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Reduced cardiac ischemia/reperfusion injury by hypothermic reperfusion *via* activation of transient receptor potential M8 channel

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ABSTRACT

Aims: To investigate the cardioprotective effects of hypothermic (25 °C) reperfusion on ischemia/reperfusion injury and the role of transient potential channel M8 (TRPM8) in this process.

Main methods: Western blot and real-time PCR were used to monitor the expression of TRPM8 in myocardium. Myocardial ischemia/reperfusion injury was induced by 30 min of global ischemia followed by 120 min of reperfusion in Langendorff-perfused hearts from Sprague-Dawley rats. The reperfusion was either normothermic (37 °C) or hypothermic (25 °C). Infarct size and left ventricular function were assessed, and lactate dehydrogenase (LDH), superoxide dismutase (SOD), and malondialdehyde (MDA) in the coronary effluent were measured spectrophotometrically, and cardiomyocyte apoptosis was detected by TUNEL assay. The expression of TRPM8, Bcl-2, Bax, cleaved capspase-3, RhoA, and ROCK2 was quantified.

Key findings: TRPM8 protein and mRNA were expressed in rat myocardium. Hypothermic reperfusion decreased the infarct size, LDH activity, MDA content, apoptosis, and expression of Bax, cleaved caspase-3, RhoA, and ROCK2 compared with normothermic reperfusion. These effects were associated with improved recovery of left ventricular contractility, and were reduced by BCTC, a TRPM8 antagonist. Ischemia/reperfusion injury and the increased expression of Bax, caspase-3, RhoA, and ROCK2 induced by normothermic reperfusion were reduced by Icilin, a TRPM8 agonist.

Significance: Hypothermic reperfusion at 25 °C has cardioprotective effects against ischemia/reperfusion injury *via* activation of TRPM8 to inhibit the oxidative stress-related RhoA/ROCK2 signal pathway.

1. Introduction

It is well known that prolonged myocardial ischemia leads to heart damage, myocardial cell apoptosis, and cell death. One of the important means of treating acute myocardial ischemia is myocardial reperfusion [1]. However, restoration of the blood supply can itself induce cardiomyocyte death, known as myocardial ischemia/reperfusion injury (MIRI), for which there is still no effective therapy. Although ischemic preconditioning has been shown to protect against MIRI [2], its clinical application is limited due to its invasiveness.

Recently, many animal and clinical studies have shown that hypothermic reperfusion is beneficial to the ischemic brain, heart, and other vital organs [3–6], suggesting that hypothermic reperfusion may be an important potential means of treating MIRI. However, the temperature for reperfusion has not been definitively recommended [7]. Because low temperatures are prone to induce ventricular fibrillation [8], perfusion at lower temperatures than but close to normal body temperature may have a protective effect without causing ventricular fibrillation.

Transient receptor potential (TRP) channels are non-selective cationic channels widely distributed in the cardiovascular system and are closely associated with many cardiovascular diseases [9] such as myocardial fibrosis [10], cardiac hypertrophy [11,12], arrhythmias, and atrioventricular block [13]. Different TRP channels are sensitive to various stimuli [14,15]. Interestingly, some TRP channels are temperature-sensitive: TRPV1–4 are thermosensitive [16,17], while TRPM8, TRPA1, and TRPC5 are cold-sensitive [15,18,19]. The TRPM8 channel is activated at 25 °C [20], at which it exhibits a significant inward current [21], and a similar temperature threshold has been demonstrated by Ca^{2+}-imaging [22]. The activation temperature of TRPA1 is 17 °C and that of TRPC5 is 20 °C [15]; they share some properties with TRPM8 [21,23]. Hypothermic reperfusion at such low temperatures may activate these TRP channels [24], but whether they participate in the cardioprotection provided by hypothermic reperfusion remains unknown. Interestingly, it has been reported that TRPM8 activation inhibits oxidative stress [25,26]. Since oxidative stress is one

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of the main causes of MIRI, activation of TRPM8 may provide cardioprotective effects by this mechanism. It is worthy of note that, among the three cold-sensitive TRP channels, the activation temperature of TRPM8 is closest to normal body temperature.

The heart undergoes oxidative stress during MIRI, and this eventually leads to apoptosis in cardiomyocytes [27]. The RhoA/ROCK (Ras homolog gene family, member A/RhoA kinase) signaling pathway is involved in oxidative stress-induced apoptosis [28–30]. RhoA is a subtype of Rho, which is a Ras homolog, while ROCK1 and ROCK2 are downstream effectors of RhoA [31,32]. The expression of ROCK1 and ROCK2 differs in different tissues. ROCK1 is expressed more in lung, spleen, liver, kidney, and testis, while ROCK2 predominates in brain and heart [33]. Studies have shown that activation of TRPM8 attenuates activation of the RhoA/ROCK2 signal pathway [34,35], suggesting that TRPM8 may participate in the inhibition of oxidative stress. However, little is known about the effects of TRPM8 activation on MIRI.

