Int J Clin Exp Pathol 2015;8(9):10239-10249
www.ijcep.com /ISSN:1936-2625/IJCEP0013426

Original Article

Cardiac sodium/calcium exchanger preconditioning promotes anti-arrhythmic and cardioprotective effects through mitochondrial calcium-activated potassium channel

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Received July 28, 2015; Accepted August 28, 2015; Epub September 1, 2015; Published September 15, 2015

Abstract: Background: Reverse-mode of the Na^+/Ca^{2+} exchanger (NCX) stimulation provides cardioprotective effects for the ischemic/reperfused heart during ischemic preconditioning (IP). This study was designed to test the hypothesis that pretreatment with an inhibitor of cardiac delayed-rectifying K^+ channel (I_{Kr}), E4031, increases reverse-mode of NCX activity, and triggers preconditioning against infarct size (IS) and arrhythmias caused by ischemia/reperfusion injury through mitoK_{Ca} channels. Materials and methods: In the isolated perfused rat heart, myocardial ischemia/reperfusion injury was created by occlusion of the left anterior descending coronary artery for 30 min followed by 120 min reperfusion. Two cycles of coronary occlusion for 5 min and reperfusion were performed, or pretreatment with E4031 or sevoflurane (Sevo) before the 30 min occlusion with the reversed-mode of NCX inhibitor (KB-R7943) or not. Results: E4031 or Sevo preconditioning not only markedly decreased IS but also reduced arrhythmias, which was significantly blunted by KB-R7943. Furthermore, these effects of E4031 preconditioning on IS and arrhythmias were abolished by inhibition of the mitoK_{Ca} channels. Similarly, pretreatment with NS1619, an opener of the mitoK_{Ca} channels, for 10 min before occlusion reduced both the infarct size and arrhythmias caused by ischemia/reperfusion. However, these effects weren't affected by blockade of the NCX with KB-R7943. Conclusion: Taken together, these preliminary results conclude that pretreatment with E4031 reduces infarct size and produces anti-arrhythmic effect via stimulating the reverse-mode NCX, and that the mitoK_{Ca} channels mediate the protective effects.

Keywords: Potassium channels, calcium, ischemic preconditioning, myocardial ischemia/reperfusion injury

Introduction

Ischemic preconditioning (IP) is a phenomenon in which brief exposures of myocardium to ischemia render it more resistant to a subsequent and more severe insult, termed index ischemia [1]. IP protects the heart against infarction and incidence of arrhythmias caused by ischemia and reperfusion [2, 3]. IP also preserves contractile function during ischemia and reperfusion [2, 3]. It is now known that a transient increase in cytosolic Ca^{2+} during preconditioning contributes to all these protective effects [4-6]. Since the L-type Ca^{2+} channel, sarcoplasmic reticulum and NCX are important sites of Ca^{2+} handling in the heart, they may also be involved in the cardioprotection of preconditioning. It has been demonstrated that the reverse-mode NCX is activated during index ischemia, contributing to intracellular Ca^{2+} overload and thus causing cardiac injury [7-9]. On the other hand, activation of the NCX in the reverse-mode may also be responsible for a transient increase in intracellular Ca^{2+} during ischemic preconditioning. It has also been demonstrated that administration of 5-(N,N-dimethyl)-amiloride, an inhibitor of calcium influx via the Na^+/H^+ exchanger and NCX, blocks the cardioprotection of ischemic preconditioning [5] and activa-

tion of the reverse-mode NCX with sevoflurane, a volatile anesthetic agent, triggers the protective effects of IP on myocardial contractile recovery [10, 11]. The observations provided some evidence that prior stimulation of the reverse-mode NCX confers cardioprotection against ischemic insults.

It is now well known that the exchange rate or activity of NCX is regulated by a variety of intrinsic factors and pharmacological chemicals, while the driving direction for NCX only depends on the transmembrane calcium and sodium concentration gradient and membrane potential [12]. Previous studies showed that E4031, an inhibitor for the rapidly activating delayed-rectifying K^+ channel (I_{Kr}) function in the heart [13], increased myocardium calcium uptake [14]. It has been further demonstrated that E4031 increased intracellular Ca^{2+} by enhancing reverse-mode NCX activity [10, 15, 16]. As I_{Kr} makes little or no contribution to repolarization in rat ventricular myocardium [17]. Therefore, we hypothesized that pretreatment with E4031 increased reverse-mode NCX activity, and triggered preconditioning against infarct size and arrhythmias caused by ischemia and reperfusion.

Mitochondria play a central role in cell life and cell death, and their damage is implicated in both necrosis and apoptosis during myocardial ischemia and reperfusion [18]. Recently, studies reported that the large-conductance calcium-activated potassium channel (mitoK_{Ca}) is present in myocardial mitochondria, and that the opening of this channel produces cardioprotection against ischemia/reperfusion injury [19, 20]. Subsequently, mitoK_{Ca} channel opening during preconditioning and index ischemia and reperfusion was shown to reduce infarct size [21, 22], and improve myocardial contractile function [23]. Based on these findings, we hypothesized that mitoK_{Ca} channel may play a role in the cardioprotective effects of prior stimulation of the reverse-mode NCX.

