EXPERIMENTAL STUDY

The Agonist of Inward Rectifier Potassium Channel (I\textsubscript{K1}) Attenuates Rat Reperfusion Arrhythmias Linked to CaMKII Signaling

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Summary

Inward rectifier potassium channels (I\textsubscript{K1}, Kir) are known to play critical roles in arrhythmogenesis. Thus, how I\textsubscript{K1} agonist affects reperfusion arrhythmias needs to be clarified, and its underlying mechanisms should be determined. Reperfusion arrhythmias were modeled by coronary ligation (ischemia, 15 minutes) and release (reperfusion, 15 minutes). Zacopride (1.5-50 μg/kg \textit{in vivo}, or 0.1-10 μmol/L \textit{ex vivo}) was applied in the settings of pretreatment (3 minutes before coronary ligation) and posttreatment (5 minutes after coronary ligation). Hypoxia (45 minutes)/reoxygenation (30 minutes) model was established in cultured H9c2 (2-1) cardiomyocytes. Zacopride or KN93 was applied before hypoxia (pretreatment). In the setting of pre- or posttreatment, zacopride at 15 μg/kg \textit{in vivo} or 1 μmol/L \textit{in vitro} exhibited superlative protections on reperfusion arrhythmias or intracellular calcium overload. Western blot data from \textit{ex vivo} hearts or H9c2 (2-1) cardiomyocytes showed that I/R (H/R) induced the inhibition of Kir2.1 (the dominant subunit of I\textsubscript{K1} channel in ventricle), phosphorylation and oxidation of CaMKII, downregulation of SERCA2, phosphorylation of phospholamban (at Thr17), and activation of caspase-3. Zacopride treatment (1 μmol/L) was noted to strikingly restore the expression of Kir2.1 and SERCA2 and decrease the activity of CaMKII, phospholamban, and caspase-3. These effects were largely eliminated by co-application of I\textsubscript{K1} blocker BaCl\textsubscript{2}. CaMKII inhibitor KN93 attenuated calcium overload and p-PLB (Thr17) in an I\textsubscript{K1}-independent manner. I\textsubscript{K1}-dependent inhibition of CaMKII activity is found to be a key cardiac salvage signaling under Ca\textsuperscript{2+} dyshomeostasis and reactive oxygen species (ROS) stress. I\textsubscript{K1} might be a novel target for pharmacological conditioning of reperfusion arrhythmia, especially for the application after unpredictible ischemia.

Key words: Calcium overload, Oxidative stress, Hypoxia/reoxygenation, Preconditioning, Postconditioning

Coronary artery obstruction or acute myocardial infarction (AMI) has been identified as the leading cause of cardiac death and disability worldwide.\textsuperscript{1} Thus, timely restoring the blood perfusion to previous ischemic tissue is essential and definitive therapy in order to reduce cell damage and improve clinical outcomes in patients. However, some cases suffered deterioration with decreased cardiac function, even lethal arrhythmias. This phenomenon is known as ischemia-reperfusion injury (IRI), and the concomitant arrhythmias are named reperfusion arrhythmias. Clinically, reperfusion arrhythmias consist of idioventricular rhythms and manifest as premature ventricular contraction (PVC), ventricular tachycardia (VT), and ventricular fibrillation (VF). Although the pathogenesis of reperfusion injury is not yet fully understood, intracellular calcium overload\textsuperscript{2-4} and an overproduction of oxygen-derived free radicals or oxidative stress\textsuperscript{5} are confirmed to be key contributors. In particular, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) is identified as a main determinant of early reperfusion arrhythmias.\textsuperscript{6}

Ischemic preconditioning\textsuperscript{7} and postconditioning\textsuperscript{8} are performed by exposure of the heart to transient episodes of ischemia/reperfusion before ischemia or upon reperfusion, respectively. Both treatments provide powerful resistance to major ischemia events and share similar pathways.\textsuperscript{9} Along with the clarification of inner mechanism, pharmacological strategies, such as adenosine agonist,\textsuperscript{10} β-blocker,\textsuperscript{11} and K\textsubscript{ATP} channel agonist,\textsuperscript{12} are developed to reproduce the cardioprotection of conditioning, accordingly called pharmacological pretreatment or posttreatment. Regarding the unpredictability of AMI, posttreatment is an...

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ticipatory and is more applicable in clinic than pretreatment.