Based on the above evidence, we hypothesized that hypothermic reperfusion at 25 °C protects the heart against MIRI by activating TRPM8 to inhibit the oxidative stress-related RhoA/ROCK2 pathway. In this study, we tested this hypothesis using the isolated Langendorff-perfused rat heart.

2. Methods

2.1. Experimental animals

Male Sprague-Dawley rats weighing 200–250 g were provided by the China Three Gorges University Laboratory Animal Center, and housed in a temperature-controlled room (22–25 °C) at 60% humidity, under a 12-h light/12-h dark cycle with water and food freely available.

2.2. Chemicals

Krebs-Henseleit (K-H) solution (in mmol/L: 118 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 $C_6H_{12}O_6$), polyformaldehyde, xylene, and ethanol (100, 95, 90, 80, 75, and 70%) were from Sinopharm Chemical; triphenyltetrazolium chloride (TTC) from Sigma; the LDH, SOD, and MDA detection kits from Nanjing Jiancheng Bioengineering Institute; the hematoxylin-eosin and TUNEL staining kits from Beyotime; the TRIzol kit from Invitrogen (Carlsbad, CA); the PrimeScript RT reagent kit and SYBR Premix from Takara (Shiga, Japan); GelRed from Biotium (Hayward, CA, USA); the SDS-PAGE kit from Servicebio; TRPM8 antibody (1:500) from Supan Bio of Shanghai; RhoA (1:1000) and ROCK2 (1:1500) antibodies from Absin; Bcl-2 (1:500), Bax (1:1000), and cleaved caspase-3 (1:1000) antibodies from Wanleibio; GAPDH antibody (1:10000) from CST; and ECL solution from Applygen.

2.3. Preparation of Langendorff-perfused rat hearts

Each rat was anesthetized with pentobarbital sodium, (50 mg/kg, i.p.) and sacrificed by decapitation. The heart was isolated, mounted to the Langendorff apparatus, and then perfused retrogradely at constant pressure of 76 mmHg with K-H buffer gassed with 95% O_2/5% CO_2, equilibrated at pH 7.3–7.4, and maintained at 37 °C. A latex balloon introduced into the left ventricle *via* the left atrium and mitral valve was connected to a pressure transducer, resulting in a left ventricular end-diastolic pressure (LVEDP) between 8 and 12 mmHg. The left ventricular developed pressure (LVDP), LVEDP, and maximum rate of increase/decrease (± dp/dtmax) were recorded using PowerLab (ADInstruments Ltd., Australia). Each heart was allowed to stabilize for at least 20 min.

2.4. Perfusion protocol for isolated hearts

Each isolated heart received 30 min of global ischemia by stopping

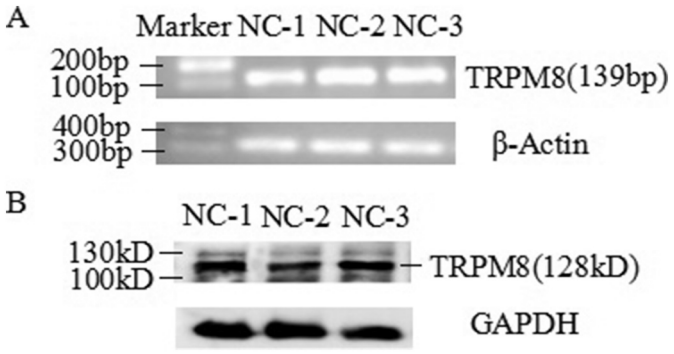

Fig. 1. TRPM8 expression in myocardial tissue. A, TRPM8 mRNA expression bands. B, TRPM8 protein expression bands. NC, normal control.