The purpose of this study was, firstly, to determine the effects of pretreatment with E4031 on cardioprotection and anti-arrhythmias, and then, to delineate the underlying signaling mechanism. At first, we stimulated with E4031 and detected the reverse-mode NCX activity by measuring the Fura-2 fluorescence signal, an indicator of NCX activity, in isolated ventricular myocytes. Secondly, we determined the effects of IP and stimulation of reverse-mode NCX with E4031 in the absence and presence of its antagonist, KB-R7943 on infarct size and arrhythmias induced by ischemia in the isolated perfused rat heart. We also preconditioned the heart with sevoflurane, and compared its effect with E4031. Thirdly, we determined whether the cardioprotection resulting from E4031 pretreatment was reduced or eliminated by blockade of the mitoK_{Ca} channel with its inhibitor, paxilline, during preconditioning or during subsequently lethal ischemia and reperfusion (index ischemia and reperfusion). Finally, we determined whether the beneficial effects of activation of the mitoK_{Ca} channel were abolished by blockade of the reverse-mode NCX during preconditioning. These results suggested that prior stimulation of the reverse-mode NCX and opening mitoK_{Ca} channel mediated the cardioprotective and anti-arrhythmic effects via E4031 pretreatment and that mitoK_{Ca} channel was located downstream of reverse-mode NCX.

Materials and methods

All procedures involving animals were approved by the Fourth Military Medical University Laboratory Animal Resources Committee.

Isolation of ventricular myocytes

Ventricular myocytes were isolated from male Sprague-Dawley rats (200-250 g), by a collagenase perfusion method described previously [24]. Briefly, the hearts were rapidly removed after decapitation and retrogradely perfused at 37°C for 5 min with oxygenated Tyrode solution containing (in mM): 140 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 1.25 $CaCl_2$, 1.2 KH_2PO_4, 10 glucose, and 10 HEPES (adjusted to pH 7.4 with NaOH), followed by 5 min with Ca^{2+}-free Tyrode solution. Collagenase was then added to the same solution to a concentration of 0.5 mg/mL with 0.1% (wt/vol) bovine serum albumin. After 25-30 min of perfusion with this solution, the atria were discarded. The ventricular tissue was cut into small pieces in high K^+ solution (KB) containing (in mM): 10 KCl, 10 KH_2PO_4, 120 K-glutamate, 10 taurine, 1.0 $MgSO_4$, 10 HEPES, 20 glucose, and 0.5 EGTA (adjusted to pH 7.2 with KOH). After gentle stirring with a glass rod for 5 min, the residue was filtered through a 250 μm mesh screen. Myocytes were stored in KB solution for 1 h, and then re-suspended in normal Tyrode solution with 0.2% bovine serum albumin.

Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in ventricular myocytes

Myocytes were placed in a 0.3 mL microperfusion chamber mounted on an inverted microscope, and perfused at 1.5 mL/min. $[Ca^{2+}]_i$ was measured by the fluorescent calcium indicator Fura-2 in a dual-fluorescence, calcium ion-sensing system (IonOptix, Milton, MA) [24, 25]. Cells were loaded with Fura-2/AM as described previously [25]. Myocytes were incubated with 4 μM Fura-2/AM for 30 min in Tyrode solution containing 1.25 mM $CaCl_2$ and 1% bovine serum albumin. Fluorescent signals obtained at 340 nm (F_{340}) and 380 nm (F_{380}) excitation wavelengths were recorded and stored in a computer for data processing and analysis. As there is significant compartmentalization of fluorescent Ca^{2+} indicators into organelles, and the degree of the compartmentalization varies from cell to cell and prevents the use of a standard calibration curve [26, 27], The F_{340}/F_{380} ratio was taken as an index of $[Ca^{2+}]_i$ changes in the ventricular myocyte.

Na^+ withdrawal in isolated ventricular myocytes

We adopted the procedures as described previously to assess reverse-mode NCX activity [28, 29]. Briefly, cells were pretreated for 5 min with 10 μM ryanodine and 1 μM thapsigargin, known to block the sarcoplasmic reticulum function [29, 30]. They were then exposed for a period of 3 min with Na^+-free solution containing (in mM): 125 N-methyl-D-glucamine (NMDG), 2.6 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.25 $CaCl_2$ and 10 HEPES (adjusted to pH 7.4 with HCl) (Schafer et al., 2001), followed by reperfusion with normal Tyrode solution for 5 min. The change of $[Ca^{2+}]_i$ in myocytes upon exposed to Na^+-free solution was measured and used to represent the reverse-mode NCX activity.

Langendorff perfused isolated rat heart preparation

We adopted the method routinely used in our lab as described previously [31, 32]. The heart was removed immediately after the rat was killed, mounted to the Langendorff apparatus and perfused retrogradely under a constant pressure of 100 cm H_2O with a Krebs-Ringer solution containing (in mM) 115 NaCl, 5 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 1.5 $CaCl_2$, 25 $NaHCO_3$, and 11 glucose. The solution was aerated with 95% O_2-5% CO_2 at pH 7.4. The temperature of the perfusion solution was maintained at 36°C. Total coronary arterial flow (CF) was measured by timed collection of the venous effluent in a graduated cylinder. A 2-0 silk suture on a tapered needle was passed around the left main coronary artery close to its origin, and the ends were passed through a vinyl tube to form a snare. The coronary artery was occluded by pulling the snare, which produced myocardial ischemia. Ischemia was confirmed by regional cyanosis and a substantial fall in CF. Reperfusion occurred following release of the snare. Each heart was allowed to stabilize for the first 15 min of perfusion. Any heart with a CF >15 mL/min or exhibiting arrhythmias during this period was discarded.