Herafore, clinical antiarrhythmic agents act mainly on ion channels that constitutively determine the action potential (AP) in cardiomyocytes, such as voltage-gated calcium channel, sodium channel, and potassium channel. Inward rectifier potassium channels (IK1, Kir), which are present in all atrial and ventricular cardiomyocytes, are critical for stabilizing resting potential (RP), establishing excitability threshold, and regulating the end stage of AP repolarization.11,12 In 2012 and 2017, we have reported a selective agonist of IK1, Zacopride, and showed its protection against aconitine-induced or MI-induced arrhythmias in rats. Of which, zacopride attenuated lethal arrhythmias post MI.

In this present study, attempts were made to clarify whether IK1 agonist might attenuate reperfusion arrhythmias in the setting of pre- and posttreatment. Further efforts were focused on the involvement of calcium-activated and oxidation-activated CaMKII signaling in the cardiac salvage of pharmacological conditioning.

**Methods**

**Animals:** Healthy adult Sprague-Dawley rats (male, 8 weeks old) were provided by the Laboratory Animal Research Center of Shanxi Medical University (Taiyuan, China). The adult rats were housed under standard conditions and fed standard chow and water ad libitum. The investigation conformed to the guidelines for the Care and Use of Laboratory Animals (NIH, revised 2011) and followed the approval of Ethics Committee of Shanxi Medical University.

**Reperfusion-induced arrhythmias:**

In vivo study Reperfusion arrhythmias were induced by ligating and loosening the left main coronary artery. After being anesthetized with sodium pentobarbital (65 mg/kg, i.p.), rats were ventilated at 60 strokes/minute and stroke volume of 30 mL/kg body weight using a small-animal respirator (DH-1, Chengdu Instrument Factory, China). The right femoral vein was cannulated for drug administration. After thoracotomy in the fourth intercostal space and removal of the pericardium to expose the heart, the coronary artery emergence was drawn out for reperfusion. The Tyrode’s solution. Simulated lead II ECG recording was performed using a waveform data analysis software (RM6240, BiopacSystem, Chengdu Instrument Factory, China). The hearts exhibiting spontaneous arrhythmias were discarded. After equilibrated for 1 hour, the left main coronary artery was ligated across a small cotton roll within 2 mm from the artery emergence and adjacent to the left atrium. Post 15 minutes, the cotton roll was drawn out for reperfusion. The Tyrode’s solution was as follows (in mmol/L): NaCl 135.0, KCl 5.4, CaCl2 1.8, MgCl2 1.0, NaH2PO4 0.33, HEPES 10.0, and glucose 10.0 (pH 7.3-7.4 adjusted with NaOH). The concentration of zacopride ranged from 0.1 to 10 μmol/L, as per Zhai, et al. and preliminary studies. Zacopride was applied 3 minutes before coronary occlusion (pretreatment) or 5 minutes after coronary occlusion (posttreatment). BaCl2 at 1 μmol/L was used as an IK1 antagonist.

Evaluation of arrhythmias: ECGs were continuously recorded prior to and during ischemia and reperfusion using a waveform data analysis software (RM6240, BiopacSystem, Chengdu Instrument Factory, China). The ventricular arrhythmias were evaluated according to the diagnostic criteria by Lambeth Convention.10

Establishment of hypoxia/reoxygenation (H/R) in cultured H9c2 (2-1) cells: H9c2 (2-1) cardiomyocytes are derived from embryonic rat heart tissue and were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). To mimic the myocardial I/R in vitro, H9c2 (2-1) cells at 80% confluence were incubated in a hypoxic C-chamber in 2% O2 and 98% N2 at 37°C for 45 minutes; then, they were converted immediately to the incubator in 21% O2 and 5% CO2 for a 30minutes reoxygenation. The oxygen rate during hypoxia was maintained at 2% using a compact gas oxygen controller (ProOx 110, Biospherix, Ltd., Paris, NY). In view of the uncontrollability for the setting of posttreatment, the cells were pretreated with zacopride in the criteria of pretreatment. The control dishes were kept in 21% O2 and 5% CO2 at 37°C for 75 minutes. The dishes were randomly subdivided into 10 groups as follows: normoxia control, H/R, H/R + Zac (0.1 μmol/L), H/R + Zac (1 μmol/L), H/R + Zac (10 μmol/L), H/R + Zac (1 μmol/L) + BaCl2: (1 μmol/L), H/R + KN93 (0.5 μmol/L), KN93(0.5 μmol/L), Zac (1 μmol/L), and BaCl2: (1 μmol/L). After the trial, the cells were lysed and collected for Western blotting assay. In parallel, cells were seeded in 24-well plates for 24 hours and then performed the same H/R protocol as above for assessment of intracellular resting [Ca2+]i fluorescence.