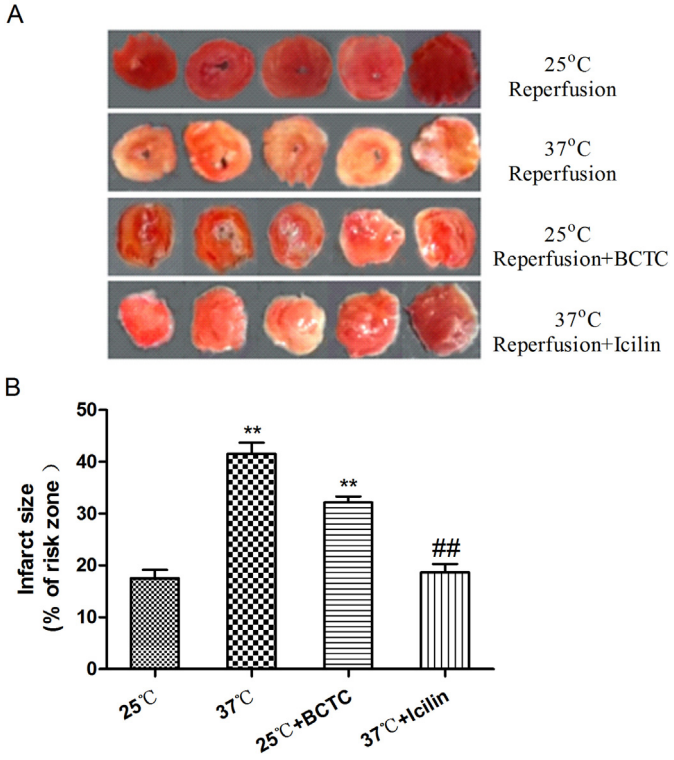

Fig. 2. Effect of hypothermic reperfusion on myocardial infarct size of isolated hearts. A, representative images of TTC-stained myocardial infarcts in each group. B, myocardial infarct size in each group. Data are expressed as mean ± S.E.M. (n = 6; $^{**}P < 0.01$ *versus* 25 °C group, $^{\#\#}P < 0.01$ *vs* 37 °C group; BCTC, TRPM8 antagonist; Icilin, TRPM8 agonist).

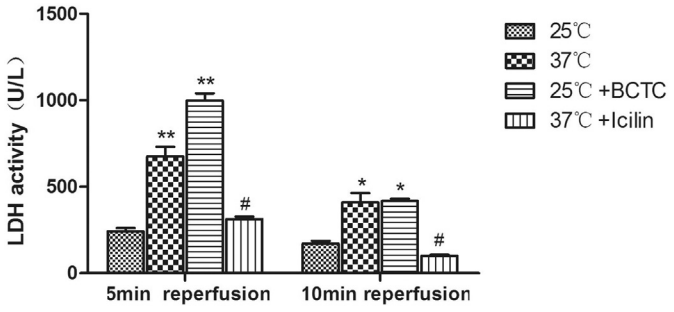

Fig. 3. LDH release in coronary effluent from isolated hearts. Data are expressed as mean ± S.E.M. (n = 6; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* 25 °C group, $^{\#}P < 0.05$ *vs* 37 °C group; BCTC, TRPM8 antagonist; Icilin, TRPM8 agonist).