Experimental protocol

After an initial stabilization period of 15 min, the heart was subjected to 30 min regional ischemia and 120 min reperfusion. **Figure 3A** shows the experimental protocols for studying the role of reverse-mode NCX in the cardioprotection of IP. Hearts were preconditioned by two cycles of 5 min regional ischemia followed by 5 min reperfusion (IP), and KB-R7943, a selective inhibitor of the reverse-mode NCX [33-35], was perfused for a period of 10 min before the first ischemic episode to 5 min after the second ischemic episode. Hearts were also pretreated with E4031 and sevoflurane respectively, both are the reverse-mode NCX activator. For E4031 pretreatment, hearts received two cycles of 5 min drug treatment, while for sevoflurane treatment, hearts received 15 minutes infusion with normal Krebs-Ringer solution saturated with 3.8 vol% sevoflurane for 30 minutes [11]. To study the relationship between mitoK$_{Ca}$ channel and reverse-mode NCX in IP, paxilline, a selective blocker of the mitoK$_{Ca}$ channel [36], was either infused for the period of 10 min before the first episode of treatment with E4031 to 5 min after the second episode of treatment with E4031, or given from 5 min before 30 min of ischemia until 10 min after reperfusion (**Figure 4A**), and NS1619, a selective activator of the mitoK$_{Ca}$ channel [36], was administered 20 min before ischemia for 10 min, KB-R7943 was administered for 30 min before ischemia (**Figure 5A**).

Measurement of ischemic (risk) zone and infarct size

At the end of each experiment, 0.25% Evans blue was infused into the heart, which was then

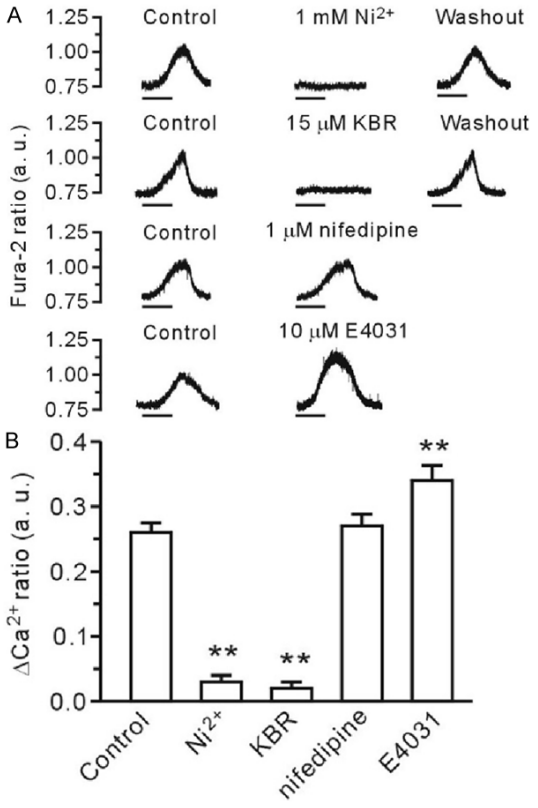

Figure 1. Effects of Ni^{2+}, KB-R7943, nifedipine, or E4031 on Fura-2 ratio response during Na^+-free exposure in ventricular myocytes. A. Representative tracing of the changes of intracellular Ca^{2+}. Cells were subjected to a Na^+-free medium, which was achieved by perfusion with NMDG solution for 3 min followed by reperfusion with normal Tyrode solution. The next Na^+-free medium exposure was separated by 15 min recovery period with normal Tyrode solution. Myocytes were pretreated for 5 min with 10 μM ryanodine and 1 μM thapsigargin to block sarcoplasmic reticulum function. 1 μM Ni^{2+}, 15 μM KB-R7943 (KBR), or 1 μM nifedipine, was administered 5 min before and during perfusion with the NMDG solution. For 10 μM E4031, it was administered 10 min before. "-" represents 3 min Na^+-free medium exposure. B. Group results on the increase of intracellular Ca^{2+}. ΔCa^{2+} ratio means the increase of Fura-2 fluorescence ratio (peak-baseline). Data are expressed as mean ± S.E.M. N = 4 hearts in each group. The Fura-2 fluorescence ratio (340/380 nm) signals in two or three cells from each rat heart were measured, and the mean value was used as a single entity. $^{**}P<0.01$ *vs.* Control.

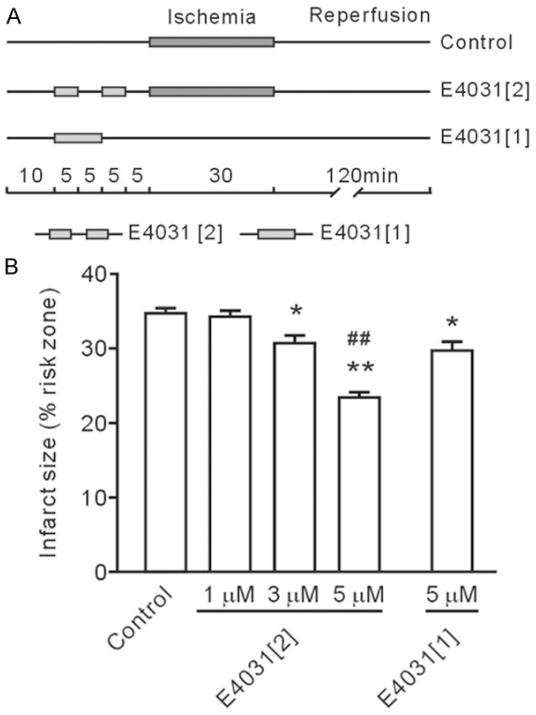

Figure 2. Effect of pretreatment with E4031 on infarct size caused by ischemia and reperfusion in isolated perfused rat hearts. A. Experimental protocol. E4031 was infused at different concentrations for two cycles of 5 min (E4031[2]) or at 5 μM for one cycle of 10 min (E4031[1]). B. Infarct size. Data are expressed as mean ± S.E.M. N = 6 hearts in each group. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* Control, $^{\#\#}P<0.01$ *vs.* 3 μM and E4031 [1].

