Effects of [5-(2-Methoxy-5-fluorophenyl)furan-2-ylcarbonyl]guanidine (KR-32560), a Novel Sodium/Hydrogen Exchanger-1 Inhibitor, on Myocardial Infarct Size and Ventricular Arrhythmias in a Rat Model of Ischemia/Reperfusion Heart Injury

Jung-Woo Park¹, Hui-Yul Roh¹, In-Sang Jung¹, Yeo-Pyo Yun², Kyu-Yang Yi³, Sung-Eun Yoo³, Suk-Hyung Kwon⁴, Hun-Jong Chung⁵, and Hwa-Sup Shin¹,*

¹Department of Applied Biochemistry, Division of Life Science, College of Biomedical and Health Science, Konkuk University, Chungju 380-701, Korea
²College of Pharmacy, Chungbuk National University, Cheongju 361-763, Korea
³Medical Science Division, Korea Research Institute of Chemical Technology, Taeejon 305-600, Korea
⁴Rexgene Biotech Co., Ltd., Seoul 137-070, Korea
⁵Pediatric Department, Chungju Hospital, Konkuk Medical School, Konkuk University, Chungju 380-701, Korea

Received January 11, 2005; Accepted June 23, 2005

Abstract. The cardioprotective effects of the novel sodium/hydrogen exchanger-1 (NHE-1) inhibitor KR-32560 {[5-(2-methoxy-5-fluorophenyl)furan-2-ylcarbonyl]guanidine} were studied in an anesthetized rat model of 30-min ischemia / 2.5-h reperfusion heart injury. KR-32560 (0.01 – 1 µM) dose-dependently inhibited NHE-1-mediated rabbit platelet swelling induced by intracellular acidification. KR-32560 at 0.1 and 1.0 mg/kg (i.v. bolus, given 10 min before ischemia) reduced infarct size from 65.9% (control) to 49.7% and 32.7%, respectively, while reducing the extension of myocardial injury (mm³/g of left heart weight) from 405.1 (control) to 302.9 and 185.4, respectively (all P<0.05 vs control). KR-32560 dose-dependently reduced the total number of ventricular premature beats (VPBs) during ischemia from 510.2 (control) to 353.8 and 134.2 beats (all P<0.05, n = 6), while reducing ventricular tachycardia (VT) incidence from 49.3 (control) to 26.8 and 4.3 and VT duration from 249.2 s (control) to 150.5 and 26.7 s (all P<0.05, n = 6). KR-32560 dose-dependently reduced ventricular fibrillation (VF) incidence from 19.0 (control) to 9.2 and 1.2 and VF duration from 88.0 s to 34.5 and 2.8 s (all P<0.05, n = 6). KR-32560 also exerted similar effects on reperfusion arrhythmias, except for VPBs. These results indicate that KR-32560 may exert significant cardioprotective effects in ischemia/reperfusion heart injury.

Keywords: KR-32560, sodium hydrogen exchanger isoform-1, cardioprotection, infarction, arrhythmia

Introduction

Myocardial ischemia and reperfusion-induced heart injury accompanied by reparative or replacement heart surgery such as coronary artery bypass graft results in myocardial stunning (reversible injury), myocardial infarction (necrosis), or myocardial apoptosis, symptoms which are clinically associated with hypotension and low cardiac output acutely or heart failure and ventricular remodeling chronically (1). Although the exact mechanisms underlying ischemia/reperfusion heart injury remain obscure, accumulating evidence points out that its etiology resides in intracellular Ca²⁺ overload during ischemia/reperfusion and oxidative stress induced by reactive oxygen species released at the onset of reperfusion (1–5).

