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KR-32570, a novel Na^+/H^+ exchanger-1 inhibitor, attenuates hypoxia-induced cell death through inhibition of intracellular Ca^{2+} overload and mitochondrial death pathway in H9c2 cells

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Abstract

A novel Na^+/H^+ exchanger-1 (NHE-1) inhibitor [5-(2-methoxy-5-chloro-5-phenyl)furan-2-ylcarbonyl]guanidine (KR-32570) has been previously demonstrated to elicit cardioprotective effect against ischemic injury in rat heart. In the present study, we examined the effects of KR-32570 on cell death induced by hypoxic insult in heart-derived H9c2 cells. Treatment with KR-32570 (1–10 μM) significantly reduced hypoxia-induced necrotic cell death (lactate dehydrogenase release) and apoptotic cell death (TUNEL-positivity, caspase-3 activity). KR-32570 also decreased the cytosolic and mitochondrial Ca^{2+} overload induced by hypoxia. Inhibition of mitochondrial Ca^{2+} overload by ruthenium red mimicked the anti-apoptotic effect of KR-32570. In addition, KR-32570 significantly recovered the large reduction in mitochondrial membrane potential ($\Delta\Psi_m$) and cytochrome *c* release induced by hypoxia. Taken together, our results suggest that a new NHE-1 inhibitor KR-32570 elicits potent cardioprotective effects in H9c2 cells, and its effects may be mediated by inhibition of intracellular Ca^{2+} overload and mitochondrial death pathway during hypoxia.
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Keywords: KR-32570; Na^+/H^+ exchanger; H9c2 cardiomyocyte; Hypoxia1

1. Introduction

Characteristic features of myocardial ischemia include intracellular acidosis resulting from anaerobic glycolysis during ischemia (Tani and Neely, 1989). Intracellular acidosis is known to stimulate several pH regulating systems including Na^+/H^+ exchanger (NHE) (Grinstein et al., 1992; Noel and Pouyssegur, 1995). Among the multiple subtypes of NHE, NHE-1 is the predominant isoform in cardiomyocytes (Karmazyn et al., 1999). NHE-1 activation is physiologically essential for maintaining intracellular pH and regulating intracellular Na^+ level. However, excessive activation of NHE-1 during cardiac ischemia leads to a remarkable elevation of intracellular Na^+ concentration, subsequently causing cytosolic Ca^{2+} overload through the Na^+/Ca^{2+} exchanger (NCX) (Masereel et al., 2003). Therefore, inhibition of NHE-1 activation has been thought to protect the heart from ischemic injury by reducing cytosolic Ca^{2+} overload (Hurtado and Pierce, 2001). Indeed, diverse NHE-1 inhibitors have been developed and demonstrated to produce cardioprotective effects in various models of myocardial ischemia/reperfusion with a marked improvement in functional recovery, attenuation of arrhythmias (Wang et al., 2002; Masereel et al., 2003). Recently, a well-known NHE-1 inhibitor cariporide has showed anti-apoptotic effect against hypoxic injury through mitochondrial Ca^{2+}-dependent manner in cultured cardiomyocyte (Sun et al., 2004).

On the basis of previous reports that the acylguanidine moiety is a pharmacophore of NHE-1 inhibitors (Shimada et al., 1996), we have focused on finding novel NHE-1 inhibitory guanidine analogues. In such attempts, we found that a new

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guanidine derivative, [5-(2-methoxy-5-chloro-5-phenyl)furan-2-ylcarbonyl]guanidine (KR-32570) showed a greater potency against NHE-1 activity compared to cariporide, and markedly improved cardiac contractile function in isolated rat heart ischemic model (Lee et al., 2005a,b). Furthermore, it has been demonstrated that KR-32570 has a potent cardioprotective effect in rat infarct model in vivo (Lee et al., in press) and that its cardioprotective effects is mediated by inhibition of NHE-1 (Lee et al., 2005a). In vitro effects of KR-32570, however, have not been elucidated in cultured cardiomyocytes. Therefore, the present study was designed to investigate the effects of KR-32570 on hypoxia-induced cell death in heart-derived H9c2 cells.

2. Methods

2.1. Cell culture and induction of hypoxia

H9c2 cells, myoblasts cell line originally derived from rat heart, were purchased from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5.5 mM glucose supplemented with 10% fetal bovine serum. For hypoxic challenges, H9c2 cells were transferred into an anaerobic chamber (Forma Scientific, Marietta, OH, USA) maintained at 37 °C with a humidified atmosphere of 5% CO_2, 10% H_2 and 85% N_2 as previously described (Moon et al., 2000).

