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Effects of KR-32570, a new Na^+/H^+ exchanger inhibitor, on functional and metabolic impairments produced by global ischemia and reperfusion in the perfused rat heart

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Abstract

The present study was performed to evaluate the cardioprotective effects of [5-(2-methoxy-5-chloro-5-phenyl)furan-2-ylcarbonyl]guanidine (KR-32570) on ischemia/reperfusion-induced mechanical and metabolic dysfunction in isolated rat hearts. In addition, the effects of KR-32570 on the Na^+/H^+-exchanger (NHE) and lipid peroxidation were also evaluated. KR-32570 strongly inhibited the recovery from acidosis induced by an NH_4Cl prepulse in PS120 fibroblast cells expressing the human NHE-1 isoform (IC_{50}: 0.05 and 1.16 μM for KR-32570 and cariporide, respectively). In isolated perfused rat hearts subjected to 30-min ischemia/30-min reperfusion, KR-32570 (1–10 μM) significantly and concentration dependently improved cardiac contractile function and severe contracture in conjunction with causing a marked reduction in lactate dehydrogenase release. Additionally, it (1–10 μM) significantly increased the content of ATP, creatine phosphate and glycogen as well as decreased the tissue lactate content in heart homogenates following ischemia and reperfusion. KR-32570 (1–10 μM) significantly decreased the concentration of 8-*iso*-prostaglandin $F_{2\alpha}$, a reliable marker for oxidant stress, in perfusates from rat hearts subjected to ischemia and reperfusion. In separate experiments, KR-32570 significantly lowered the concentration of malondialdehyde in rat liver homogenate and inhibited Cu^{2+}-induced peroxidation of low-density lipoprotein. Taken together, these results suggest that KR-32570 possesses potent cardioprotective effects in perfused rat hearts, and its effects may be mediated by inhibition of NHE-1, preservation of high-energy phosphates, and inhibition of lipid peroxidation.

Keywords: KR-32570; Cardioprotective effect; Na^+/H^+ exchanger; Lipid peroxidation

1. Introduction

Intracellular Ca^{2+} overload during ischemia and reperfusion is an important pathophysiological factor that contributes to reduced post-ischemic recovery of mechanical function. It has been demonstrated that the Na^+/H^+ exchanger (NHE) is largely responsible for the excessive Ca^{2+} influx during ischemia and reperfusion (Karmazyn et al., 1999; Gumina et al., 2001; Doggrell and Hancox, 2003; Masereel et al., 2003). To date, eight isoforms of NHE have been identified and designated NHE-1 to NHE-8 (Goyal et al., 2003; Doggrell and Hancox, 2003). NHE-1 is the most predominant isoform expressed in the heart, where it contributes to cardiomyocyte pH homeostasis (Doggrell and Hancox, 2003; Masereel et al., 2003). Although NHE activation is essential for the restoration of physiological pH, hyperactivation of NHE-1 during cardiac ischemia/reperfusion leads to a dramatic increase in intracellular Na^+ concentration, which subsequently causes a marked increase in intracellular Ca^{2+} concentration through the Na^+/Ca^{2+} exchanger (Karmazyn et al., 1999; Gumina et al., 2001; Doggrell and Hancox, 2003; Masereel et al., 2003). The resulting intracellular Ca^{2+} overload has multiple potential deleterious effects, but could theoretically be reduced or prevented by NHE-1 inhibition. Actually, inhibition of

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NHE-1 has been shown to provide significant protection in a variety of models of myocardial ischemia/reperfusion with consistent improvement in functional recovery, metabolic status, attenuation of arrhythmias, preservation of cellular ultrastructure and inhibition of apoptosis (Wang et al., 2002; Masereel et al., 2003). In the past, amiloride and its derivatives have been extensively used to study NHE and its role during myocardial ischemia and reperfusion (Karmazyn, 1988). Although efficacious as cardioprotective agents, amiloride and its derivatives lack sufficient potency and selectivity to be used as therapeutic agents. Recently, cariporide and eniporide have been shown to be potent and selective inhibitors of NHE-1 with potential for the treatment of acute myocardial infarction (Scholz et al., 1995; Baumgarth et al., 1997). To date, however, there are no clinically successful drugs against ischemia/reperfusion injury (Kloner and Rezkalla, 2004).