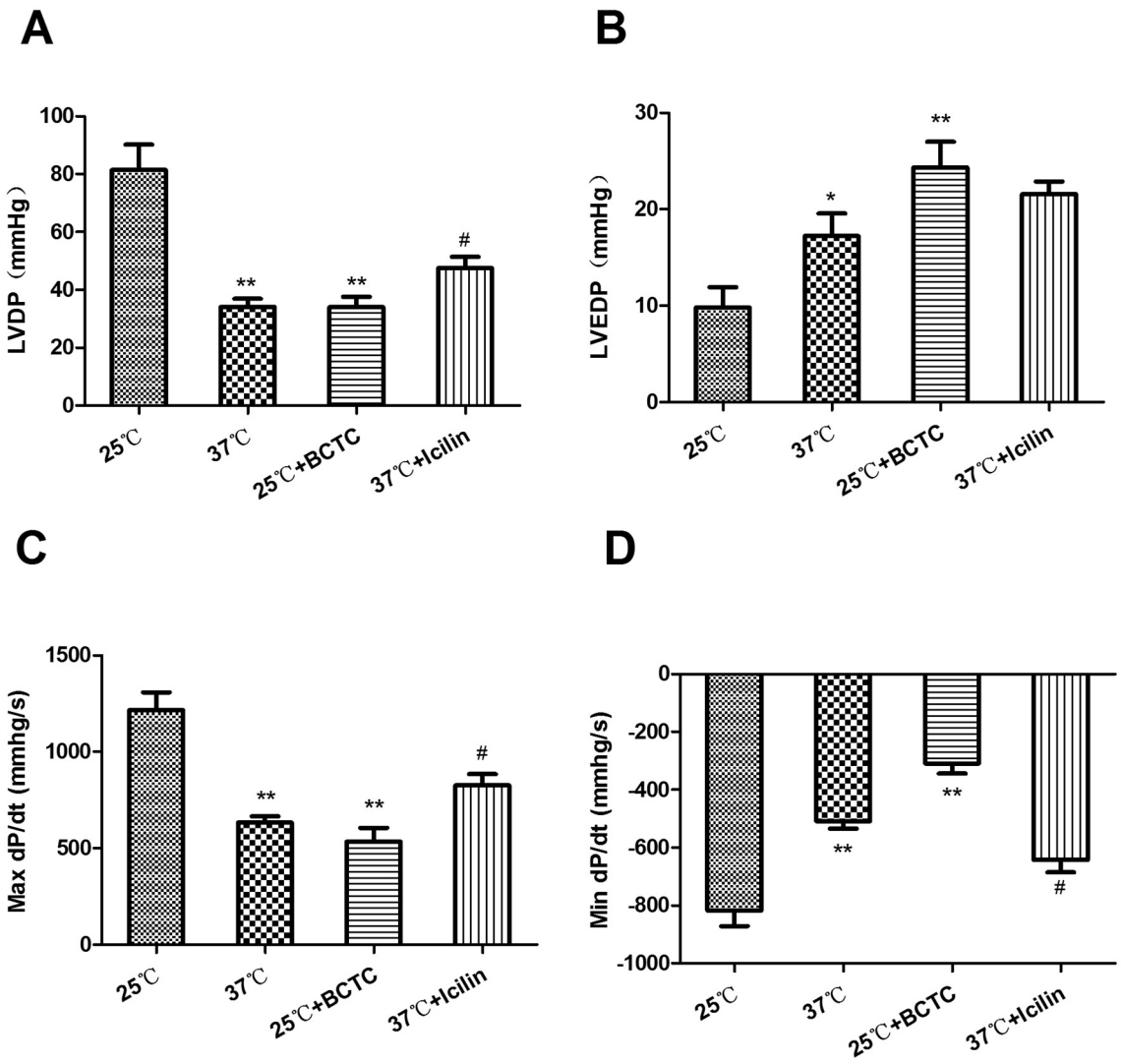

Fig. 4. Hemodynamic changes. A, LVDP. B, LVEDP. C, +dp/dtmax. D, −dp/dtmax. Data are expressed as mean ± S.E.M. (n = 6; *P < 0.05, **P < 0.01 *vs* 25 °C, #P < 0.05 *vs* 37 °C; BCTC, TRPM8 antagonist; Icilin, TRPM8 agonist).

the perfusion of K-H buffer, and this was followed by 120 min of K-H buffer reperfusion. Hypothermic reperfusion was achieved by switching the K-H buffer from 37 °C to 25 °C. In order to assess the effects of TRPM8 blockade on 25 °C reperfusion, during the first 15 min of reperfusion at 25 °C, BCTC (a specific TRPM8 antagonist) was administered at 8 μmol/L, which is known to block TRPM8 channels [36,37]. To assess the effects of TRPM8 activation on the MIRI induced by 37 °C reperfusion, during the first 15 min of reperfusion at 37 °C, Icilin (a TRPM8 agonist) was administered at 2 μmol/L, a concentration known to activate TRPM8 channels [22,38,39].

2.5. Infarct size measurement

At the end of 120 min reperfusion, each heart was frozen at −20 °C for 3 h. The frozen heart was cut into 2-mm transverse slices, which were incubated in 0.4% w/v TTC phosphate buffer (pH 7.4 and 37 °C) for 3–5 min for staining, then fixed in 4% paraformaldehyde for 8–10 min. The risk zone stained red, and the infarcted area was pale. Infarct and risk areas were measured using planimetry with Image/J (National Institutes of Health, Bethesda, MD). Infarct size was expressed as a percentage of the risk zone.

2.6. TUNEL staining

After 2 h reperfusion, each heart was fixed in 4% paraformaldehyde. Myocardial sections were dehydrated in xylene and an ascending ethanol series, then cleared in xylene and embedded in paraffin. Paraffin sections were cut at 4 μm, dewaxed with xylene, and stained for TUNEL analysis. DNase-free proteinase K (20 μg/mL) was added to the sections, reacted at 20–37 °C for 15–30 min (optimal temperature and time adjusted according to the tissue), and washed 3 times for 5 min each in phosphate-buffered saline (PBS) at 37 °C. The sections were blocked for 20 min with 3% H_2O_2 in PBS at 25 °C and washed 3 times for 5 min each in PBS at 37 °C. TUNEL reaction mixture (50 μL) was added to the sections, which were incubated for 1 h at 25 °C in a dark, humidified environment. Then the sections were washed 3 times in PBS for 5 min each at 37 °C. Streptavidin-HRP reaction mixture (50 μL) was added to the sections and incubated for 30 min in a humidified environment at 25 °C, then the sections were washed 3 times for 5 min each in PBS at 37 °C. DAB reaction mixture was added to the sections and incubated for 10 min at 25 °C. The sections were then washed 3 times for 5 min each in PBS 37 °C, stained with hematoxylin for 3 min at 37 °C, and finally cleared with xylene and sealed with neutral balata gum. The nuclei of apoptotic cells stained brownish yellow and normal nuclei stained blue; cells were counted using Image-