frozen and cut into 2 mm slices. After removal of the right ventricle and connective tissue, the slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) in pH 7.4 buffer for 15 min at 37°C. The slices were immersed in 10% formalin overnight. The areas of infarct (TTC negative) and risk zone (TTC positive) were determined by computerized planimetry (Sigma-Scan program 4). The infarct size and risk zone were calculated by multiplying each area by slice thickness and summing the products. Infarct size was expressed as a percentage of the area at risk.

Evaluation of arrhythmias

Fine platinum electrodes were placed on the right atrium and the apex of the left ventricle to record an epicardial electrogram. To quantify arrhythmias occurring within 30-min ischemia, a scoring system developed by Curtis and Walker [37] and modified [38] was used. In view of the fact that arrhythmias induced by ischemia were mainly premature ventricular contractions (PVC) and ventricular tachycardia (VT), the emphasis for scoring was placed on ventricular arrhythmias. Therefore, the scoring system adopted was as follows: 0, no arrhythmias; 1, 1 to 30 premature ventricular contractions; 2, >30 premature ventricular contractions; 3, < three episodes of ventricular fibrillation (VF)/VT; 4, three to five episodes of VF/VT;

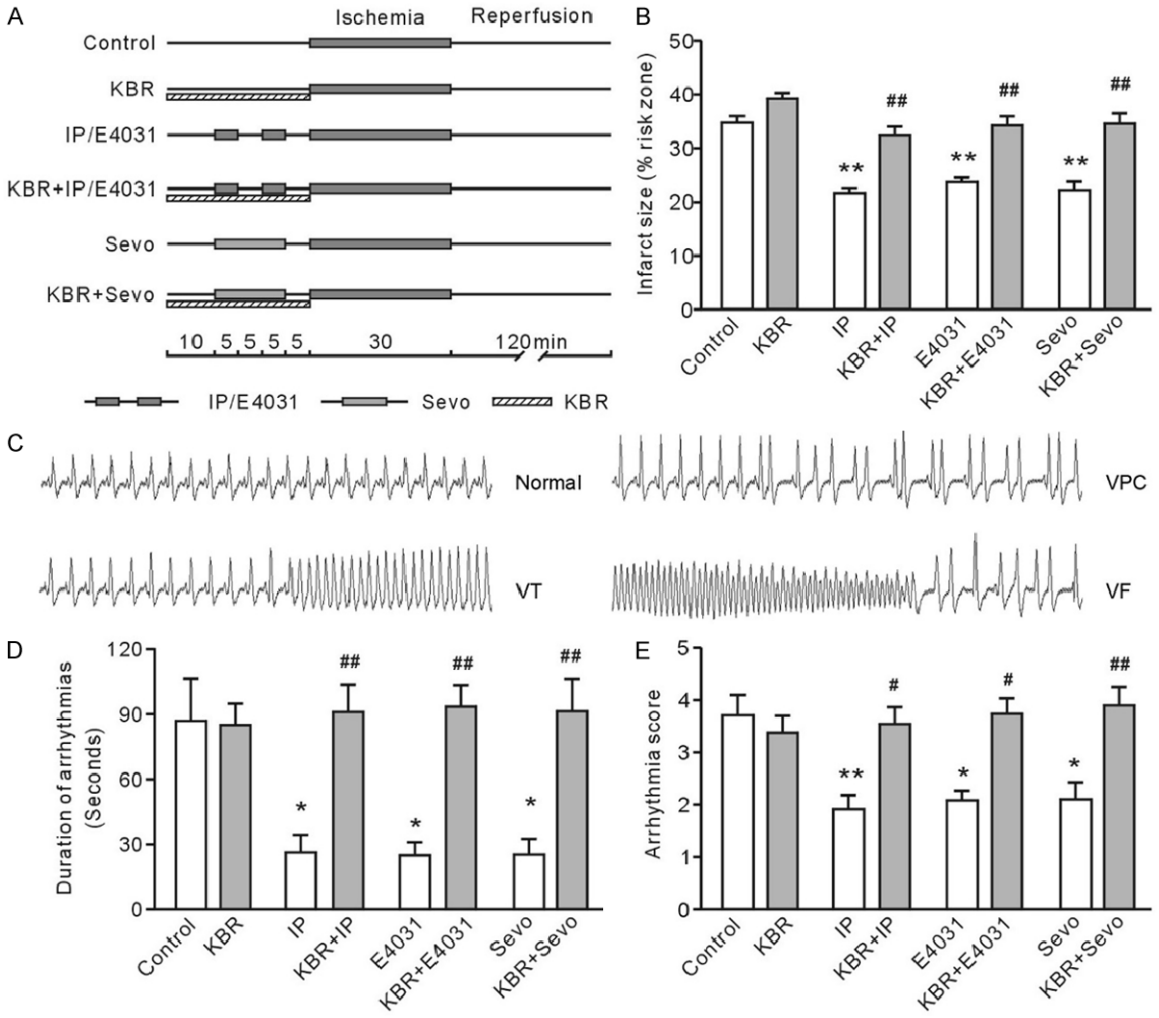

Figure 3. Effects of IP, E4031 or sevoflurane pretreatment with or without KB-R7943 on infarct size and arrhythmias caused by ischemia and reperfusion in isolated perfused rat hearts. A. Experimental protocol. B. Group results on infarct size. C. Representative ECG trace including normal, PVC, VT and VF. D and E. Results on the durations of arrhythmias and arrhythmia scores. Data are expressed as mean ± S.E.M. N = 6 hearts in each group. **P*<0.05, ***P*<0.01 *vs.* Control; #*P*<0.05, ##*P*<0.01 *vs.* corresponding group without KB-R7943; E4031, E4031 pretreatment; Sevo, sevoflurane pretreatment; KBR, KB-R7943.

and 5, > five episodes of VF/VT. VT was defined as a successive run of at least four PVCs with uniform QRS complexes, and VF was a single event for which individual QRS deflections could no longer be distinguished from one another and a rate could not be measured. For irreversible VF, tapping by fingers was used to revert to sinus rhythm. Any hearts with failure to revert to sinus rhythm within 2 min were excluded from this study.

Drugs and chemicals

Ryanodine, thapsigargin paxilline, 2-[2-[4-(4-Nitrobenzyloxy) phenyl]ethyl] isothiourea mesylate (KB-R7943), and N-[4-[[1-[2-(6-Methyl-2-pyridinyl)ethyl]-4-piperidinyl]carbonyl]phenyl] methanesulfonamide dihydrochloride (E4031) were purchased from Tocris. Other chemicals were from Sigma Company. All chemicals were dissolved in distilled water except Fura-2/AM, KB-R7943, and paxilline, which were dissolved in DMSO. The final concentration of DMSO was < 0.1%, at which no effect was observed.

Concentrations of nifedipine and $NiCl_2$ [28] were based on previous studies. KB-R7943 (5 μM), the selective inhibitor of the reverse-mode NCX, was used to inhibit NCX stimulation in isolated perfused heart [35, 39, 40]. NS1619 at