Western blotting: Proteins from isolated I/R rat hearts or cultured H9c2 (2-1) cardiomyocytes were loaded on 10-15% acrylamide gels by 40 μg per lane. After electropho-
The representative ECGs illustrating multiple ventricular reperfusion arrhythmias. The elevation of ST segment indicates the establishment of cardiac ischemia. VT indicates ventricular tachycardia; VF, ventricular fibrillation; and PVC, premature ventricular contraction.

retic transfer and incubated with 5% nonfat milk in Tris-buffered saline, the nitrocellulose membranes were incubated overnight at 4°C with target protein antibodies. The relative levels of Kir2.1 (1:1000, rabbit monoclonal, Abcam), CaMKII (1:1000, rabbit polyclonal, Abcam), phosphorylated CaMKII (1:500, rabbit polyclonal, Abcam), oxidized CaMKII (1:1000, rabbit polyclonal, Millipore), p-Thr17-phospholamban (1:1000, rabbit monoclonal, Abcam), phospholamban (1:1000, rabbit monoclonal, Abcam), and cleaved caspase-3 (1:1000, rabbit monoclonal, Cell Signaling Technology) were quantified by Western blotting, respectively. The GAPDH (1:2000, rabbit monoclonal, Sigma) was applied as the loading control.

Intracellular calcium measurement: The intracellular Ca$^{2+}$ fluorescence in cultured H9c2(2-1) cells were indicated either by a single-wavelength Ca$^{2+}$ indicator Fluo-4 AM (Dojindo Laboratories, Japan) or by a dual-wavelength Ca$^{2+}$ indicator Fura-2 AM (Dojindo Laboratories, Japan). H9c2 (2-1) cells were incubated with 5 μM Fluo-4 AM or 3 μM Fura-2 AM in the dark at 37°C for 45 minutes. The loaded cells were then washed three times with Hanks’ Balanced Salt Solution (HBSS) and kept in HBSS for another 30 minutes to allow de-esterification of calcium indicator in the cell.

The Ca$^{2+}$ fluorescence indicated by Fluo-4 was measured using an FV1000 laser scanning confocal microscope (Olympus). Thereafter, 494 nm wavelength was applied to excite the samples at 30-second intervals and collect fluorescence emission at 516 nm. Gain and laser intensity were adjusted to obtain fine images. The data was collected and analyzed using FluoView 1.7a software (Olympus). The measured fluorescence was subtracted from the background and autofluorescence.

The Ca$^{2+}$ fluorescence indicated by Fura-2 was measured as fluorescence ratios (excitation at 340 and 380 nm; emission at 510 nm) from single cells using an Olympus IX71 inverted fluorescence microscope (Olympus, Tokyo, Japan) and a Luca EMCCD camera, and these were collected at 2-second intervals. The data were recorded and analyzed using MetaFluor® Fluorescence Ratio Imaging System (Molecular Devices, USA).

Statistics: All statistical analyses were performed using SPSS 17.0 software. All data were presented as mean ± standard error (SEM) and analyzed using the least significant difference and Games-Howell tests of analysis of variance. Differences in the incidence of VT or VF among groups were analyzed using the χ²-test. P < 0.05 was considered statistically significant.
Table I. The Suppressive Effects of Zacopride Treatments on Acute Reperfusion Arrhythmias in Anesthetized Rats in vivo (Mean ± SEM)