It has been shown that selective sodium/hydrogen exchanger isoform-1 (NHE-1) inhibitors such as cari-
porin, enporin, and zonopiride hold considerable promise for the near future as a novel therapeutic modality to protect against ischemia/reperfusion heart injury (1, 6–10). The mechanism by which NHE-1 inhibitors protect hearts against ischemia/reperfusion is as follows (11, 12): during cardiac ischemia, cytosolic acidosis-stimulated NHE-1 causes Na⁺ influx in exchange for H⁺ efflux, resulting in Na⁺ overload within the cardiac cell, with the concurrent inhibition of Na⁺/K⁺ ATPase during ischemia. Intracellular Na⁺ accumulation through the excessive activation of NHE and other mechanisms such as Na⁺-HCO₃⁻ symporter (13) leads to a reverse mode of functioning of Na⁺/Ca²⁺ exchanger (NCX), contributing to Ca²⁺ overload accompanied by the injury of cardiomyocytes. It has been repeatedly reported that blockade of the NHE-1 before ischemia or only after the onset of reperfusion protects cardiomyocytes against ischemia/reperfusion by successively inhibiting the increase in cytosolic Na⁺ and then Ca²⁺, with the resultant prevention of Ca²⁺ overload, a necrotic and apoptotic signal in ischemia/reperfusion injury (1). Alternatively, NHE-1 blockers may exert cardioprotective effects by delaying mitochondrial matrix acidification and ATP exhaustion during ischemia (14).

Myocardial injury associated with restoration of blood flow into previously ischemic heart has been termed reperfusion injury consisting of four main components of injury such as vascular injury, arrhythmias, myocardial stunning, and extension of the mass of tissue injury (lethal myocyte injury: infarct) (15). Although our understanding of the molecular events that modulate the reperfusion injury is limited, more than several mechanisms have been proposed. Among the most plausible ones are considered the free radical hypothesis and calcium overload hypothesis that can be conveniently integrated and are not mutually exclusive. The free radical hypothesis is based on the direct identification of free radicals in reperfused heart, the inverse correlation of their generation to functional recovery, and the existence of a number of potential mechanisms for generation of free radicals during reperfusion of ischemic myocardium (16). The calcium hypothesis is another possible mechanism for reperfusion heart injury that has gained significant experimental support. In this respect, it is anticipated that activation of the calcium transport system at the inner mitochondrial membrane and sarcoplasmic reticulum may contribute to increased energy expenditure during reperfusion, forming the basis for uncoupling of oxidative phosphorylation from tension generation even after myocardial oxygen consumption recovers to baseline rapidly after reperfusion (17). As in ischemia, calcium overload during early reperfusion is closely associated with the activation of NHE-1 coupled with the reverse-mode functioning of NCX and the inactivation of Na⁺/K⁺ ATPase (18). Besides these two hypotheses, the recent studies indicate that pro-apoptotic STAT-1 and anti-apoptotic STAT-3, the signal transducers and activators of transcription (STAT) factors, can modulate the apoptotic program both by direct DNA binding and via a co-activator mechanism following ischemia/reperfusion induced damage of the myocardium (19).

Recently, [5-(2-methoxy-5-fluorophenyl)furan-2-ylcarbonyl]guanidine (KR-32560, Fig. 1) has been synthesized by Korea Research Institute of Chemical Technology (KRICT, Taejeon, Korea) as a new therapeutic candidate for cardioprotection. This newly synthesized guanidine derivative was shown to inhibit intracellular pH recovery in PS120/NHE-1 (h NHE-1 transfected) cells with a significantly greater potency than cariporide (IC₅₀: 0.7 and 1.6 µM, respectively, personal communication). The main purpose of the present study was to evaluate the cardioprotective effects of KR-32560 on myocardial infarction and various types of ventricular arrhythmias in a rat in vivo model of ischemia/reperfusion-induced heart injury.

Materials and Methods

Animals
Male Sprague-Dawley rats (350–380 g) and male New Zealand White rabbits (2–3 kg) were purchased from Samtako Bio Korea (Osan, Korea). The animals were conditioned for 2 weeks in an animal storage room, where a constant temperature (22.5 ± 1.0°C) and a constant humidity (55 ± 5%) were maintained under a cycle of 12-h light/dark illumination, and free access to food and tap water was allowed. This study conformed to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health.

Chemicals and Drugs
KR-32560 was synthesized at the Medical Science Division, KRICT (Taejeon, Korea), and it was dissolved in 5% dimethylsulfoxide in saline for intravenous administration (20). Sodium pentobarbital was pur-
chased from Hanlim Pharmaceutical Co. (Seoul, Korea).