It is known that the acylguanidine moiety is a pharmacophore of NHE-1 inhibitors (Shimada et al., 1996), although there are several exceptions. It has been suggested that a charged acylguanidine group at physiological pH may mimic a sodium ion at the binding site of the exchanger (Vigne et al., 1982). During the course of our efforts to discover novel inhibitors of NHE-1, we found that a series of analogues of (5-phenylfuran-2-carbonyl)guanidine exhibited potent inhibitory effects on NHE-1 (submitted to Journal of Medicinal Chemistry). Especially [5-(2-Methoxy-5-chloro-5-phenyl)furan-2-ylcarbonyl]guanidine (KR-32570) showed a greatly improved potency against NHE-1 activity compared to cariporide, and significantly improved cardiac contractile function. The present study was performed to evaluate the cardioprotective effects of KR-32570 on ischemia/reperfusion-induced mechanical and metabolic dysfunction in isolated rat hearts. In addition, effects of KR-32570 on lipid peroxidation were also evaluated.

2. Materials and methods

2.1. Measurement of Na^+/H^+-exchange activity in PS120/NHE-1 cell lines

NHE-1 inhibitory activity was used as our primary screen and was measured as the rate of NHE-1-mediated recovery of intracellular pH (pH_i) in a 96-well microplate using a pH-sensitive fluorescent dye, 2′,7′-bis-2-carboxyethyl-5-(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Sigma-Aldrich, MO, USA) (Russ et al., 1996; Ahmad et al., 2001). PS120 fibroblast cells expressing human NHE-1 (PS120/NHE-1 cell) were obtained from Professor J. Pouyssegur et al. (1984) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin/streptomycin (100× solution), 1% L-glutamine (200 mM solution), and 10% fetal bovine serum at 37 °C, in a humidified atmosphere of 5% CO_2 and 95% air. Cells were grown to confluency and then harvested from 100-mm culture dishes using Trypsin-EDTA solution. Cells were washed twice with Na-free buffer (composition in mM: choline chloride, 138.2; KCl, 4.9; $CaCl_2 \cdot 2H_2O$, 1.5; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; D-glucose, 15; HEPES, 20; pH 7.4) and incubated with 10 μM of BCECF-AM and 20 mM NH_4Cl at 37 °C for 30 min. The BCECF- and NH_4Cl-loaded cells were then washed, resuspended in Na-free buffer, and kept on ice.

Then 10 μl of the BCECF- and acid-loaded cells (2.5×10^4 cells) was added to 180 μl of HBS buffer containing NaCl (composition in mM: NaCl, 137; KCl, 4.9; $CaCl_2 \cdot 2H_2O$, 1.5; $MgSO_4 \cdot 7H_2O$, 1.2; KH_2PO_4, 1.2; D-glucose, 15; HEPES, 20; pH 7.4) with 10 μl of dimethyl sulfoxide (DMSO) or compounds in a 96-well microplate. NHE activity was initiated by NaCl, and fluorescence readings were taken every 30 s after the addition of acid-loaded cells to each microplate containing NaCl, at 444 nm excitation/535 nm emission and also at 485 nm excitation/535 nm emission, using a spectrofluorometer (GEMINI-XS, Molecular Device, CA, USA), at room temperature. The activity in the absence of NaCl was subtracted from the activity in the presence of NaCl. The initial increase in pH_i in response to NaCl was taken as an estimate of Na^+/H^+-exchange activity, and the inhibitory effect of the compounds was evaluated as the IC_{50} value (concentration required to inhibit pH_i recovery by 50%). Calibration of pH_i was accomplished using the high K^+-nigericin method (Thomas et al., 1979). The BCECF fluorescence ratio (485/444) was plotted against pH_i and fitted by linear regression.

2.2. Measurement of cardiac function in perfused rat hearts subjected to global ischemia and reperfusion

This study conformed with the Guide for the Care and Use of Laboratory animals, published by the U.S. National Institute of Health. Male Sprague–Dawley rats (weighing 380–420 g, Orient, Seoul, Korea) were anesthetized with sodium pentobarbital (60 mg/kg, i.p., Hanlim Pharm., Korea) (Lee et al., 2004). Heparin (1000 U/kg, Choongwae Pharm. Korea) was injected into the tail vein and then the trachea was intubated. While rats were mechanically ventilated with a rodent ventilator (Model 7025, Ugobasile, Italy), the chest was opened and their hearts were perfused in situ with oxygenated Krebs–Henseleit bicarbonate buffer (described herein) by retrograde aortic cannulation. The hearts were then excised and mounted on a Langendorff apparatus (Hugo Sachs Electronic, Hugstetten-March, Germany), where they were perfused with oxygenated Krebs–Henseleit bicarbonate buffer containing (in mM) NaCl 116, $NaHCO_3$ 24.9, KCl 4.7, $MgSO_4$ 1.1, KH_2PO_4 1.17, $CaCl_2$ 2.52, glucose 8.32 and pyruvate 2.0 at a constant perfusion pressure (80 mm Hg). The perfusion pressure was monitored with a pressure transducer (Isotec, Hugo Sachs Electronic) connected to the pressure amplifier (DC bridge Amp, Hugo Sachs Electronic), and a water-filled latex balloon attached to a metal cannular was placed inside the