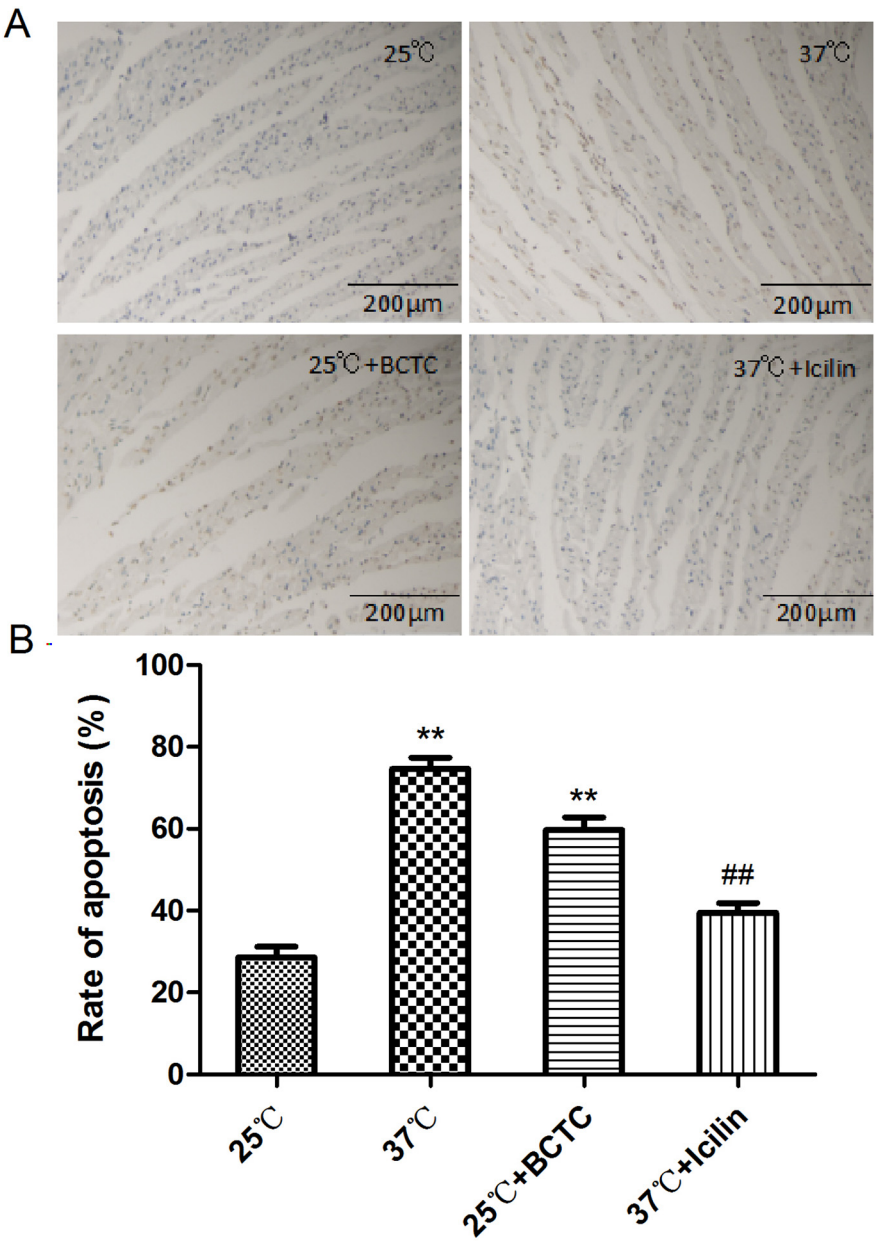

Fig. 5. Apoptosis in myocardium. A, representative TUNEL staining of myocardial tissue. B, apoptotic rate expressed as the ratio of TUNEL-positive (apoptotic) cells to the total number of cardiomyocytes. Data are expressed as mean ± S.E.M. (n = 6; $^{**}P < 0.01$ *vs* 25 °C group, $^{\#\#}P < 0.01$ *vs* 37 °C group; BCTC, TRPM8 antagonist; Icilin, TRPM8 agonist).

ProPlus 5.0. The ratio of the number of brownish-yellow cells to the total number of cells was calculated, giving the apoptotic index. TUNEL-stained images were captured with an Olympus microscope at 200× magnification.

2.7. Hemodynamic monitoring

LVDP, LVEDP, and ± dp/dtmax were recorded using the Labchart biological signal processing system.

2.8. Lactate dehydrogenase measurement

The coronary effluent from each Langendorff-perfused heart was collected at 5 and 10 min of reperfusion. The LDH activity in the effluent was determined spectrophotometrically using a kit (from Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions and expressed as units per liter.

2.9. Measurement of superoxide dismutase and malondialdehyde

After 2 h of reperfusion, the myocardial tissue was stored at −80 °C for measurements of SOD activity and MDA content using kits(from Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

2.10. Western blot analysis

Total proteins were assessed using the BCA protein assay. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene difluoride membranes after blocking with 5% non-fat milk for 1.5 h and washing with TBST for 5 min, and then incubated with primary antibodies against Bcl-2, Bax, cleaved caspase-3, RhoA, ROCK2, and GAPDH at 4 °C overnight. Proteins were detected with horseradish peroxidase-conjugated secondary antibody and visualized by electrochemiluminescence. The