**Platelet swelling assay (PSA)**

Blood samples (9/1 blood/ACD solution, vol/vol%) were withdrawn from rabbits by venipuncture of ear vein into tubes containing ACD solution (65 mM citric acid, 85 mM trisodium citrate, 2% dextrose). Each sample was centrifuged at 1300 rpm for 10 min at room temperature, and platelet-rich plasma (PRP) was obtained from the upper two-thirds of the supernatant. The remainder of the blood sample was then centrifuged at 3000 rpm for 10 min to obtain platelet-poor plasma (PPP). Platelets in PRP were counted in a hemocytometer using an optical microscope and adjusted to 1 x 10⁹ cells/ml (final counts). Platelet NHE-1 activity was measured according to the previously described method, with minor modification (21). Briefly, increases in light transmission associated with cell swelling were measured with an aggregometer (model 490 4D; Chrono-Log, Havertown, PA, USA). Sodium propionate measured with an aggregometer (model 490 4D; Chrono-Log, Havertown, PA, USA). Sodium propionate solution (250 µl, composition: 135 mM sodium propionate, 20 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, pH 6.7) in a cuvette was stirred at 1000 rpm and prewarmed for 5 min at 37°C. An increase in light transmission of PRP induced by platelet swelling was observed after application of PRP (50 µl). Mixing PRP and sodium propionate solution produces an acidic intracellular pH at which platelet NHE is activated, and the increase in Na⁺ influx associated with excretion of cytosolic H⁺ via NHE results in cellular swelling as a result of water accumulation in the cytoplasm (21). Light transmission through PRP increased since the density of the cellular component decreased with swelling (21). We used this simple assay system to evaluate the effects of drugs on NHE-1, since NHE-1 is considered to be the dominant subtype of NHE in platelets. KR-32560 was added to the cuvette 3 min before the addition of PRP. An equivalent mixture of PPP and Na propionate solution was used to correct for light transmission through the non-platelet portion of PRP. Changes in light transmission were recorded continuously at 37°C for 5 min, and rate constants were calculated from slopes generated during the first 42 s as described by Rosskopf et al. (21). The inhibitory effect of KR-32560 at various concentrations was expressed as rate constant percentage relative to the value obtained in the presence of vehicle. The half-maximal inhibitory concentration (IC₅₀) value of KR-32560 was obtained from the linear part of the relationship between the log concentration and NHE activity using linear regression analysis.

**Surgical procedure for regional ischemia and reperfusion**

After male Sprague-Dawley rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), the surgical procedure was performed as previously described (22). The trachea was intubated and connected to a rodent ventilator (SAR 830/P ventilator; CWE, Inc., Ardmore, PA, USA) for artificial ventilation with room air (stroke volume, 10 ml/kg; 60 strokes/min). The temperature of the heating pad was adjusted to 37°C by a temperature controller. Arterial blood pressure was continuously monitored via a saline-filled catheter (PE50) inserted into the left femoral artery, which was connected to an isocet pressure transducer (Hugo Sachs Electronic, Hugstetten-March, Germany) and recording system (V75-25A; Coulbourn Instruments, Allentown, PA, USA). Throughout the experiment, the blood pressure signal was saved as a file on a personal computer using WINDAQ/Lite Waveform Recording software (DATAQ Instruments, Akron, OH, USA) for later retrieval and analysis. ECG and heart rate were measured by standard limb lead II electrodes using an isolated ECG bioamplifier (V75-04; Coulbourn Instruments, Allentown, USA). The chest was opened by a left thoracotomy in the fifth to sixth intercostal space, and the pericardium was incised. The heart was gently exteriorized by applying pressure on the right side of the chest, and a ligature (5-0 silk) was placed around the left anterior descending coronary artery (LAD) for later initiation of coronary occlusion and reperfusion. The heart was quickly repositioned in the thoracic cavity with both ends of the ligature exteriorized and passed through a segment of polyethylene tube (PE 100, 2.5-cm-long). Following stabilization for 20 min, the animal was injected through the femoral vein with vehicle or KR-32560 (0.1 and 1.0 mg/kg, i.v. bolus) for monitoring hemodynamic effects for 10 min. Then, the animals were subjected to 30-min ischemia by complete tightening of the ligature around LAD with the help of the PE tube and a small hemostat. Reperfusion was allowed for 2.5 h by releasing the ligature around the LAD.