left ventricle to measure the left ventricular pressure. Left ventricular end-diastolic pressure was adjusted to about 10 mm Hg before the start of the experiment by adjusting the volume in the intraventricular balloon with the aid of a micrometer syringe. Heart rate was counted with a tachometer (Heart rate module, Hugo Sachs Electronic). Coronary perfusion flow was measured from the amount of perfusate per minute. Cardiac contractile function was calculated by subtracting left ventricular end-diastolic pressure from left ventricular peak systolic pressure, yielding developed pressure. Cardiac temperature was maintained throughout the experiment by submerging the hearts in buffer at 37 °C; the buffer was allowed to accumulate in a chamber. Hearts showing arrhythmias or a left ventricular pressure <90 mm Hg or both were not used. After a 15-min equilibration period, and 10 min before the induction of global ischemia, vehicle (0.04% DMSO) or KR-32570 (1, 3 and 10 μM) was infused for 10 min. All hearts were subjected to 30 min of global ischemia and 30 min of reperfusion. Ischemia was initiated by completely shutting off the perfusate flow. Left ventricular developed pressure, left ventricular end-diastolic pressure, heart rate and coronary perfusion flow were measured before and 10 min after infusion of the drug, 10, 20 and 30 min after the induction of global ischemia, and 10, 20 and 30 min after reperfusion. The effluent perfusate was collected 30 min after reperfusion to measure the biochemical parameters including lactate dehydrogenase (LDH) activity. LDH activity in the perfusate was measured with assay reagents supplied by Sigma-Aldrich based on the technique of Wroblewski and La Due (1995), and normalized by the wet weight of the heart.

2.3. Determination of tissue metabolites in perfused rat hearts subjected to global ischemia and reperfusion

At the end of the experimental protocol for the isolated rat heart model of global ischemia and reperfusion, hearts were rapidly frozen in liquid nitrogen until the biochemical analysis was performed. The frozen hearts were transferred into 5 ml of 6% perchloric acid and homogenized using an Ultra-turrax (Ika® Works, Model T25 basic, Japan). The homogenates were centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatant was neutralized with 3.75 M of K_2CO_3 and was centrifuged again. Adenosine 5′-triphosphate (ATP) and creatine phosphate were measured spectrophotometrically in the supernatant according to the standard enzymatic procedure. The absorbance was read at 340 nm using a UV-spectrophotometer (Shimadzu UV-1601, Japan) fitted with a temperature controller (Shimadzu, Model CPS-240A, Japan). Lactate was also determined spectrophotometrically with a commercially available assay kit (absorbance at 550 nm, Randox Laboratories, UK). The unextracted pulverized tissue was used for the determination of glycogen content. The homogenates were incubated with 1 M $KHCO_3$ and amyloglycosidase solution (pH 4.8) for 2 h at 40 °C. Then, perchloric acid was added to the homogenates, which were then centrifuged at 10,000×*g* for 15 min at 4 °C. The glucose liberated after hydrolysis of glycogen was proportional to the increase in NADPH and measured by the extinction change of NADPH at 340 nm at 30 °C. The results are expressed as micromole per gram of heart weight.

2.4. Determination of 8-iso-prostaglandin $F_{2\alpha}$ in perfusates from rat hearts subjected to global ischemia and reperfusion

At the end of the experimental protocol for the isolated rat heart model of global ischemia and reperfusion, effluent perfusate was collected and stored at −20 °C until analyzed to measure of 8-*iso*-prostaglandin $F_{2\alpha}$. The level of 8-*iso*-prostaglandin $F_{2\alpha}$ in heart perfusate was determined using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Stressgen, Canada). Prior to the assay, the perfusates were hydrolyzed with 2 N NaOH for 2 h at 45 °C. Then, the perfusates were cooled and added to an equal volume of 2 N HCl, to neutralize then, and centrifuged at 10,000×*g* for 5 min at 4 °C.