| n  | Episodes of PVC | Duration of VT (second) | Incidence of VT (%) | Duration of VF (second) | Incidence of VF (%) |
|----|----------------|-------------------------|---------------------|-------------------------|---------------------|
| Sham | 6              | 0**                    | 0**                 | 0**                     | 0**                 |
| Pretreatment  |                  |                        |                     |                         |                     |
| I/R model | 9              | 15 ± 4                 | 7.6 ± 1.9           | 100                     | 25.8 ± 6.9          | 100.0               |
| Zac 1.5 μg/kg | 8              | 21 ± 10                | 6.9 ± 2.5           | 62.5                    | 1.1 ± 0.7**         | 25.0**              |
| Zac 15 μg/kg  | 9              | 15 ± 5                 | 2.0 ± 1.2**         | 33.3**                  | 0.3 ± 0.3**         | 11.1**              |
| Zac 50 μg/kg  | 8              | 12 ± 4                 | 5.7 ± 2.0           | 75                      | 0.0**               | 0**                 |
| Posttreatment |                  |                        |                     |                         |                     |                     |
| I/R model | 8              | 14 ± 2                 | 12.2 ± 1.5          | 100                     | 26.1 ± 5.0          | 87.5                |
| Zac 1.5 μg/kg | 7              | 10 ± 1                 | 9.9 ± 2.2           | 85.7                    | 7.7 ± 5.4*          | 28.6*               |
| Zac 15 μg/kg  | 8              | 6 ± 1**                | 3.2 ± 1.0**         | 87.5                    | 4.4 ± 4.4*          | 12.5**              |
| Zac 50 μg/kg  | 7              | 9 ± 2                  | 5.8 ± 2.5           | 85.7                    | 15.2 ± 7.7          | 57.1                |

*P < 0.05, **P < 0.01 versus I/R.

Table II. The Suppressive Effects of Zacopride Treatments on Acute Reperfusion Arrhythmias in ex vivo Isolated Rat Hearts (Mean ± SEM)

| n  | Episodes of PVC | Duration of VT (second) | Incidence of VT (%) | Duration of VF (second) | Incidence of VF (%) |
|----|----------------|-------------------------|---------------------|-------------------------|---------------------|
| Sham | 6              | 0                      | 0                   | 0                       | 0                   |
| Pretreatment  |                  |                        |                     |                         |                     |
| I/R model | 10             | 59 ± 20                | 45.0 ± 13.3         | 100                     | 70.4 ± 17.1         | 80                  |
| Zac 0.1 μmol/L | 10             | 42 ± 12                | 19.4 ± 7.1          | 90                      | 16.4 ± 14.2         | 40                  |
| Zac 1 μmol/L  | 10             | 33 ± 9                 | 8.4 ± 2.8           | 80                      | 0.1 ± 0.1*          | 10**                |
| Zac 10 μmol/L | 10             | 69 ± 17                | 24.6 ± 6.8          | 90                      | 6.3 ± 3.3*          | 30                  |
| Zac 1 μmol/L + BaCl2 1 μmol/L | 10 | 98 ± 21 | 88.1 ± 45.6 | 100 | 35.6 ± 14.8 | 70* |
| Posttreatment |                  |                        |                     |                         |                     |                     |
| Sham | 6              | 0                      | 0                   | 0                       | 0                   |
| I/R model | 10             | 59 ± 20                | 45.0 ± 13.3         | 100                     | 70.4 ± 17.1         | 80                  |
| Zac 0.1 μmol/L | 9              | 80 ± 16                | 29.9 ± 10.8         | 88.9                    | 12.9 ± 6.7          | 44.4                |
| Zac 1 μmol/L  | 9              | 27 ± 10                | 9.1 ± 5.5           | 66.7                    | 0.1 ± 0.1*          | 11.1*               |
| Zac 10 μmol/L | 9              | 74 ± 22                | 19.6 ± 7.7          | 88.9                    | 14.1 ± 5.7          | 66.7                |
| Zac 0.1 μmol/L + BaCl2 1 μmol/L | 9 | 78 ± 36 | 51.3 ± 29.0 | 100 | 40.7 ± 13.3 | 88.9** |

PVC indicates premature ventricular contraction; VT, ventricular tachycardia; VF, ventricular fibrillation; and Zac, zacopride. *P < 0.05, **P < 0.01, versus I/R. *P < 0.05 versus 1 μmol/L Zac. **P < 0.01 versus 0.1 μmol/L Zac.

Results

Effects of zacopride on reperfusion arrhythmias:

In vivo study Severe ventricular arrhythmias including PVC, VT, or VF were noted to occur immediately after coronary release (Figure 1B). Table I demonstrates the effects of zacopride on reperfusion-induced arrhythmias in anesthetized rats. In detail, all sham rats showed normal sinus rhythm, and the cases with spontaneous arrhythmia were weeded out. All model rats (100%) developed VT and VF. Both in the setting of pretreatment and posttreatment, 1.5, 15, and 50 μg/kg zacopride strikingly inhibited the genesis of VF, with no effects on PVC. Zacopride at 15 μg/kg exhibited powerful effects on both VT and VF (Table I).