2.2. Chemicals and treatments

KR-32570, [5-(2-methoxy-5-chloro-5-phenyl)furan-2-ylcarbonyl]guanidine, and cariporide, a known NHE-1 inhibitor were synthesized at Bio-organic Division of Korea Research Institute of Chemical Technology (Daejon, Korea). KR-32570 and cariporide were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 0.1% and this concentration of DMSO was found to have no effect on H9c2 cell viability. Cells were treated with KR-32570 (1, 3 or 10 μM) or cariporide (1, 3 or 10 μM) 1 h before and during hypoxia. The concentrations and durations of drug-treatment were chosen on the basis of preliminary studies (data not shown). Fluo-3, rhod-2 and rhodamine 123 were obtained from Molecular probes (Eugene, OR, USA), and ruthenium red from Calbiochem (San Diego, CA, USA).

2.3. Lactate dehydrogenase (LDH) assay

To measure overall cell injury, we assayed the activity of LDH released into the medium 10 h after hypoxic insult as described previously (Moon et al., 2000), i.e., by spectrophotometric analysis at 340 nm.

2.4. In situ terminal deoxynucleotidyl transferase UTP nick end labeling (TUNEL) assay

To examine the extent of apoptotic cell death, we performed TUNEL-staining after 8 h of hypoxia with a commercially available ApopTag Plus kit (Oncor, Gaithersburg, MD, USA), as described previously (Jung et al., 2003).

2.5. Caspase-3 activity assay

The cells were lysed with lysis buffer (10 mM Tris/HCl, 0.32 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 1 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM EDTA, and 10 mM dithiothreitol (DTT), pH 8.0) for 30 min and the lysates were centrifuged (10,000 ×*g* for 5 min at 4 °C). Samples (200 μg) of the extracted protein were incubated with the reaction buffer (100 mM HEPES, 10% sucrose, 0.1% 3-[3-cholamidopropylammonio]-1-propanesulfonate (CHAPS), pH 7.5, 10 mM DTT and 10 μg/ml leupeptin) to 100 μl volume containing 200 μM Ac-DEVD-*p*-Na (Biomol, Plymouth meeting, PA, USA). Enzyme-catalyzed release of *p*-nitroanilide was measured at 405 nm using microplate reader (Molecular Devices, Palo Aldo, CA, USA).

2.6. Measurement of cytosolic Ca^{2+} level

The cytosolic Ca^{2+} level was measured after loading cells with the fluo-3 acetoxymethyl ester (Molecular probes, Eugene, OR, USA), a selective fluorescent indicator for cytosolic Ca^{2+} (Yao et al., 1997). H9c2 cells plated on glass-bottom dishes were washed three times with HEPES-buffered control salt solution (HCSS) containing 120 mM NaCl, 5 mM KCl, 1.6 mM $MgCl_2$, 2.3 mM $CaCl_2$, 15 mM glucose, 20 mM HEPES, and 10 mM NaOH. The cells were loaded with 5 μM fluo-3 for 20 min at 37 °C in HCSS. After washing with HCSS, the cells were observed with a confocal microscope (Olympus, Japan) and fluo-3 fluorescence was excited at 480 nm and emitted at 520 nm. The fluorescence intensity of fluo-3 was quantified using image-analysis computer software (Fluoview FV300; Olympus, Japan).

2.7. Measurement of mitochondrial Ca^{2+} level

The mitochondrial Ca^{2+} level was measured after loading cells with the Ca^{2+} fluorophore rhod-2 acetoxymethyl ester (Molecular probes, Eugene, OR, USA), a selective fluorescent indicator for mitochondrial Ca^{2+} (Trollinger et al., 1997). The loading solution was then replaced by HCSS containing 2 μM rhod-2 and the cells were incubated for 30 min at 4 °C and then incubated for 30 min at 37 °C (Sato et al., 2005). The cells were observed with a confocal microscope (Olympus, Japan) and rhod-2 fluorescence was excited at 560 nm and emitted fluorescence was collected through a 590 nm long pass barrier filter. The fluorescence intensity of rhod-2 was quantified using image-analysis computer software (Fluoview FV300; Olympus, Japan).

2.8. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was assayed by measuring the accumulation of rhodamine 123 (Molecular probes, Eugene, OR, USA), a membrane-permeable cationic fluorescent dye (Emaus et al.,

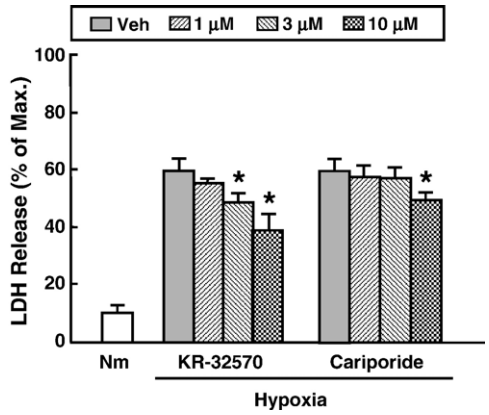

Fig. 1. Effect of KR-32570 on hypoxia-induced necrotic cell death. H9c2 cells were treated with KR-32570 (1, 3 and 10 μM) and cariporide (1, 3 and 10 μM) 1 h before and during hypoxia. Lactate dehydrogenase (LDH) release was measured 10 h after hypoxic insult. Percent LDH was calculated from the maximum LDH release (100%) induced by 0.1% Triton X-100. The data are mean±S.D. (n=5). Veh, Vehicle; Nm, Normoxia. *P<0.05 vs. Veh-hypoxia.