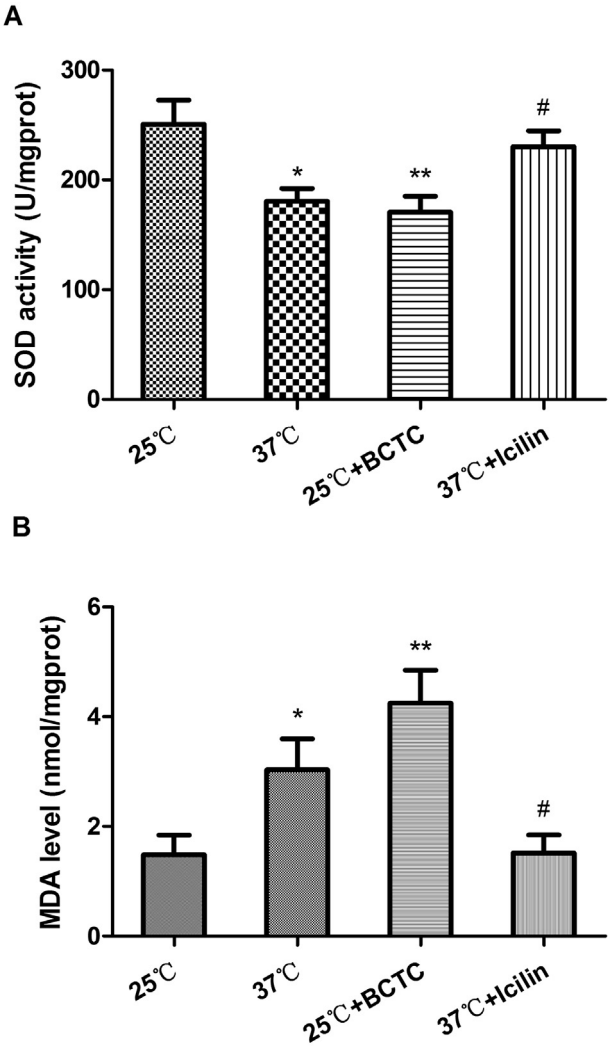

Fig. 6. A, B, effects of hypothermic reperfusion on changes in SOD (A) and MDA (B) in myocardium from each group of rats. Data are expressed as mean ± S.E.M. (n = 6; *P < 0.05, **P < 0.01 *vs* 25 °C, #P < 0.05 *vs* 37 °C; BCTC, TRPM8 antagonist; Icilin, TRPM8 agonist).

band densities were normalized to GAPDH.

2.11. Reverse transcription and real-time PCR

Total RNA in myocardial tissue was extracted using a TRIzol kit (from Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription was performed using a PrimeScript RT reagent kit (from Takara, Shiga, Japan) according to the manufacturer's instructions. The following primers were used for real-time PCR: β-actin, forward: 5′-GAG ACC TTC AAC ACC CCA GCC-3′ and reverse: 5′-GGC CAT CTC TTG CTC GAA GTC-3′; TRPM8, forward: 5′-GCA GTG GTA CAT GAA CGG AGT-3′ and reverse: 5′-TGA AGA GTG AAG CCG GAA TAC-3′. Reactions were carried out on a real-time PCR thermocycler, using SYBR Premix as the fluorescent dye. Real-time PCR products were analyzed by gel electrophoresis on 2% agarose containing GelRed nucleic acid stain and visualized under a UV illuminator.

2.12. Statistical analysis

Data are expressed as mean ± S.E.M. Statistical comparisons were performed by one-way analysis of variance and the Newman–Keuls test. Differences of $P < 0.05$ were regarded as significant.

3. Results

3.1. Expression of TRPM8 in rat myocardium

The mRNA expression of TRPM8 was detected using real-time PCR (Fig. 1A), and TRPM8 protein expression was assessed using western blot analysis of isolated left-ventricular cardiomyocyte homogenates (Fig. 1B).

3.2. Effect of hypothermic reperfusion on infarct size

Hypothermic reperfusion at 25 °C reduced the infarct size in the isolated perfused rat heart compared with reperfusion at 37 °C, and this effect was abolished by BCTC (8 μmol/L), an antagonist of TRPM8. Administration of the TRPM8 agonist Icilin (2 μmol/L) reduced the infarct size induced by reperfusion at 37 °C (Fig. 2).

3.3. Effect of hypothermic reperfusion on LDH release

After 30 min of global ischemia, the LDH level peaked at 5 min of reperfusion and then began to decrease. Here we selected 2 time points (5 and 10 min) during reperfusion to assess LDH release. The results showed that the LDH release was lower with 25 °C reperfusion than with 37 °C reperfusion, and this was abolished by BCTC. Administration of Icilin reduced the LDH release induced by reperfusion at 37 °C (Fig. 3).

3.4. Effect of hypothermic reperfusion on hemodynamic parameters

LVDP and ± dp/dtmax were significantly higher in hearts with reperfusion at 25 °C than at 37 °C, while LVEDP was markedly lower. However, these effects were reversed by BCTC, whereas Icilin improved the LVDP and ± dp/dtmax (Fig. 4).

3.5. Effect of hypothermic reperfusion on myocardial apoptosis

Myocardium receiving 25 °C reperfusion showed less cardiomyocyte apoptosis than at 37 °C reperfusion, and this effect was reduced by BCTC, whereas administration of Icilin reduced the apoptosis induced by reperfusion at 37 °C (Fig. 5).