Effects of zacopride on heart rate and arterial blood pressure in anesthetized rats Baseline HR and BP values did not differ between sham, I/R, and zacopride treatment groups (Supplemental Table). In I/R groups, left coronary artery ligation induced significant decline in HR and mean arterial pressure (MAP) (P < 0.05 or P < 0.01, versus sham); reperfusion attenuated the abnormality of HR and MAP. In the setting of pretreatment, 1.5-50 μg/kg zacopride has largely maintained HR and MAP, whereas 15 μg/kg zacopride showed the most striking protection (P < 0.05 or P < 0.01, versus I/R). In the setting of posttreatment, zacopride had no significant effects on HR and MAP, although 1.5 and 15 μg/kg zacopride showed a trend of elevation in HR and MAP.

Ex vivo study To further clarify the involvement of IK1 activation in cardioprotection on reperfusion arrhythmias, the effects of zacopride are monitored in Langendorff-perfused rat hearts. The protocols for pretreatment and posttreatment were the same as that shown in Figure 1A. All sham rat hearts showed normal sinus rhythm, and the cases with spontaneous arrhythmia were weeded out. All isolated rat hearts (100%) developed VT, of which 80% developed VF post-reperfusion. Both pretreatment and posttreatment with 0.1-10 μmol/L Zacopride treatment showed promising prevention on VF, but had no significant effects on PVC and VT (Table II). Zacopride at 1.0 μmol/L exhibited the most striking antiarrhythmic effects as evidenced by the reduction in the duration (P < 0.05) and incidence (P < 0.01 or P < 0.05) of VF. The effects
The expression of Kir2.1 and CaMKII in *ex vivo* study. A: In the setting of pretreatment. B: In the setting of posttreatment. Both in the setting of pre- and posttreatment, zacopride restored the expression of Kir2.1 and inhibited the phosphorylation and oxidation of CaMKII. All data were normalized to sham. Zac indicates zacopride. Data were presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, versus sham. #P < 0.05, ##P < 0.01, versus I/R. &P < 0.05, &&P < 0.01, versus 1 μmol/L Zac. Could be largely counteracted by 1 μmol/L BaCl₂, which is a non-specific Iₖ,₁ blocker (*P < 0.05 or P < 0.01). All the data suggest that Iₖ,₁ is involved in the genesis of reperfusion arrhythmia, and the antiarrhythmic effects of zacopride are mediated by Iₖ,₁ activation.

The expression of Kir2.1 and CaMKII in the setting of pretreatment and posttreatment

In rat ventricles, Kir2.1 (KCNJ2) is the predominant isoform of native Iₖ,₁ channels.¹⁶) Kir2.1 proteins were significantly inhibited in isolated rat hearts that have underwent I/R injury compared with sham (Figure 2, *P < 0.01). Both in the setting of pre- and posttreatment, 0.1, 1, and 10 μmol/L zacopride counteracted the inhibition of Kir2.1 (*P < 0.01 or P < 0.05). The superlative activation by 1 μmol/L zacopride was reversed by 1 μmol/L BaCl₂ (*P < 0.01).

CaMKII autophosphorylation at serine 287 (p-CaMKII) and oxidation at mt 281/282 (ox-CaMKII) was detected by Western blotting. As shown in Figure 2, I/R significantly increased p-CaMKII and ox-CaMKII levels compared with sham (*P < 0.05 or P < 0.01). Both in the setting of pre- and posttreatment, the phosphorylation and oxidation of CaMKII were depleted using Iₖ,₁ agonist zacopride. It is noteworthy that the total CaMKII was also upregulated by I/R and downregulated by zacopride pre- or posttreatment (*P < 0.05 or P < 0.01). These effects were largely eliminated by low-dose Iₖ,₁ blocker BaCl₂ (*P < 0.05 or P < 0.01). This suggests that upregulation of Kir2.1, which carries Iₖ,₁, mediated the modification of CaMKII expression and activity.