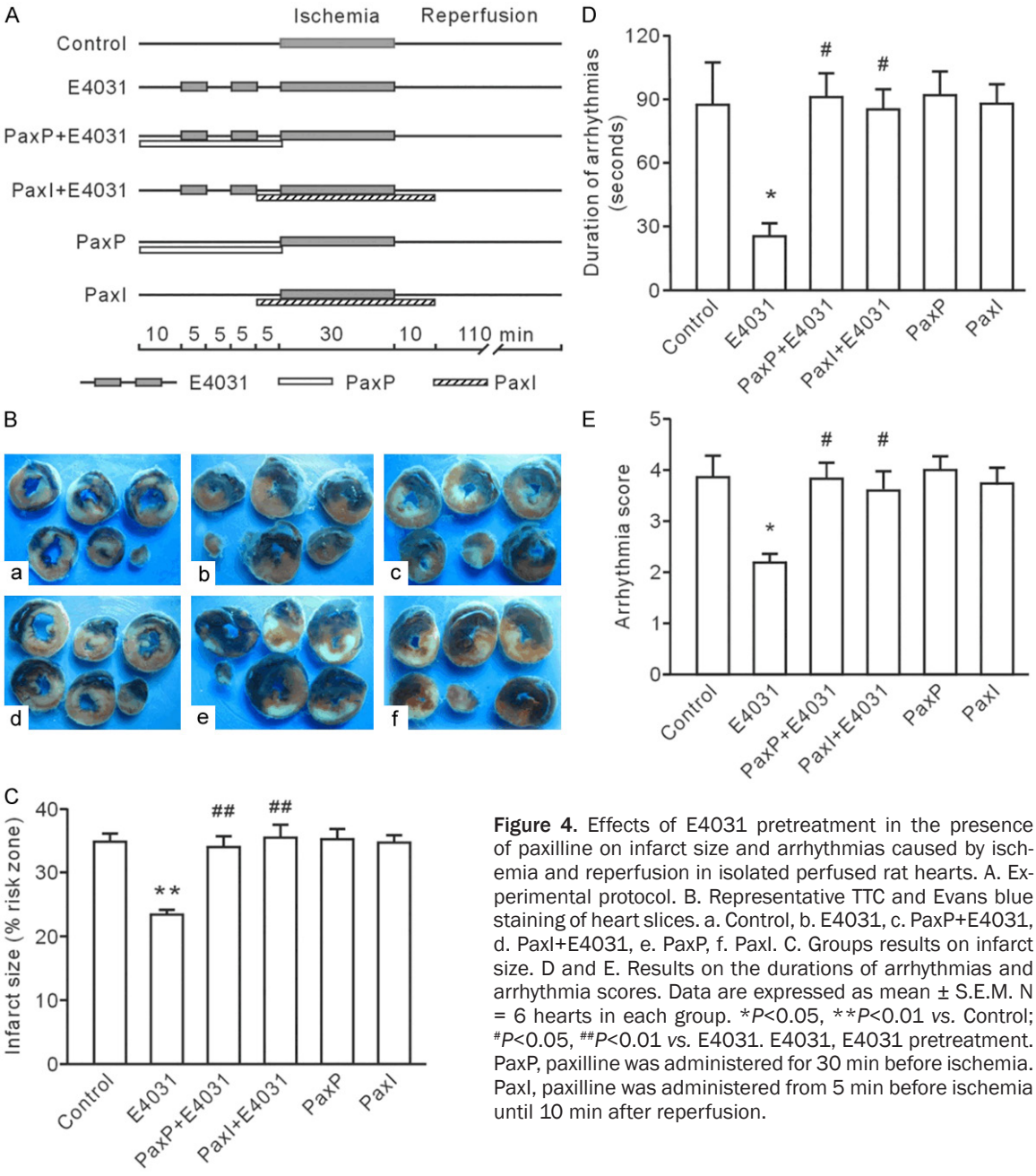

Figure 4. Effects of E4031 pretreatment in the presence of paxilline on infarct size and arrhythmias caused by ischemia and reperfusion in isolated perfused rat hearts. A. Experimental protocol. B. Representative TTC and Evans blue staining of heart slices. a. Control, b. E4031, c. PaxP+E4031, d. PaxI+E4031, e. PaxP, f. PaxI. C. Groups results on infarct size. D and E. Results on the durations of arrhythmias and arrhythmia scores. Data are expressed as mean ± S.E.M. N = 6 hearts in each group. *P<0.05, **P<0.01 *vs.* Control; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs.* E4031. E4031, E4031 pretreatment. PaxP, paxilline was administered for 30 min before ischemia. PaxI, paxilline was administered from 5 min before ischemia until 10 min after reperfusion.

10 μM [19, 21] or paxilline at 1 μM [19, 21] were used to open or block the mitoK$_{Ca}$ channel, respectively.

Statistical analysis

Values presented are mean ± standard error of mean (S.E.M.). Differences in heart rate and CF before and after drug treatment were tested by paired Student's t-test. Differences between preconditioning conditions with control were tested by unpaired t-test. For the differences in infarct size and arrhythmias, One-way ANOVA and the post hoc (Bonferroni's correction) test were used for multiple comparisons at a minimal significance level of P<0.05.

Results

Effects of Ni^{2+}, KB-R7943, nifedipine, or E4031 on Fura-2 fluorescence ratio response during Na^+-free exposure in ventricular myocytes

After a myocyte was exposed to a Na^+-free medium, $[Ca^{2+}]_i$ significantly increased. The elevation in $[Ca^{2+}]_i$ was completely blocked by the