3.6. Effect of hypothermic reperfusion on SOD activity and MDA levels in myocardial tissue

SOD activity in myocardium with 25 °C reperfusion was higher, but the MDA level was lower, than that with 37 °C reperfusion, and these effects were reduced by BCTC. The decrease in SOD activity and the increase in MDA in myocardium re-perfused at 37 °C were reversed by Icilin (Fig. 6).

3.7. Effect of hypothermic reperfusion on the expression of Bax, Bcl-2, cleaved caspase-3, and RhoA/ROCK2

In myocardium with 25 °C reperfusion, the expression of Bax, cleaved caspase-3 and RhoA/ROCK2 was lower, while the Bcl-2 expression was markedly higher than that with 37 °C reperfusion, and these effects were abolished by BCTC. Administration of Icilin reversed the effects induced by 37 °C reperfusion (Fig. 7).

4. Discussion

Our results indicate that: (1) hypothermic reperfusion at 25 °C reduces myocardial ischemia-reperfusion injury by activating TRPM8; and (2) activation of TRPM8 attenuates oxidative stress by inhibiting the RhoA/ROCK2 pathway.

Using western blot and real time PCR, we demonstrated the

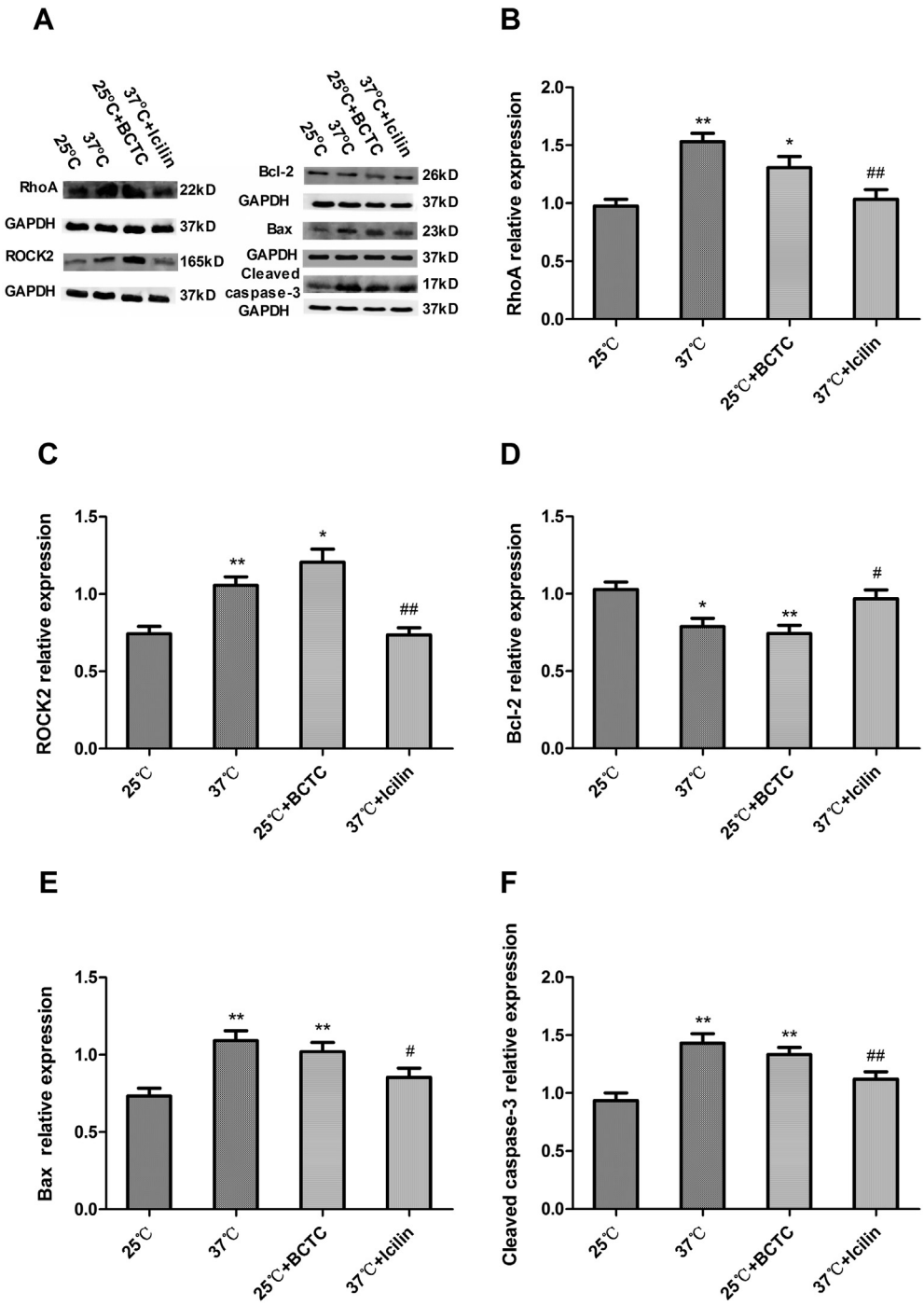

Fig. 7. Expression changes of Bax, Bcl-2, cleaved caspase-3, and RhoA/ROCK2. A, representative protein expression bands in myocardium from each group. B–F, relative expression of RhoA (B), ROCK2 (C), Bcl-2 (D), Bax (E), and cleaved caspase-3 (F). Data are expressed as mean ± S.E.M. (n = 6; *P < 0.05, **P < 0.01 *vs* 25 °C, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 *vs* 37 °C; BCTC, TRPM8 antagonist; Icilin, TRPM8 agonist).

expression of TRPM8 in the myocardium of the Sprague-Dawley rat. Since Demir et al. [40] reported that TRPM8 mRNA is not expressed in the cardiac tissue of Wistar rats, we suggest that TRPM8 expression may differ in different strains of rat.