The expression of calcium-handling proteins and activation of caspase-3 in the setting of pretreatment and posttreatment

Ca²⁺-ATPase 2 (SERCA2) pumps, located on the sarcoplasmic reticulum (SR) in cardiomyocytes, play a critical role in regulating intracellular Ca²⁺. The Ca²⁺ sequestrating activity of SERCA2 is regulated by phospholamban (PLB), with alternating basal conditions (dephosphorylation) or phosphorylation by c-AMP-dependent protein kinase A (PKA) at Ser-16 or by CaMKII at Thr-17. Compared with sham, I/R reduced the expression of SERCA2 (*P < 0.05 or P < 0.01) and induced phosphorylation of PLB (*P < 0.01). Both in the setting of pre- and posttreatment, zacopride at 1 μmol/L normalized the expression of SERCA2 (*P < 0.05 or P < 0.01) and inhibited the phosphorylation of PLB (*P < 0.01). These effects
The expression of calcium-handling proteins and activation of caspase-3 in *ex vivo* study. A: In the setting of pretreatment. B: In the setting of posttreatment. Both in the setting of pre- and posttreatment, zacopride increased the expression of SERCA2, inhibited the phosphorylation of PLB, and decreased the activity of caspase-3. All data were normalized to sham. PLB indicates phospholamban; and Zac, zacopride. Data were presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, versus sham. *P < 0.05, **P < 0.01, versus 1 μmol/L Zac.

were largely reversed by BaCl2 (P < 0.05 or P < 0.01).

The IRI also featured with the activation of caspase-3 which suggests the increase of apoptosis (Figure 3, P < 0.05 compared with sham). Both in the setting of pre- and posttreatment, 1 μmol/L zacopride reduced the activity of caspase-3 (P < 0.05 or P < 0.01). These effects were largely eliminated by I_{K1} blocker BaCl2. This suggests that regulation of I_{K1} mediated the anti-apoptosis following I/R insult.

**Effect of zacopride and KN93 on H/R in cultured H9c2 (2-1) cells:**

The expression of Kir2.1 and CaMKII in the setting of pretreatment Compared with normoxic control, H/R-induced Kir2.1 downregulation in H9c2 (2-1) cells (Figure 4A and B, P < 0.01). Zacopride at 1 μmol/L counteracted the inhibition of Kir2.1 (P < 0.05), and the effect could be reversed by 1 μmol/L BaCl2 (P < 0.05). KN93, a CaMKII inhibitor, had no significant effect on Kir2.1, regardless if under normoxia or H/R condition.

As shown in Figure 4A, C, and D, H/R significantly increased p-CaMKII and ox-CaMKII levels compared with normoxia (P < 0.05). The phosphorylation and oxidation of CaMKII could be depleted by 1-10 μmol/L zacopride or 0.5 μmol/L KN93 (P < 0.05). The protection of 1 μmol/L zacopride was largely eliminated by low-dose I_{K1} blocker BaCl2 (P < 0.05 or P < 0.05). This suggests that upregulation of Kir2.1 mediated the modification of CaMKII activity. The total CaMKII did not differ in all groups.

The expression of calcium-handling proteins in the setting of pretreatment As shown in Figure 5, compared with normoxia, H/R reduced the expression of SERCA2 (P < 0.05) and induced phosphorylation of PLB (P < 0.01). Zacopride at 0.1-10 μmol/L largely normalized the expression of SERCA2 (P < 0.05 or P < 0.01) and inhibited the phosphorylation of Thr17 site in PLB (P < 0.01). Zacopride at 1 μmol/L showed the most striking protection, and the effects were reversed by BaCl2 (P < 0.01). CaMKII inhibitor KN93 had no significant effect on SERCA2, but it attenuated the phosphorylation of PLB in the scenario of H/R (P < 0.01).

The effect of zacopride and KN93 on resting [Ca^{2+}]i in the setting of pretreatment The calcium fluorescence indicated by single-wavelength Ca^{2+} indicator Fluo-4 (Figure 6A) or dual-wavelength Ca^{2+} indicator Fura-2 (Figure 6B) showed that 0.1-10 μmol/L zacopride alleviated H/R-induced [Ca^{2+}]i overload in H9c2 (2-1) cells (P < 0.01). The superlative protection by 1 μmol/L zacopride was reversed using I_{K1} blocker BaCl2 (P < 0.01). As an inhibitor of CaMKII, KN93 significantly suppressed H/R-induced calcium overload (P < 0.01). In normal cardiomyocytes, KN93 at 0.5 μmol/L also slightly decreased [Ca^{2+}]i. The data confirmed that the activation of CaMKII is involved in H/R-induced [Ca^{2+}]i overload.