**Area at risk and myocardial infarct size determination**

The area at risk of infarction and the infarct size were measured according to the principle and procedure of the original method using Evans blue (Sigma-Aldrich Korea, Ltd., Yongin, Korea) and 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich Korea) (23, 24). Two and half hours after reperfusion, LAD was reocluded and 2 ml of a 1% Evans blue solution was administered into the femoral vein via a catheter. Then, the heart was removed and trimmed of the right ventricle and both atria. The left ventricle immediately below the
ligature was cut into 5 transverse slices from the apex with a thickness of about 2 mm. Each slice was weighed and the images of heart slices were captured by a high resolution CCD camera and analyzed for the calculation of area at risk of infarction (AAR) using an image analysis program (Image Pro Plus; Media Cybernetics, Silver Spring, MD, USA). Normal myocardium was stained blue by Evans blue and AAR unstained blue. To determine the infarct zone, the heart slices were incubated with 1% solution of TTC in phosphate buffer (pH 7.4) at 37°C for 15 min and then fixed overnight in 10% formalin solution. The images of the heart slices were captured and analyzed again. The area of necrotic myocardium (infarct zone, IZ) was unstained and the area of viable myocardium stained brick red by TTC. The area at risk of infarction (AAR/LV, risk mass/left ventricular mass) and the infarct size (IZ/AAR, infarct mass/risk mass) were calculated as described by other researchers (25). In each heart, the total volume of the damaged myocardium (infarct zone) was calculated as the sum of the partial values of the different slices based on the area of IZ and the thickness of each slice. To compare the extension of myocardial injury between hearts of different sizes, the total volume of the damaged myocardium was divided by the total weight of 5 slices for each heart (26).

**Evaluation of arrhythmias**

Throughout the experiment, a standard limb lead II ECG was continuously recorded and saved as a file on a personal computer using WINDAQ/Lite Waveform Recording software (DATAQ Instruments, Akron, OH, USA). Following the experiment, the ECG waveform was retrieved and analyzed in 10-s intervals for ventricular arrhythmias associated with myocardial ischemia and reperfusion throughout the ischemia and reperfusion (27, 28). Total number of episodes for ventricular premature beats (VPBs), total number of episodes, and total duration for ventricular tachycardia (VT) and ventricular fibrillation (VF) were evaluated according to the guidelines of the Lambeth Conventions (29). Briefly, VT was recognized as three or more consecutive premature ventricular contractions and VF as an irregular modulating baseline. A heart was considered in normal sinus rhythm when normal sinus complexes occur at a regular rate. VPBs were recognized as faster heart beats compared to the normal sinus complex.

**Statistical analysis**

All values are expressed as the mean ± S.E.M. Data were analyzed by one-way analysis of variance followed by the Dunnett’s test for multiple comparisons (Sigma Stat; Jandel Co., San Rafael, CA, USA). In all comparisons, the difference was considered to be statistically significant at P<0.05.

**Results**

**Inhibition of NHE-1-mediated rabbit platelet swelling**

KR-32560 (0.01 – 1.0 µM) inhibited NHE-1-mediated platelet swelling induced by intracellular acidification of rabbit platelet-rich plasma in a concentration-dependent manner (% control of swelling rate constant: 83.4 ± 6.9, 67.2 ± 7.4, 39.0 ± 13.4, 0, and 7.2 ± 7.2 at 0.01, 0.03, 0.1, 0.3, and 1.0 µM, respectively, Fig. 2). The IC_{50} (concentration of KR-32560 required to decrease the rate of rabbit platelet swelling by 50%) was 0.052 ± 0.01 µM.

**Effects on myocardial infarct size in a rat model of ischemia/reperfusion heart injury**

The effects of KR-32560 on the mean arterial blood pressure and the heart rate are shown in Table 1. KR-32560 (0.1 and 1.0 mg/kg, i.v. bolus) had no significant effects on the pre-ischemic heart rate and blood pressure. In the control group, the heart rate and the blood pressure were on the gradual decrease at 30-min ischemia and 150-min reperfusion, the pattern of which was unaltered by KR-32560 at 0.1 mg/kg, i.v., but weakened by KR-32560 at 1.0 mg/kg, i.v. The effects of intravenously administered KR-32560 on the myocardial infarct zone in anesthetized rats are shown in Fig. 3. The area at risk (risk mass/left ventricular mass) in the control vehicle group was 50.3 ± 2.0%, which was similar to those for the two KR-32560-treated groups (48.6 ± 1.1% and

![Fig. 2. Effects of KR-32560 on endogenous NHE-1-mediated rabbit platelet swelling induced by application of Na propionate, expressed as percentage of swelling rate constant relative to the control rate constant. Values are the mean ± S.E.M. (n = 3 – 4).](image)
Cardioprotective Activity of KR-32560