We found that hypothermic reperfusion at 25 °C reduced MIRI by reducing myocardial infarct size, LDH, and cardiomyocyte apoptosis, which resulted in an improvement of cardiac function, showing that hypothermic reperfusion has cardioprotective effects. In order to determine whether TRPM8 participates in these effects, we blocked TRPM8 with its specific antagonist BCTC, and the results showed that the cardioprotective effects were significantly attenuated, indicating that TRPM8 is involved. More importantly, the TRPM8 agonist Icilin reduced the MIRI induced by reperfusion at 37 °C, further confirming that TRPM8 activation is involved in the cardioprotection provided by hypothermic reperfusion and suggesting that TRPM8 activation may be an effective potential therapy to reduce MIRI.

Oxidative stress plays an important role in MIRI, which produces large amounts of active oxygen and oxygen free radicals [27,41]. MDA is one of the most important products of membrane lipid peroxidation, and SOD is an important endogenous oxygen free radical scavenger. Their levels indirectly reflect resistance to oxygen free radical damage [42], so the commonly-used indicators for evaluating cardiac oxidative

stress include SOD and MDA [43]. When MIRI occurs in the heart, its MDA content increases and the activity of SOD decreases [30,44], reflecting the degree of myocardial tissue damage. Our results showed that hypothermic reperfusion increased the SOD activity and decreased the MDA content in coronary effluent, whereas inhibition of TRPM8 with its antagonist BCTC abolished these effects, suggesting that activation of TRPM8 can reduce oxidative stress, consistent with published reports [26,45]. Our further studies showed that activation of TRPM8 reversed the SOD reduction and MDA elevation induced by reperfusion at 37 °C, further indicating that activation of TRPM8 mediates the inhibition of oxidative stress.

The RhoA/ROCK2 signaling pathway plays an important role in many cellular functions, including contraction, movement, proliferation, and apoptosis, and its excessive activity induces oxidative stress [33,46–48]. In addition, it has been reported to be activated in MIRI [49,50]. To investigate the mechanism underlying the inhibition of oxidative stress by activation of TRPM8, we further investigated the contribution of the RhoA/ROCK2 signaling pathway in this process. Our results showed that reperfusion at 25 °C reduced RhoA/ROCK2 expression in the myocardium compared to reperfusion at 37 °C, and this was reversed by inhibition of TRPM8, suggesting that activation of TRPM8 inhibits the RhoA / ROCK2 signaling pathway.

Apoptosis is thought to play an important role in MIRI [51,52], and the RhoA/ROCK2 signaling pathway is widely involved in oxidative stress-induced cardiomyocyte apoptosis [53,54]. Oxidative stress-related activation of the RhoA/ROCK2 signaling pathway ultimately leads to increased apoptosis in cardiomyocytes [55]. Apoptosis is mediated by cleaved caspase 3; in addition, the Bax family promotes apoptosis, and Bcl-2 plays a key role in the anti-apoptotic process [56]. The balance between Bax and Bcl-2 determines the inhibition or activation of apoptosis [57]. In our study, reperfusion at 37 °C was associated with high expression of cleaved caspase 3 and Bax, but low expression of Bcl-2, suggesting greater apoptosis than at 25 °C, whereas these effects were attenuated by treatment with the TRPM8 agonist Icilin. In addition, the TRPM8 antagonist BCTC reversed the expression of Bax, cleaved caspase 3, and Bcl-2 induced by reperfusion at 25 °C, and increased the number of TUNEL-positive cardiomyocytes, suggesting that activation of TRPM8 during hypothermic reperfusion attenuates MIRI by inhibiting apoptosis.

In addition to TRPM8, TRPA1 and TRPC5 are also sensitive to cold stimuli. In the present study, we did not investigate their potential roles in cardioprotection by hypothermic reperfusion. Since it is unclear whether these two cold-sensitive channels are involved in this process at their activation temperatures, they will be investigated in subsequent studies.

5. Conclusions

In summary, hypothermic reperfusion at 25 °C protects the myocardium against MIRI *via* activating TRPM8, and inhibition of the oxidative stress-related RhoA/ROCK2 signaling pathway mediated by TRPM8 is involved in this process.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Ethics approval

All procedures were approved by the Ethics Committee for the Use of Experimental Animals in China Three Gorges University, and complied with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA.

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