2.5. Measurement of lipid peroxidation in liver homogenates from rat

Lipid peroxidation was determined by measuring the concentration of malondialdehyde in the liver homogenates according to the method of Ohkawa et al. (1979). Liver homogenates from normal rats were mixed with 0.2 ml of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid (pH adjusted to 3.5 with NaOH), and 1.5 ml of a 0.8% aqueous solution of thiobarbituric acid. The volume of the resultant solution was made up to 4.0 ml with distilled water and heated for 60 min at 95 °C. After cooling under tap water, 1.0 ml of distilled water and 5.0 ml of a mixture of *n*-butanol and pyridine (15:1, v/v) were added and the samples were shaken vigorously. After centrifugation (3500×*g*, 10 min), the absorbance at 532 nm of the organic layer was measured with a spectrophotometer (model DU 600, Beckman Coulter, Fullerton, CA, USA). Lipid peroxidation was calculated from the standard curve using the malondialdehyde tetrabutylammonium salt and expressed as concentration of nmol malondialdehyde per mg of protein.

2.6. Measurement of Cu^{2+}-mediated low-density lipoprotein oxidation

The formation of conjugated diene by oxidation of low-density lipoprotein (LDL) was measured using a Shimadzu UV-1601 UV-spectrophotometer (Japan) equipped with an auto-cell holder and controlled by the temperature controller. Human LDL (50 μg protein) was incubated with $CuSO_4$ (final concentration: 1.67 mM) in 0.1 M potassium phosphate buffer (pH 7.4) at 30 °C. The increase in conjugated diene as a result of Cu^{2+}-induced oxidation was measured continuously for 5 h in a 1 cm quartz cuvette at 234 nm.

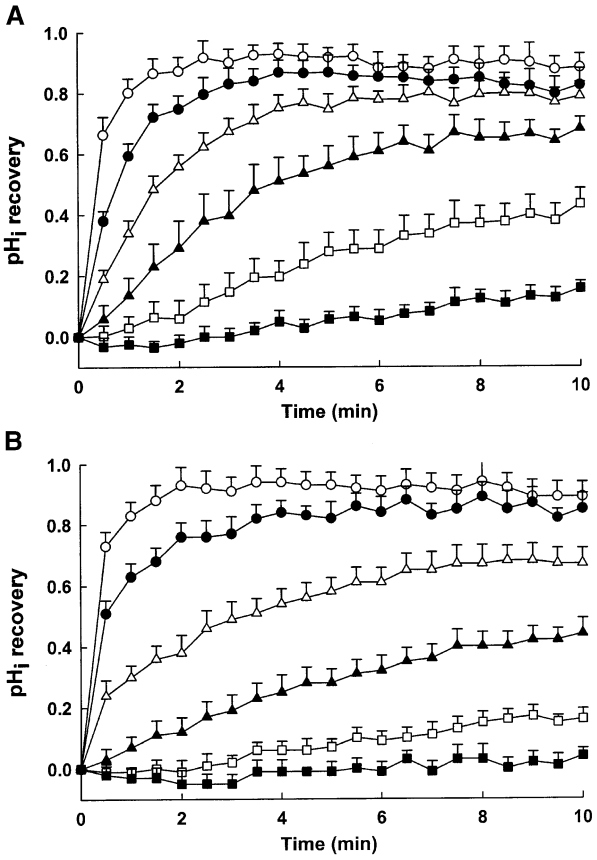

Fig. 1. Time course of pH_i (BCECF ratio) recovery from acidosis after NH_4Cl prepulse in PS120/NHE1 cell lines. Cariporide (A): vehicle (open circles), 0.1 (closed circles), 0.3 (open triangles), 1.0 (closed triangles), 3.0 (open square), and 10.0 μM (closed square). KR-32570 (B): vehicle (open circles), 0.01 (closed circles), 0.03 (open triangles), 0.1 (closed triangles), 0.3 (open square), and 1.0 μM (closed square). Values are expressed as means±S.E.M. (n=4).