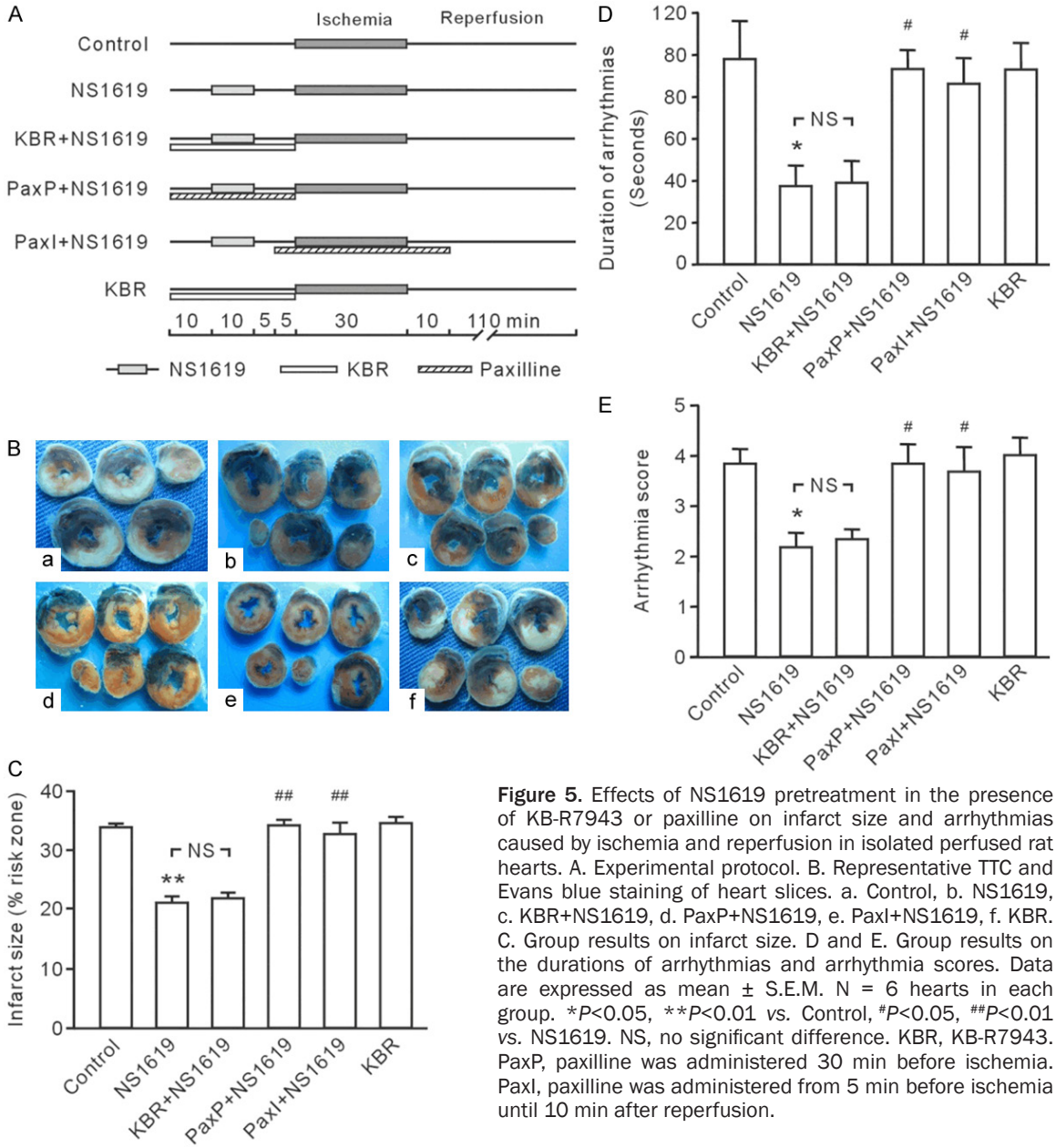

Figure 5. Effects of NS1619 pretreatment in the presence of KB-R7943 or paxilline on infarct size and arrhythmias caused by ischemia and reperfusion in isolated perfused rat hearts. A. Experimental protocol. B. Representative TTC and Evans blue staining of heart slices. a. Control, b. NS1619, c. KBR+NS1619, d. PaxP+NS1619, e. PaxI+NS1619, f. KBR. C. Group results on infarct size. D and E. Group results on the durations of arrhythmias and arrhythmia scores. Data are expressed as mean ± S.E.M. N = 6 hearts in each group. *P<0.05, **P<0.01 *vs.* Control, $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs.* NS1619. NS, no significant difference. KBR, KB-R7943. PaxP, paxilline was administered 30 min before ischemia. PaxI, paxilline was administered from 5 min before ischemia until 10 min after reperfusion.

NCX inhibitors Ni^{2+} (1 mM) (**Figure 1A** and **1B**) or 15 μM KB-R7943 (**Figure 1A** and **1B**), but not by 1 μM nifedipine, a selective inhibitor of the L-type calcium channel (**Figure 1A** and **1B**). The observations indicate that the elevation in $[Ca^{2+}]_i$ upon exposure to a Na^+-free medium was due to the reversed-mode NCX. Treatment of myocytes with 10 μM E4031 for 10 min significantly increased the change of $[Ca^{2+}]_i$ upon Na^+ withdrawal (**Figure 1A** and **1B**), which was also blocked by 15 μM KB-R7943 (data not shown). The data suggest that E4031 stimulates reverse-mode NCX and increases $[Ca^{2+}]_i$ level. Therefore, we chose E4031 as an activator of reverse-mode NCX.

Heart rate and coronary flow in the isolated perfused rat heart

Under baseline conditions, the heart rate and CF in the control group were 267±11 beats/min and 13.5±0.7 mL/min respectively, and both heart rate and CF before index ischemia were comparable in all groups studied (including control, IP, and E4031, sevoflurane and the blockers pretreatment group). One minute after administering 5 μM E4031 and 3.8 vol% sevo-

flurane, the heart rate was reduced to 223±12 and 218±10 beats/min respectively (P<0.05 *vs.* baseline). The effects of both E4031 and sevoflurane on heart rate were followed by full recovery after washout of the drugs. Coronary artery occlusion resulted in a marked reduction in CF in all of the experimental groups. On reperfusion, the CF was immediately restored to normal. There were no significant differences in heart rate and CF among all groups at the end of reperfusion.