**Discussion**

The generation and severity of reperfusion arrhythmia can largely depend on the duration of preceding ischemia. The preliminary experiment showed that occlusion of the left coronary artery in rats for 15 minutes succeeded by release was more prone to induce reperfusion arrhythmias, and the arrhythmias were burst on the early stage of reperfusion. Thus 15-minute ischemia plus 15-minute reperfusion was set as the model criteria. In this present study, we confirmed that the inhibition of Kir2.1, the dominant subunit of I_{K1} in ventricles, was involved in the genesis of reperfusion arrhythmias. Moreover, I_{K1} ago-
The expression of Kir2.1 and CaMKII in cultured H9c2 (2-1) cells. A: Representative Western blotting images showed the expression of Kir2.1, CaMKII, and GAPDH in the setting of pretreatment. B: Kir2.1 protein expression levels relative to GAPDH. C: Phosphorylation of CaMKII levels relative to total CaMKII. D: Oxidization of CaMKII levels relative to total CaMKII. All data were normalized to normoxia. Zac, zacopride. Data were presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, versus normoxia. #P < 0.05, ##P < 0.01, versus H/R. &P < 0.05, versus 1 μmol/L Zac.

The expression of SERCA2 and PLB in cultured H9c2 (2-1) cells. A: SERCA2 protein expression levels relative to GAPDH in the setting of pretreatment. B: Phosphorylation of PLB levels relative to total PLB. All data were normalized to normoxia. Zac, zacopride. Data were presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, versus normoxia. P < 0.05, **P < 0.01, versus H/R. &P < 0.05, versus 1 μmol/L Zac.

Kir2.1 inhibition and CaMKII activation are involved in the genesis of reperfusion arrhythmias: As regards the genesis of tachyarrhythmias, reentry, increased automaticity, and triggered activity are probably the ultimate electrophysiological mechanisms based on membrane damage and ionic disorder. The pathogenesis of reperfusion arrhythmias is much similar to that during ischemia, but there still are some characteristic differences. During ischemia, non-reentry excitation including abnormal automaticity and triggered activity has been determined to be critical initiators for fatal arrhythmia. As progressing to reperfusion, cell injury and ionic disturbance were superlative. Intracellular Ca^2+ overload is a central event accounting for triggered arrhythmias. Oxydative stress, overactivated Na^+-Ca^2+ exchanger (NCX reverse mode), as well as Na^+/H^+ exchanger (for amending intracellular acidosis) facilitate Ca^2+ overload and DADs. Throughout the ischemia and reperfusion, lysophospholipids and catecholamine release have been identified to be responsible for the inhibition of I_{K1}, I_{R}, inhibition and resultant RP depolarization enhance cardiac excitability.
and autorhythmicity, rendering the ventricular myocardium susceptible to early afterdepolarizations (EADs) or DADs. \( I_{\text{K1}} \) inhibition also decreases the repolarizing reserve \( K^+ \) current, postpones late repolarization phase, and thereafter increases the vulnerable window for reactivation of voltage-gated \( Ca^{2+} \) channels.

\( \text{CaMKII} \) is a multimeric serine/threonine kinase responsible for the phosphorylation of downstream cardiac proteins. It has emerged as an important pro-arrhythmogenic signaling molecule responsible for arrhythmias. \( \text{CaMKII} \) activation occurs upon binding of calcium-activated calmodulin (\( Ca^{2+}/CaM \)) to the autoregulatory domain and autophosphorylation across subunits at Thr287. An alternative activated mechanism is oxidization at methionines 281 and 282 by the ROS. Phosphorylated or oxidized \( \text{CaMKII} \) regulates multiple ion channels and \( Ca^{2+} \) handling proteins such as voltage-gated Na\(^+\) channel, L-type \( Ca^{2+} \) (LTCC), phospholamban (PLB), and ryanodine receptor 2 (RyR2), which ultimately induce the prolongation of AP, depolarization of RP, deteriorative \( Ca^{2+} \) overload, and triggered activities.

Synchronization of SERCA2 and RyR2 fine-tunes SR \( Ca^{2+} \) uptake and release in cardiomyocytes. RyR2 mediates \( Ca^{2+} \) release from SR, after the depolarization-induced \( Ca^{2+} \) influx through the LTCC. The \( Ca^{2+} \) sequestering activity of SERCA is mainly regulated by phosphorylation status of PLB. Dephosphorylated PLB reduces the affinity of SERCA2 to \( Ca^{2+} \), while phosphorylated PLB increases SERCA2 activity and the rate of \( Ca^{2+} \) uptake. \( \text{CaMKII} \)-dependent phosphorylation of LTCC, RyR2 (Ser2814), and PLB (Thr17) might be highly related to intracellular \( Ca^{2+} \) overload. Moreover, PLB phosphorylation at Thr17 was mapped as a marker of \( \text{CaMKII} \) activation during acute ischemia and reperfusion.