1986). H9c2 cells were loaded with 1 μM rhodamine 123 in HCSS. The cells were incubated for 10 min at 37 °C and washed three times with HCSS. The fluorescence intensity of the cell was then measured by flow cytometry (BD Biosciences, Lexington, KY, USA).

2.9. Separation of cytosolic and mitochondrial fractions and Western blot analysis for cytochrome c release

The cells for lysate preparation were washed twice with ice-cold phosphate buffered saline (PBS) and collected by centrifugation at 1000 ×g for 10 min at 4 °C. The cell pellets were washed once with ice-cold PBS and resuspended in lysis buffer (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1.0 mM sodium EDTA, 1.0 mM sodium EGTA, 1.0 mM DTT, 0.1 mM PMSF, and 250 mM sucrose) supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin). The cells were then homogenized in a glass homogenizer, and the nuclei and cell debris were removed by centrifugation at 1000 ×g for 15 min at 4 °C. The supernatants were further centrifuged at 10,000 ×g for 15 min at 4 °C, and the resulting mitochondrial pellets were resuspended in lysis buffer. The supernatants created from the 10,000 ×g centrifugation were centrifuged once more at 100,000 ×g for 1 h at 4 °C, and the supernatant was collected and designated the cytosolic fraction. To quantify cytochrome c release, Western blot analysis in the mitochondrial and the cytosolic fractions was performed as described previously (Kim et al., 2004). We verified the mitochondrial fraction with the mitochondrial marker cytochrome oxidase subunit IV (COX IV, Molecular Probes, Eugene, OR, USA), and cytosolic fractions with the cytosol marker actin (Sigma, St. Louis, MO, USA).

2.10. Statistical analysis

All data were expressed as mean±S.D. The numerical data were compared using a one-way analysis of variance (ANOVA) followed by a posttest such as Bonferoni test. A P-value of <0.05 was considered significant.

3. Results

3.1. Effect of KR-32570 on hypoxia-induced cell death

To evaluate whether KR-32570 protects H9c2 cells from hypoxia, we measured LDH activity released during hypoxia, as an indicator for necrotic cell death (Filipovic et al., 1999). In H9c2 cells exposed to hypoxia for 10 h, LDH release was increased up to about 59.5±4.3% compared to that in the normoxic condition (8.8±3.8%). As shown in Fig. 1, hypoxia-induced cell death (59.5±4.3%) was inhibited by treatment with 3 and 10 μM KR-32570 (48.3±3.1% and 38.9±5.2%, respectively) and 10 μM cariporide (43.6±3.0%). Fig. 2A shows the protective effects of KR-32570 and cariporide against hypoxia-induced cell death by TUNEL staining, as a well-known indicator of apoptotic cell death. The number of TUNEL-positive cells was 43.5±1.8% in vehicle-treated cells exposed to hypoxia for 8 h. When cells were treated with

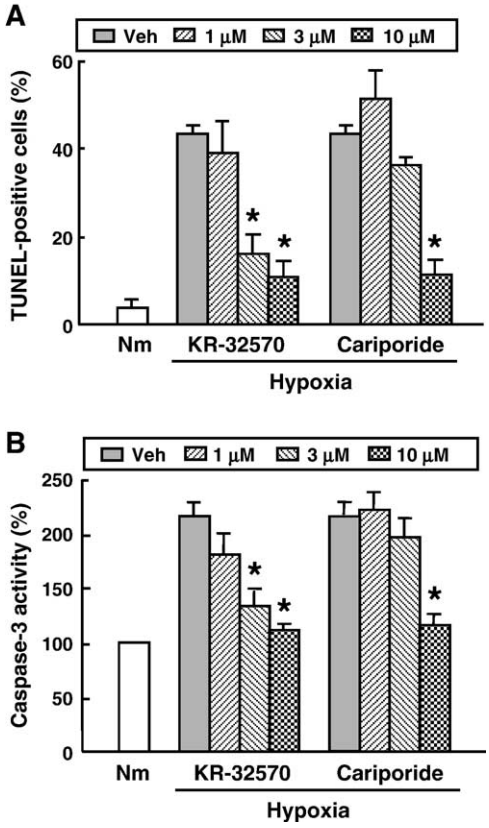

Fig. 2. Effect of KR-32570 on hypoxia-induced apoptotic cell death. (A) Quantitative analysis of TUNEL-positive cells. H9c2 cells were treated with KR-32570 (1, 3, and 10 μM) and cariporide (1, 3, and 10 μM) 1 h before and during hypoxia. Cell death (%) was calculated by dividing the number of TUNEL-stained cells by total number of cells 8 h after hypoxic insult. (B) Effect of KR-32570 on hypoxia-induced caspase-3 activation. H9c2 cells were treated with KR-32570 (1, 3, and 10 μM) and cariporide (1, 3, and 10 μM) 1 h before and during hypoxia (8 h). Data shown are mean±S.D. (n=5). Veh, Vehicle; Nm, Normoxia. *P<0.05 vs.Veh-hypoxia.

