*. Late sodium current inhibition reverses electromechanical dysfunction in human hypertrophic cardiomyopathy. *Circulation* 2013; 127: 575-84.
- 32 Wagner S, Rokita AG, Anderson ME, Maier LS. Redox regulation of sodium and calcium handling. *Antioxid Redox Signal* 2013; 18: 1063-77.
- 33 Anzai K, Ogawa K, Kuniyasu A, Ozawa T, Yamamoto H, Nakayama H. Effects of hydroxyl radical and sulfhydryl reagents on the open probability of the purified cardiac ryanodine receptor channel incorporated into planar lipid bilayers. *Biochem Biophys Res Commun* 1998; 249: 938-42.