In rats treated with vehicle alone, the infarct size (infarct mass/risk mass) caused by ischemia (30 min)/reperfusion (2.5 h) was 65.9 ± 1.6%. KR-32560 given as an i.v. bolus 10 min before ischemia significantly reduced the myocardial infarct size in a dose-dependent manner (49.7 ± 0.5% and 32.7 ± 0.9% at 0.1 and 1.0 mg/kg, respectively, all P < 0.05 vs control) when compared with the control group. KR-32560 also dose-dependently reduced the extension of myocardial injury (mm$^3$/g of heart weight) from 405.1 ± 29.8 (control) to 302.9 ± 18.7 and 185.4 ± 15.1 at 0.1 and 1.0 mg/kg, respectively (P < 0.05 and P < 0.01 vs control, respectively) (Fig. 4).

**Table 1. Changes in the heart rate and blood pressure**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Stabilization (Pre-drug)</th>
<th>Pre-ischemia (Post-drug)</th>
<th>End of ischemia</th>
<th>150-min reperfusion</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/min)</td>
<td>Control</td>
<td>364 ± 17</td>
<td>357 ± 18</td>
<td>344 ± 9</td>
<td>317 ± 7*</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>KR-32560 0.1 mg/kg</td>
<td>401 ± 15</td>
<td>404 ± 10</td>
<td>373 ± 9</td>
<td>330 ± 13*</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1 mg/kg</td>
<td>371 ± 15</td>
<td>390 ± 6</td>
<td>388 ± 3</td>
<td>399 ± 9*</td>
<td>6</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mmHg)</td>
<td>Control</td>
<td>84.2 ± 13.3</td>
<td>77.9 ± 9.5</td>
<td>66.5 ± 2.7*</td>
<td>42.9 ± 5.7*</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>KR-32560 0.1 mg/kg</td>
<td>97.5 ± 9.0</td>
<td>89.8 ± 9.1</td>
<td>69.1 ± 7.7*</td>
<td>46.2 ± 9.8*</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1 mg/kg</td>
<td>88.7 ± 7.3</td>
<td>92.6 ± 3.4</td>
<td>81.7 ± 5.3</td>
<td>65.3 ± 5.6*</td>
<td>6</td>
</tr>
</tbody>
</table>

Values are each a mean ± S.E.M. Control: vehicle only was administered i.v. after stabilization; KR-32560, 0.1 and 1 mg/kg: KR-32560 was administered i.v. after stabilization; Stabilization: values immediately before administration of vehicle or KR-32560; Pre-ischemia: values 10 min after administration of vehicle or KR-32560 and immediately before the onset of ischemia; End of ischemia: values 30 min after ischemia; 150-min reperfusion: values 150 min after reperfusion; n: number of rats in each group. *P < 0.05 versus stable time.

**Fig. 3.** Effects of KR-32560 on the area at risk (AAR) expressed as percentage of the left ventricular (LV) mass and infarct size (IZ) as percentage of the risk mass (AAR) in anesthetized rats subjected to 30-min occlusion of the left anterior coronary artery (LAD) followed by 2.5-h reperfusion. Vehicle or drug was administered intravenously 10 min before ischemia. Values are each a mean ± S.E.M. (n = 6). *P < 0.05: significantly different from the vehicle control group.

**Fig. 4.** Effects of KR-32560 on the extension of left ventricular myocardium with ischemia/reperfusion-induced injury in anesthetized rats subjected to 30-min occlusion of the LAD followed by 2.5-h reperfusion. The extension of left ventricular myocardium was evaluated by computer assisted morphometry on heart stained with TTC and expressed as the total volume of the damaged myocardium divided by the total weight of 5 slices for each heart. Vehicle or drug was administered intravenously 10 min before ischemia. Values are each a mean ± S.E.M. (n = 6). *P < 0.05, **P < 0.01: significantly different from the vehicle control group.