Effect of E4031 pretreatment on infarct size and arrhythmias caused by I/R

Exposure of the heart to E4031 at 3-5 μM for two cycles of 5 min each reduced the infarct size caused by index ischemia in a concentration-dependent manner. The effect was highest at 5 μM. The effect of 5 μM E4031 administered for two cycles of 5 min each was significantly higher than that of 5 μM E4031 administered for one cycle of 10 min (**Figure 2**). Although 10 μM E4031 also induced cardioprotective effects, heart rate did not reversibly return to normal 5 min after pretreatment with 10 μM E4031 (data are not shown). So we chose 5 μM E4031 administered for two cycles in the subsequent experiments.

Preconditioning with two cycles of 5 min ischemia each significantly reduced both the infarct size (**Figure 3B**), which is consistent with previous observations [10, 41] and arrhythmias in terms of duration and score (**Figure 3D** and **3E**), which is also in agreement with the previous observation [41]. More importantly, KB-R7943 at 5 μM, which itself had no effect on the heart, not only attenuated the infarct-reducing, but also the anti-arrhythmic effects of IP (**Figure 3B**, **3D** and **3E**). Pretreatment with 5 μM E4031 or 3.8 vol% sevoflurane mimicked the effects of IP on the heart, and the effects were also blocked by 5 μM KB-R7943 (**Figure 3B**, **3D** and **3E**).

Effect of E4031 pretreatment on myocardial injury caused by I/R upon blockade of the MitoK$_{Ca}$ channel with paxilline

Administration of 1 μM paxilline to the heart 30 min before ischemia abolished the beneficial effects of 5 μM E4031 on infarct size (**Figure 4B** and **4C**). Administration of 1 μM paxilline from 5 min before the 30-min ischemia until 10 min after reperfusion also abolished the cardioprotective effect of E4031 (**Figure 4B** and **4C**). Furthermore, the anti-arrhythmic effect of E4031 pretreatment was also blocked by 1 μM paxilline (**Figure 4D** and **4E**), which itself had no effects on these two parameters, nor on the CF.