2.7. Statistical analysis

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

3. Results

3.1. Inhibitory effects on Na^+/H^+-exchanger in PS120/NHE-1 cell lines

The Na^+/H^+ exchange activity in PS120/NHE-1 cell was measured as the recovery of pH_i following acidosis induced by the NH_4Cl prepulse technique. An NH_4Cl prepulse in the absence of Na^+ induced an immediate decrease in pH_i (from ~7.2 to ~6.4). Addition of NaCl increased the pH_i to ~7.2 through Na^+/H^+-exchange activation. The rate of pH_i recovery was almost linear during the first 60 s of the reaction. As shown in Fig. 1, KR-32570 and cariporide inhibited the pH_i recovery in a concentration-dependent fashion and almost completely inhibited the recovery at 0.3 and 10.0 μM for KR-32570 and cariporide, respectively. The IC_{50} value of KR-32570 was 0.05±0.01 μM. In this assay system, the IC_{50} value of cariporide, an inhibitor of Na^+/H^+-exchange, was 1.16±0.16 μM.

3.2. Cardioprotective effect of KR-32570 against injury induced by global ischemia and reperfusion in the perfused rat heart

The basal value of left ventricular developed pressure after vehicle treatment before the induction of global ischemia was 118±3 mm Hg in the rat heart (n=56). Global ischemia for 30 min abolished the left ventricular developed pressure within approximately 2–5 min after the start of global ischemia (Fig. 2A). Although the reduced cardiac contractility gradually recovered following reperfusion, the recovery of left ventricular developed pressure was very poor (13.3±1.3% in vehicle group), which is indicative of severe ischemia/reperfusion damage. KR-32570 significantly and concentration dependently restored the reduction in left ventricular developed pressure during reperfusion (E_{max}: 28.2±4.4%, 32.6±5.7% and 41.0±6.2% at 1.0, 3.0 and 10.0 μM, respectively, P<0.05) compared with that of the vehicle-treated group (13.3±1.3%), without changing the basal contractility. An increase in left ventricular end-diastolic pressure, which is an indicator of cardiac contracture, was observed approximately 20 min after coronary perfusion was stopped and the pressure continued to increase during global ischemia in the vehicle group (Fig. 2B). Reperfusion of the heart was associated with a further elevation of the left ventricular end-diastolic pressure, suggesting the existence of reperfusion injury, and the left ventricular end-diastolic pressure still remained high even 30 min after reperfusion. Although KR-32570 did not attenuate the cardiac contracture during global ischemia even at the highest concentration, it concentration dependently inhibited progression of the cardiac contracture after reperfusion (Fig. 2B). The LDH released into the coronary perfusate of the vehicle group (28.3±1.8 U/g) was clearly increased 30 min after reperfusion (not detected in baseline), indicating that cardiac myocytes had been injured by the global ischemia and reperfusion (Fig. 2C). KR-32570 also reduced the release of LDH from injured cardiac cells in a concentration-dependent manner (22.1±2.4, 17.2±1.6 and 14.1±3.3 U/g at 1.0, 3.0 and 10.0 μM, respectively). The heart rate was abolished by global ischemia, but it rapidly recovered to 90–95% of the pre-ischemic value. Heart rate was not significantly influenced by treatment with KR-32570 as compared with vehicle. Coronary perfusion flow in the vehicle group decreased 30 min after reperfusion compared with the baseline value, without any significant difference among groups (84.9±5.7%, 84.2±4.1%, 91.7±6.0%, 92.2±8.9% in vehicle, 1.0, 3.0 and 10.0 μM of KR-32570, respectively).

3.3. Protective effects on tissue metabolites

Figs. 3 and 4 show the concentration of metabolites in rat hearts subjected to global ischemia and reperfusion. KR-32570 significantly increased the content of high-energy phosphates—ATP and creatine phosphate. KR-32570 also increased glycogen stores as well as decreased the tissue lactate content following global ischemia and reperfusion.

3.4. Inhibitory effects on 8-iso-prostaglandin $F_{2\alpha}$

As shown in Fig. 5A, KR-32570 significantly decreased the level of 8-*iso*-prostaglandin $F_{2\alpha}$, a reliable marker of oxidant stress, in a concentration-dependent manner (141.41±0.07, 141.14±0.27, 140.91±0.08 and 140.55±