Effects on various types of ischemia-induced ventricular arrhythmias in a rat model of ischemia/reperfusion heart injury

In anesthetized rats subjected to 30-min coronary occlusion followed by 2.5-h reperfusion, various types of ventricular arrhythmias were observed to occur almost during ischemia and the first 10 min period following reperfusion. The total number of VPBs during ischemia was 510.2 ± 39.8 beats in the vehicle control group, which was dose-dependently reduced to 353.8 ± 34.7 and 134.2 ± 10.2, respectively (P < 0.05 and 0.01 vs
control, respectively) by KR-32560 at 0.1 and 1.0 mg/kg (i.v.) given 10 min before ischemia (Fig. 5A). The total number of episodes (incidence) and total duration of VT in the vehicle group were 49.3 ± 4.4 and 249.2 ± 32.4 s, respectively (Fig. 5: B and C). KR-32560 at 0.1 and 1.0 mg/kg (i.v.) given 10 min before ischemia dose-dependently reduced the incidence of VT to 26.8 ± 4.6 (P < 0.05) and 4.3 ± 1.7 (P < 0.01) (Fig. 5B) and the duration of VT to 150.5 ± 38.8 (P < 0.05) and 26.7 ± 11.5 s (P < 0.01 vs control), respectively (Fig. 5C). The total number of episodes (incidence) and total duration of VF in the vehicle group were 19.0 ± 3.8 and 88.0 ± 21.6 s, respectively (Fig. 5: D and E). KR-32560 at 0.1 and 1.0 mg/kg (i.v.) given 10 min before ischemia dose-dependently reduced the incidence of VF to 9.2 ± 3.0 (P < 0.05) and 1.2 ± 1.0 (P < 0.01) (Fig. 5D) and the duration of VF to 34.5 ± 10.2 (P < 0.05) and 2.8 ± 2.3 s (P < 0.01 vs control), respectively (Fig. 5E).

**Effects on various types of reperfusion-induced ventricular arrhythmias in a rat model of ischemia/reperfusion heart injury**

In anesthetized rats subjected to 30-min coronary occlusion followed by 2.5-h reperfusion, various types of ventricular arrhythmias were observed to occur almost during the first 10 min period of reperfusion,
although the severity of arrhythmogenesis was markedly reduced compared with that during ischemia. The total number of VPBs during reperfusion was $8.0 \pm 2.2$ beats in the vehicle control group, which was slightly increased to $10.2 \pm 2.0$ and significantly to $19.2 \pm 5.4$, respectively ($P < 0.05$ vs control) by KR-32560 at 0.1 and 1.0 mg/kg (i.v.) given 10 min before ischemia (Fig. 6A). The total number of episodes (incidence) and total duration of VT during reperfusion in the vehicle group were $0.5 \pm 0.2$ and $1.3 \pm 0.7$ s, respectively (Fig. 6: B and C). KR-32560 at 0.1 and 1.0 mg/kg (i.v.) given 10 min before ischemia dose-dependently reduced the incidence of VT to $0.3 \pm 0.1$ and $0.3 \pm 0.1$ (all $P < 0.05$) (Fig. 6B) and the duration of VT to $0.8 \pm 0.4$ ($P < 0.05$ vs control) and $1.0 \pm 0.5$ s, respectively (Fig. 6C). The total number of episodes (incidence) and total duration of VF in the vehicle group were $0.5 \pm 0.3$ and $2.2 \pm 1.8$ s, respectively (Fig. 6: D and E). KR-32560 at 0.1 and 1.0 mg/kg (i.v.) given 10 min before ischemia dose-dependently reduced the incidence of VF to $0.3 \pm 0.1$ and 0 ($P < 0.05$) (Fig. 6D) and the duration of VF to $1.3 \pm 0.8$ and $0.1 \pm 0.1$ s ($P < 0.05$ vs control), respectively (Fig. 6E).
Discussion