In this present study, phosphorylated PLB (Thr17) was increased in the scenario of I/R in isolated hearts or H/R in H9c2 (2-1) cells and decreased by zacopride in an \( I_{\text{K1}} \)-dependent manner. Moreover, \( \text{CaMKII} \) inhibitor KN93 abolished the phosphorylation of the Thr17 site of PLB in an \( I_{\text{K1}} \)-independent manner. Phosphorylation of PLB in I/R (H/R) might have a dual effect. Beneficially, phosphorylation of PLB was found to be essential for cardiac \( Ca^{2+} \) transients and contractility during reperfusion. Detrimentally, during early reperfusion, increased reuptake of \( Ca^{2+} \) leads to excessive SR \( Ca^{2+} \) load and, consequently, to \( Ca^{2+} \) release through phosphorylated RyR. It makes a futile cycle between SR \( Ca^{2+} \) uptake and release, generating \( Ca^{2+} \) oscillations. In particular, we found that the expression of SERCA2 in I/R (H/R) was significantly downregulated, which may counteract the beneficial effect of PLB phosphorylation and exacerbate cytosolic \( Ca^{2+} \) overload.

Pharmacological upregulation of Kir2.1 and resultant inhibition of \( \text{CaMKII} \) might be a novel cardioprotective strategy against calcium dyshomeostasis and arrhythmias. Moderately upregulating Kir2.1 is a regression to the physiological status or compensation for \( I_{\text{K1}} \) deficiency. Via upregulating Kir2.1 and inhibiting \( \text{CaMKII} \), as well as \( \text{CaMKII} \)-dependent phosphorylation of \( Ca^{2+} \) handling proteins, zacopride might restore intracellular \( Ca^{2+} \) homeostasis and, ultimately, eliminate all causes of abnormal automaticities.
Relief of cell injury interacts with the improvement of electrical disorder upon reperfusion: Zacopride pretreatment and posttreatment effectively eliminated malignant reperfusion arrhythmias such as VT or VF. The optimal dose is 1.0 μmol/L, ex vivo or 15 μg/kg in vivo. The dose-response relationships were consistent with our previous observations in AMI-induced ischemic arrhythmia. Zacopride suppressed AMI-induced ischemic arrhythmia, either by preventive or therapeutic application. The protection, such as stabilization of RP, attenuation of ionic disorder, and relief of cell injury during ischemia, may expand to reperfusion. It might be the most important mechanism accounting for the cardioprotection of zacopride pretreatment and posttreatment.

During the early moments of reperfusion, superlative Ca\(^{2+}\) overload, rapid genesis of ROS, rapid correction of tissue pH, and opening of the mPTP fundamentally aggrava the cell injury and electrical disorder. Besides Ca\(^{2+}\) overload, oxidative stress also increases the risk of ventricular arrhythmias, which are generally mediated by abnormalities in cardiomyocyte ionic channels, gap junction remodeling, and multiple Ca\(^{2+}\)-handling proteins. Oxidative stress and ROS stress. Relief of cell injury might interact with the improvement of electrical disorder upon reperfusion. A research from Fuwai Hospital (Beijing, China) demonstrated that application of 1 μmol/L zacopride posttreatment, the antioxidative potential of Zacopride pretreatment, and rescue of cell injury might improve electrical dysfunction. Van der Weg, et al. proposed that arrhythmias and fatal injury induced by reperfusion are two outcomes proceeded from one process around calcium overload. This viewpoint is highly in concert with our findings.

**Clinical relevance and prospecton:** Upregulating Kir2.1 which carries I\(_{K1}\), and consequently maintaining RP and shortening APD might be a promising strategy to attenuate reperfusion arrhythmias caused by Ca\(^{2+}\) overload and oxidative stress. Pharmacologically upregulating I\(_{K1}\) as soon as possible during the ongoing ischemia will shed light on the prevention of reperfusion arrhythmias. Meanwhile, although Kir2.1 channel is an attractive target in the development of ventricular-specific drugs, the molecular mechanisms underlying the modification of I\(_{K1}\) and CaMKII need to be further clarified in the future.

**Disclosure**

**Conflicts of interest:** The authors declare no conflict of interest.

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Supplemental Files
Supplemental Table
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