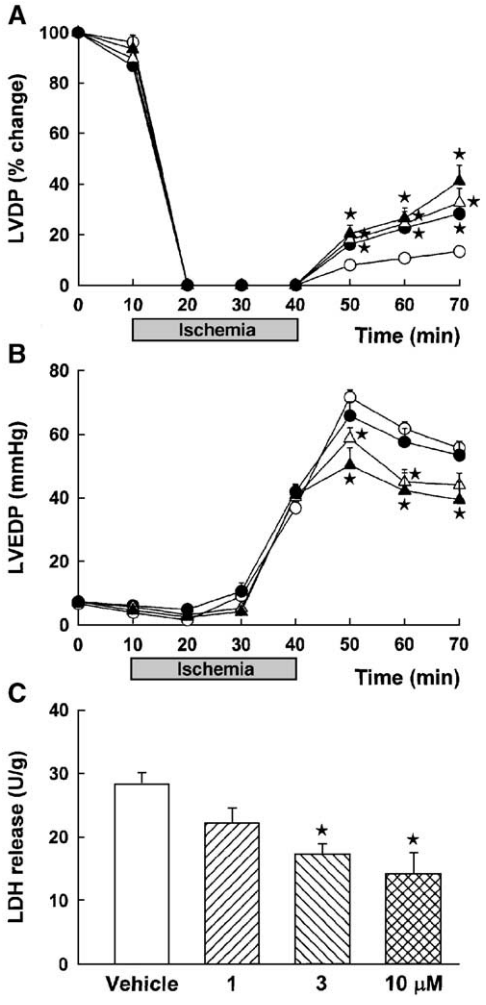

Fig. 2. Cardioprotective effects of KR-32570 on changes in the left ventricular developed pressure (LVDP, A), left ventricular end-diastolic pressure (LVEDP, B), and lactate dehydrogenase (LDH) release (C) induced by global ischemia and reperfusion in perfused rat hearts. The time course of changes in left ventricular developed pressure and left ventricular end-diastolic pressure was measured before, during, and after global ischemia. LDH release from the injured heart was measured 30 min after reperfusion. Vehicle (open circles, n=56) and 1 (closed circles, n=17), 3 (open triangles, n=11), and 10 μM (closed triangles, n=10) KR-32570. Values are expressed as means±S.E.M. ★P<0.05, significantly different from the vehicle group.

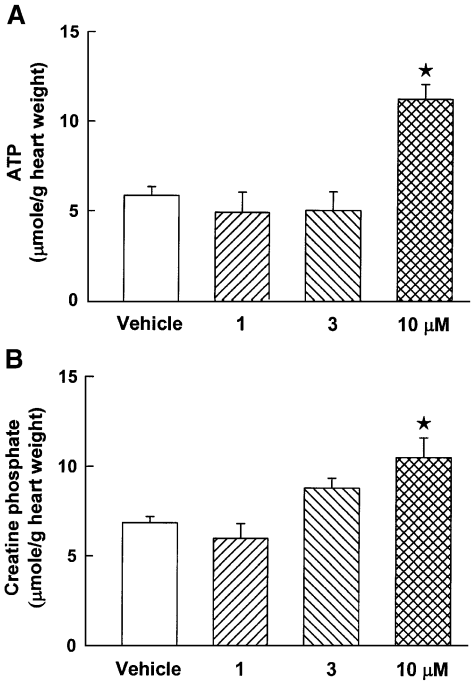

Fig. 3. Effects of KR-32570 on changes in tissue ATP (A) and creatine phosphate (B) contents induced by global ischemia and reperfusion in perfused rat hearts. Vehicle (n=16 and 17 for ATP and creatine phosphate, respectively) and 1 (n=9 and 7 for ATP and creatine phosphate, respectively), 3 (n=8), and 10 μM (n=8) KR-32570. Values are expressed as means±S.E.M. ★P<0.05, significantly different from the vehicle group.

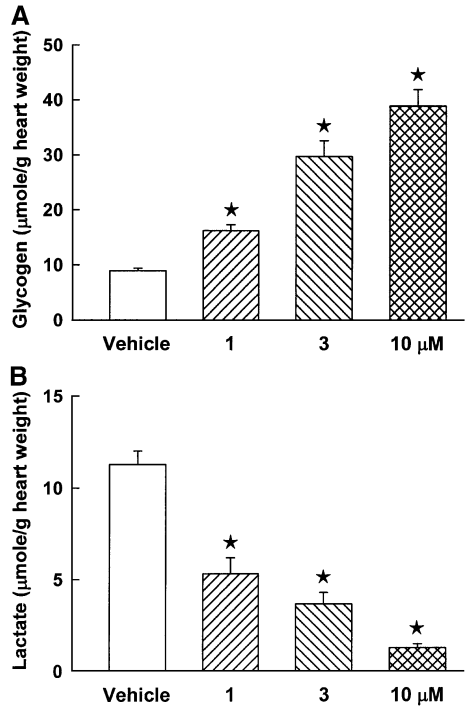

Fig. 4. Effects of KR-32570 on changes in tissue glycogen (A) and lactate (B) contents induced by global ischemia and reperfusion in perfused rat hearts. Vehicle (n=16 and 14 for glycogen and lactate, respectively) and 1 (n=9), 3 (n=8), and 10 μM (n=8) KR-32570. Values are expressed as means±S.E.M. ★P<0.05, significantly different from the vehicle group.