The present study was designed to evaluate the effects of KR-32560, a newly synthesized NHE-1 inhibitor, on myocardial infarction and various types of arrhythmias in a rat in vivo model of ischemia/reperfusion heart injury. It is well known that swelling of platelets from human, rat, and rabbit in the presence of intracellular acidosis is mediated by the activation of NHE-1 (30–32). Thus, the results from the rabbit platelet swelling assay indicate that KR-32568 is a potent NHE-1 inhibitor, its IC50 (0.052 µM) being in the similar or smaller range compared with those for cariporide and eniporide reported for rat and human platelets in the literature (e.g., IC50 values for cariporide and eniporide: 0.075 and 0.044 µM, respectively; in rat platelets and 0.023 and 0.04 µM, respectively, in human platelets; refs. 30–32). In rats subjected to a 30-min occlusion of the coronary artery followed by 2.5-h reperfusion, the area at risk and the infarct size for the control group were 50.3% and 65.9%, whereas those values were 50% and 28%, respectively, in a similar rat model adopting a protocol of 20-min ischemia/2-h reperfusion (7), indicating the induction of a more severe cardiac injury by our experimental protocol. KR-32560 administered by i.v. bolus 10 min prior to the initiation of ischemia dose-dependently reduced the infarct size (infarct mass/risk mass) from 65.9% in controls to 49.7% and 32.7% at 0.1 and 1.0 mg/kg, respectively (P<0.05). The reduction of cell death by KR-32560, particularly at the higher dose, appears to be associated with improved hemodynamic (MBP) recovery during ischemia/reperfusion, as reported for cariporide (20) and epigallocatechin-3-gallate (19) in the similar rat model of ischemia/reperfusion heart injury. The infarct-reducing effects of KR-32560 were in line with the earlier findings on the prototype selective NHE-1 inhibitor cariporide, which demonstrated its potent cardioprotective activity in various animal models of ischemia/reperfusion heart injury (7, 25, 33–35).

In our experimental model with a protocol of 30-min ischemia/2.5-h reperfusion, the significant occurrence of various types of arrhythmias (VPBs, VT, and VF) was observed during the 30-min ischemic period, and the arrhythmogenicity appeared to be comparable to that reported by others in the similar rat model of ischemia/reperfusion adopting a 30-min ischemic time protocol (20). KR-32560 dose-dependently and significantly reduced the occurrence of all three types of ventricular arrhythmia during ischemia, as reflected in the total number of VPBs, total events and duration of VT and VF. Unlike the high arrhythmogenicity during the ischemic period, the occurrence of those three types of arrhythmias was markedly reduced during the reperfusion period with most of the VPBs, VT, and VF being observed to occur up to the first 10 min of reperfusion in our model as reported by others (7, 28). The less severe arrhythmogenicity during the reperfusion period may be related to the somewhat long duration of the preceding ischemic period, considering other researchers’ findings that reperfusion of ischemic myocardium in anesthetized animal models with normal coronary arteries often causes ventricular arrhythmias, particularly if performed abruptly after 15–20 min of ischemia, and that the frequency of malignant arrhythmias drops substantially with more prolonged ischemia and staged gradual reflow (36–38). Despite the less severe arrhythmogenicity during reperfusion, KR-32560 significantly reduced the occurrence of most of the reperfusion-induced ventricular arrhythmias, except for the total number of VPBs with a dose-dependent increase, the underlying mechanism for which remains to be resolved. The significant antiarrhythmic activities of KR-32560 during both ischemia and reperfusion, in conjunction with reduction of the infarct size, may contribute to the efficient cardioprotective effect of this compound.

Treatment timing of prospective agents to protect against ischemia/reperfusion heart injury is among one of major considerations for new drugs under development. NHE-1 is classically known to be stimulated by cytosolic acidosis during ischemia, leading to the increase in intracellular Na+ with the concomitant malfunctioning of Na+/K+-ATPase due to the deficiency of ATP synthesis, whereas the blockade of the NHE-1 before ischemia abolishes the increase in intracellular Na+ during this period (8, 39). These ionic mechanisms during cardiac ischemia are essentially different from those underlying the reperfusion heart injury. Thus, the sarcolemmal NHE-1 may be stimulated in several ways during reperfusion. Firstly, on reperfusion following a period of ischemia, H+ is rapidly washed out from the extracellular space, resulting in a sudden increase in the H+ gradient across the sarcolemma of the cardiomyocytes. The increase in the H+ gradient will further stimulate the NHE-1 to remove H+ from the cell in exchange for extracellular Na+. Secondly, reperfusion stimulates phosphoinositide hydrolysis via a PLC-βγ, in a Gq protein-dependent pathway (40, 41). This leads to PKC activation that stimulates NHE-1 directly through PKC-dependent phosphorylation (41–44). Thirdly, the activation of NHE-1 at the onset of reperfusion is linked to the increase in reactive oxygen species (ROS) (3–5), probably through the ROS-activated mitogen-activated protein kinase pathway that phosphorylates ERK 1/2 and the cytosolic tail of the NHE-1 (45). These
findings support the normal functioning of NHE-1 for the increase in intracellular Na\(^+\) during reperfusion. The increase in intracellular Na\(^+\) during reperfusion drives the NCX in a reverse mode to pump out Na\(^+\) in exchange for extracellular Ca\(^{2+}\), which finally causes intracellular Ca\(^{2+}\) overload, leading to subsequent cellular damage and lethal arrhythmias (1). The contribution of NHE-1 to reperfusion heart injury has been demonstrated by experimental evidences showing that the heart becomes protective against ischemia/reperfusion injury when the NHE-1 or the NCX is blocked only after the onset of reperfusion (46, 47). Intracellular Ca\(^{2+}\) overload during early reperfusion is achieved by the cooperation of the NHE-1 and NCX functioning in a reverse mode until the Na\(^+/K\(^+\)-ATPase is resupplied with ATP and the NCX changes to its forward mode of operation, eventually leading to the normal recovery of cellular Ca\(^{2+}\) homeostasis (18). Intracellular Ca\(^{2+}\) increase during early reperfusion leads to rigor-type contracture or Ca\(^{2+}\) overload contracture depending on the slow or rapid recovery of ATP, the development of contracture triggering reperfusion injury including necrosis and arrhythmias (18, 40).