Effect of NS1619 pretreatment on infarct size and arrhythmias caused by I/R upon inhibition of reverse-mode NCX activity

Opening the mitoK$_{Ca}$ channel with 10 μM NS1619 significantly reduced the infarct size (**Figure 5B** and **5C**), consistent with previous finding [21]. Furthermore, NS1619 pretreatment also reduced the incidence of arrhythmias (**Figure 5D** and **5E**). The protective effects of NS1619 pretreatment on infarct size and arrhythmias were blocked by blockade of the mitoK$_{Ca}$ channel with 1 μM paxilline, which was administered either during preconditioning or index ischemia and reperfusion. The last but not least, the protective effects of NS1619 still occurred when blocking the reverse-mode NCX with 5 μM KB-R7943 (**Figure 5B-E**).

Discussion

The most important findings in the present study are 1) pretreatment with E4031, increasing intracellular Ca^{2+} via the reverse-mode NCX, not only reduced the effects of ischemia on the infarct, but also on the arrhythmias in isolated perfused rat heart, which mimicked the effect of IP. Prior stimulating the reverse-mode NCX via sevoflurane, mimicked the similar effects; 2) the reverse-mode NCX inhibitor, KB-R7943, not only abolished the ameliorating effects of IP on the infarct and arrhythmias induced by ischemia and reperfusion, but also attenuated the cardioprotection effects of pretreatment with E4031 or sevoflurane; and 3) the cardioprotective effects of pretreatment with E4031 were attenuated by paxilline, a selective inhibitor of the mitoK$_{Ca}$ channel, administered either during preconditioning or index ischemia/reperfusion. These observations suggest that prior stimulation of the reverse-mode NCX with E4031 is anti-arrhythmic and cardioprotective, and stimulation of the exchanger activates the mitoK$_{Ca}$ channel during preconditioning and the subsequent index ischemia/reperfusion, leading eventually to protection against myocardial infarct and arrhythmias.

In the present study, we chose both E4031 and sevoflurane to increase the reverse-mode NCX activity. We found that pretreatment with

E4031 or sevoflurane decreased the severity of ischemia/reperfusion-induced infarct or arrhythmias as did IP, and the effects were blocked by KB-R7943, a selective inhibitor of the NCX. Our results suggested that E4031 pretreatment could stimulate reverse-mode NCX activity, which produce preconditioning effect in the heart.

Another important observation is that paxilline, a selective inhibitor of the mitoK$_{Ca}$ channel, administered during preconditioning, blocked the protective effects of E4031 pretreatment. This is in agreement with the previous finding that mitoK$_{Ca}$ channel opening triggers the protective effects of IP [21, 22, 42, 43] or pharmacological preconditioning [42]. We concluded that prior reverse-mode NCX stimulation with E4031 results in an increase of intracellular calcium level, leading to opening of mitoK$_{Ca}$ channel, which reduces cardiac injury and arrhythmias.

In this study, we also observed that paxilline, administered from 5 min before index ischemia until 10 min after reperfusion, blocked the infarct-reducing effects of E4031 pretreatment. This indicated that the mitoK$_{Ca}$ channel was also a mediator in the protective effects of prior stimulation of the reverse-mode NCX. This finding is consistent with observations in tumor necrosis factor-α-induced cardioprotection [44], but is not in agreement with a previous finding by Cao et al., who found that 1 μM paxilline, when given during index ischemia, did not block the infarct-reducing effect of IP [21]. This discrepancy may be related in part to the different types of preconditioning.

Interestingly, blockade of mitoK$_{Ca}$ channel with paxilline also attenuated the anti-arrhythmic effects of E4031 pretreatment in addition to its effect on infarct reduction. This observation suggests that the two effects may share the same downstream signaling pathways.

It has been pointed out that there is a direct connection between mitochondrial function and the myocardial action potential [45]. The fact that mitoK$_{Ca}$ mediated the anti-arrhythmic effects of E4031 pretreatment suggests that opening of the mitoK$_{Ca}$ channel by preconditioning results in protection of mitochondrial function, which prevents the change of the cellular action potential, thus reducing the incidence of arrhythmias. Further studies are needed to delineate the mechanism.

KB-R7943 is a well-known inhibitor of the reverse-mode NCX in heart, it also produces non-specific effects in non-heart tissues, i.e., Sobolevsky and Khodorov reported that KB-R7943 blocked *N*-methyl-D-aspartate channels in acutely isolated hippocampal neurons [46]. In this study we performed the experiments in the isolated rat heart preparation. We observed that KB-R7943, administered during preconditioning, did not alter the beneficial effects of NS1619 pretreatment. These results indicate that NCX is located upstream from the mitoK$_{Ca}$ channel. As to the mechanisms behind the activation of mitoK$_{Ca}$ channel, because Ca^{2+} is an important activator of mitoK$_{Ca}$ channel [19], we suggest that prior stimulation of reverse-mode NCX increase intracellular Ca^{2+}, which directly activate mitoK$_{Ca}$ channel, thus leading to cardioprotection. On the other hand, PKC is an important downstream mediating the cardioprotection afforded by Ca^{2+} preconditioning [11, 47], therefore, whether PKC is involved in the activation of mitoK$_{Ca}$ channel needs further studied.

In conclusion, this study has provided the first evidence that prior stimulation of the reverse-mode NCX not only reduces the infarct size but also attenuates arrhythmias induced by ischemia/reperfusion, and mitoK$_{Ca}$ channel mediates the cardioprotective effects.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 81100082; No. 31371181).

Disclosure of conflict of interest

None.

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