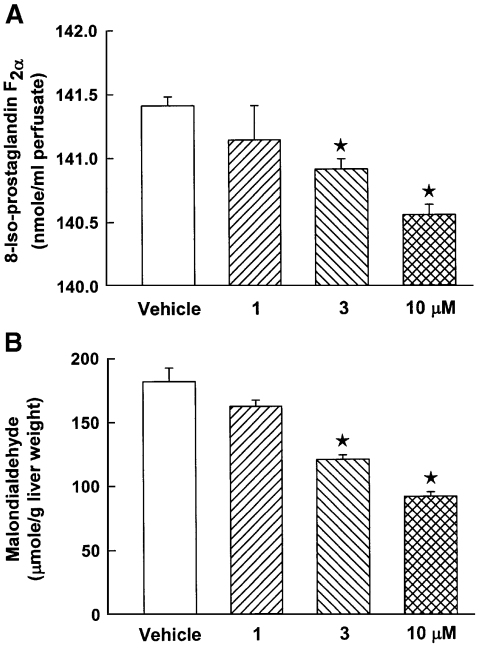

Fig. 5. Inhibitory effects of KR-32570 on the concentration of 8-*iso*-prostaglandin $F_{2\alpha}$ (A) and malondialdehyde (B). The concentration of 8-*iso*-prostaglandin $F_{2\alpha}$ was measured in heart perfusates from isolated rat hearts subjected to global ischemia and reperfusion. Malondialdehyde was measured in rat liver homogenates. Vehicle (n=7 and 10 for 8-*iso*-prostaglandin $F_{2\alpha}$ and malondialdehyde, respectively) and 1 (n=9), 3 (n=8), and 10 μM (n=10) KR-32570. Values are expressed as mean±S.E.M. ★P<0.05, significantly different from the vehicle group.

0.08 nmol/ml perfusate for vehicle, 1, 3 and 10 μM, respectively) in heart perfusate from rat hearts subjected to global ischemia and reperfusion.

3.5. Inhibitory effects of lipid peroxidation in liver homogenates from rat

As shown in Fig. 5B, the concentration of malondialdehyde was significantly lower after KR-32570 (181.6±10.5, 162.1±4.7, 120.2±3.6 and 90.9±3.4 μmol/g liver weight for vehicle, 1, 3 and 10 μM, respectively) compared with vehicle in liver homogenate prepared from normal rats.

3.6. Inhibitory effects of conjugated diene formation of LDL oxidation

The oxidation of LDL catalyzed by Cu^{2+} ions proceeded in two time phases: a lag phase (~120 min) and a propagation phase (~270 min, Fig. 6). The rate of lipid peroxidation increased more rapidly in the propagation phase than in the lag phase. KR-32570 concentration dependently inhibited the oxidation of LDL during the propagation phase.

4. Discussion

This study demonstrated that KR-32570, a newly synthesized inhibitor of the NHE-1, has a potent NHE-1 inhibitory and cardioprotective effect against injury induced by global ischemia and reperfusion in the perfused rat heart.

In the assay of Na^+/H^+-exchange inhibition, we assessed the effect of KR-32570 and cariporide on Na^+/H^+-exchange in PS120 fibroblast cells expressing the human NHE-1 isoform. In this experimental set-up, pH_i recovery from acidosis is exclusively mediated by acid extrusion by Na^+/H^+-exchange. KR-32570 concentration dependently inhibited the recovery from acidosis induced by an NH_4Cl prepulse in PS120/NHE-1 cells, and its IC_{50} value was 0.05 μM, ~20 times lower than that of cariporide (1.16 μM), a well-known NHE-1 inhibitor. The effect of KR-32570 on ischemia/reperfusion injury may be due to suppression of the intracellular Ca^{2+} overload, which is a conceivable consequence of inhibition of the NHE-1 after ischemia and reperfusion. Moreover, KR-32570 has a good selectivity for NHE-3 and epithelial Na^+ channels (IC_{50} values>30 μM) and a slow dissociation pattern, as shown in a kinetic study with PS120/NHE-1 cells, indicating that inhibition of NHE-1 persists even after rinsing out of KR-32570 (personal communication with Dr. M. K. Lee, Yonsei University, Korea). This long-lasting inhibitory effect on NHE-1 was observed with sabiporide also, but not with cariporide (NHE-1 inhibitory % at 5 min after washout; 4.7%, 90.0% and 90.4% for cariporide, sabiporide and KR-32570, respectively), as has been also demonstrated by another research group (Touret et al., 2003). The unique characteristics of KR-32570, with a different kinetic profile from that of other NHE-1 inhibitors, might be helpful for obtaining better results with in vivo animal studies or clinical tests.