All these studies indicate that NHE-1 in cardiomyocytes may be activated both during ischemia and during reperfusion, contributing to the ischemic injury and reperfusion injury, respectively, through the increase in Na\(^+\), followed by the increase in intracellular Ca\(^{2+}\) via NCX working in reverse mode. Therefore, NHE-1 inhibitors appear to be an effective modality to treat ischemia/reperfusion heart injury due to their inhibitory efficacy against NHE-1, a transmembrane antport responsible for intracellular Ca\(^{2+}\) overload throughout the period of ischemia and reperfusion, although their myocardial protective effects were generally markedly larger when the agents were administered preischemia than those seen when administered immediately prior to or at the time of reperfusion (11, 12, 48). However, the blockade of NHE-1 may not completely abolish ischemia/reperfusion heart injury since ischemia/reperfusion injury may be attributed to other mechanisms such as activation of the Na\(^+\)-HCO\(_3\)- cotransport mediated by ERK1/2 during reperfusion (13, 49). Furthermore, more detailed mechanisms of action underlying the cardioprotective activity of NHE-1 inhibitors await clarification of the role of different types of NHE isoforms at subcellular locations with respect to ischemia and the reperfusion injury process and Ca\(^{2+}\) mobilization (50).

Although the results from the platelet swelling assay strongly point to the inhibition of NHE-1 on cardiomyocytes as a principal site of action for KR-32560, we could not completely exclude the involvement of other mechanisms. Preliminary data from a separate series of experiments with rabbit platelets indicate that KR32560 inhibit platelet aggregation by the blockade of both arachidonic acid release and thromboxane A\(_2\) synthase. In cardiomyocytes, it was reported that the accumulation of intracellular unesterified arachidonic acid resulting from the peroxidation of membrane lipids increased tissue injury caused by exogenous free radicals (51), whereas drugs like ibuprofen, nafazatrom, and BW755C exerted protection against myocardial ischemia/reperfusion injury by inhibiting arachidonic acid metabolic pathways (16). Thus, the cardioprotective activity of KR-32560 observed in the present study may be ascribed in part to its modulatory effect on the arachidonic acid metabolic cascade. Since the pathophysiology of ischemia/reperfusion heart injury is complex, including primarily cellular and humoral components of inflammation, as well as ionic and metabolic disturbances in cardiomyocytes and vessels (16), further investigation into the detailed mechanisms of KR-32560 need to be conducted.

In conclusion, the results from the present study indicate that KR-32560, a newly synthesized NHE-1 inhibitor, exert significant infarct size-reducing and antiarrhythmic activity, with the potential usefulness as a cardioprotective agent against ischemia/reperfusion-induced heart injury.

Acknowledgments

This work was supported by the Ministry of Commerce, Industry, and Energy through the Bio-Food and Drug Research Center at Konkuk University, Chungju, Korea.

References

6. Karmazyn M. Mechanisms of protection of the ischemic and reperfused myocardium by sodium-hydrogen exchange inhibi-


Hurtado C, Pierce GN. Inhibition of Na⁺/H⁺ exchange at the beginning of reperfusion is cardioprotective in isolated, beating adult cardiomyocytes. J Mol Cell Cardiol. 2000;32:1897–1907.