In perfused rat hearts subjected to global ischemia and reperfusion, KR-32570 significantly improved reperfusion cardiac contractile function (left ventricular developed pressure) compared with that of the vehicle treated group in a concentration-dependent manner, without changing the basal contractility. The maximal reduction in left ventricular developed pressure induced by KR-32570 was not as great as we expected (E_{max}: 41.0% at 10.0 μM) and was similar to

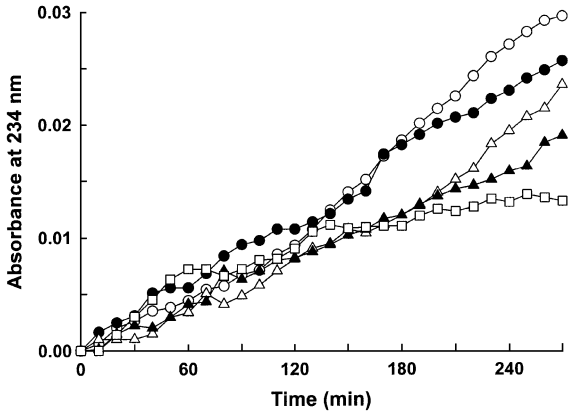

Fig. 6. Kinetics of conjugated diene formation by Cu^{2+}-mediated LDL oxidation. Vehicle (open circles) and 0.1 (closed circles), 0.3 (open triangles), 1.0 (closed triangles), and 3.0 μM (open square) KR-32570. Values are expressed as mean of four experiments.

that of cariporide (unpublished data). This result might be because our experimental conditions for inducing ischemia were too severe, resulting in severe ischemia/reperfusion damage of perfused rat hearts. KR-32570 induced a significant and concentration-dependent improvement of the severe contracture and a marked reduction in reperfusion-induced LDH release, further indicating its cardioprotective activity. After reperfusion, ATP and creatine phosphate were measured in the reperfused ischemic myocardium to estimate the production and transduction of high-energy phosphate (Ichihara and Abiko, 1984; Nakai et al., 1996), and the content of glycogen was determined as an index of anaerobic ATP production (Cross et al., 1996). KR-32570 significantly increased the content of ATP and creatine phosphate, and glycogen stores as well as decreased the tissue lactate content following global ischemia and reperfusion. These results suggest that the effects of KR-32570 on Na^+/H^+-exchange could be associated with an improved metabolic status as well as inhibition of NHE-1.

The main mechanisms causing irreversible ischemia/reperfusion injury include oxygen free radical formation as well as Ca^{2+} overload and depletion of high-energy phosphate stores (Wang et al., 2002). Since oxidative stress caused by lipid or protein oxidation through intracellular free radical generation may aggravate a perturbed membrane function and mitochondrial dysfunction which result in reperfusion injury (Siesjo et al., 1995), we investigated the effects of KR-32570 on lipid peroxidation. The level of 8-*iso*-prostaglandin $F_{2\alpha}$, a representative isoprostane, is increased in coronary artery diseases (Pratico et al., 1997; Mehrabi et al., 1999) and is used as an in vivo marker of oxidative stress-generation of free radicals and lipid peroxidation (Basu et al., 2000; Favreau et al., 2004). KR-32570 significantly decreased the concentration of 8-*iso*-prostaglandin $F_{2\alpha}$ in perfusates from rat hearts subjected to global ischemia and reperfusion. In separate experiments, KR-32570 significantly lowered the concentration of malondialdehyde, a marker of lipid peroxidation, compared with the effect of vehicle in liver homogenates prepared from normal rats. These results were confirmed again by showing that KR-32570 concentration dependently inhibited Cu^{2+}-induced peroxidation of LDL.

In conclusion, the results from the present study suggest that KR-32570 has potent cardioprotective effects in perfused rat hearts subjected to global ischemia and reperfusion. Its effects may be mediated through the inhibition of NHE-1, the preservation of high-energy phosphates, and the inhibition of lipid peroxidation.

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