several concentrations (3 and 10 μM) of KR-32570, the number of TUNEL-positive cells after 8 h of hypoxia was significantly decreased (16.3±4.2% and 10.8±3.6%, respectively), suggesting an anti-apoptotic efficacy of KR-32570 in H9c2 cells. And the number of TUNEL-positive cells during hypoxia was decreased by only 10 μM cariporide (11.5±3.1%). Caspase-3 activity was analyzed from the cytosolic fraction of H9c2 cells 8 h after hypoxic insult. As shown in Fig. 2B, hypoxia increased caspase-3 activity (215.6±13.4%) compared to the normoxic control (100%). The treatment of cells with 3 and 10 μM KR-32570 (133±17.0% and 111.1±6.4%, respectively) markedly decreased the caspase-3 activity nearly to the level of normoxia-vehicle. The treatment of cells with 10 μM cariporide (116.6±10.4%) markedly decreased the caspase-3 activity. KR-32570 and cariporide alone did not affect caspase-3 activity in the normoxic condition up to 10 μM concentration (data not shown).

3.2. Effect of KR-32570 on cytosolic Ca^{2+} overload

Preservation of intracellular ion homeostasis is a normal potential mechanism of cardioprotection by NHE inhibition (Teshima et al., 2003). As shown in Fig. 3A, the cytosolic Ca^{2+} during hypoxia began to increase at 15 min after hypoxia and further increased as maximum level (about 2.5 fold) after 30 min of hypoxia, with subsequent decrease with hypoxic time. The reason for the subsequent decrease may be due to the mechanism for cytosolic Ca^{2+} homeostasis such as extracellular Ca^{2+} efflux and mitochondrial Ca^{2+} uptake (Gunter et al., 1994). We, thereafter, observed the effect of KR-32570 on cytosolic Ca^{2+} at 30 min of hypoxia. The cytosolic Ca^{2+} increased after 30 min of hypoxia was significantly decreased by treatment with 3 and 10 μM KR-32570 and 10 μM cariporide (Fig. 3B). KR-32570 and cariporide alone did not affect in normoxic condition up to 10 μM concentration (data not shown).

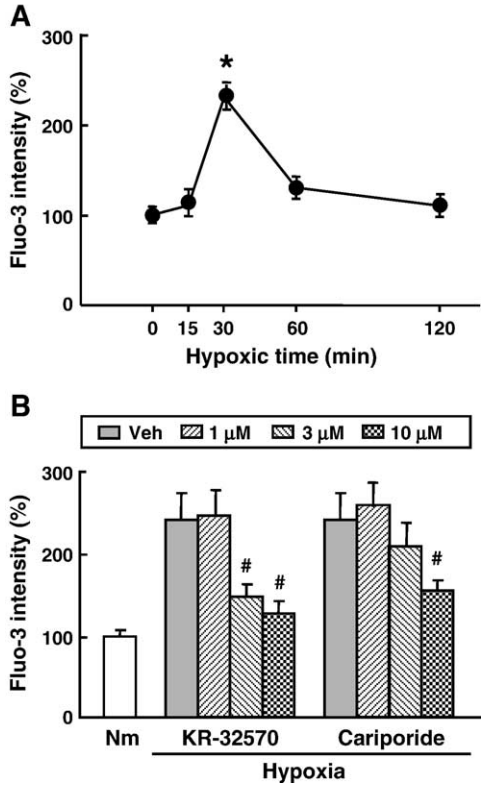

Fig. 3. Effect of KR-32570 on hypoxia-induced cytosolic Ca^{2+} overload. (A) Time course of cytosolic Ca^{2+} change during hypoxia, shown by fluorescence intensity of fluo-3 in H9c2 cells. (B) Quantitative analysis of fluo-3 intensity after 30 min of hypoxia. H9c2 cells were treated with KR-32570 (1, 3 and 10 μM) and cariporide (1, 3 and 10 μM) 1 h before and during hypoxia. The data shown are mean±S.D. ($n=5$). Veh, Vehicle; Nm, Normoxia. *$P<0.05$ vs. time 0 (control). #$P<0.05$ vs. Veh-hypoxia.

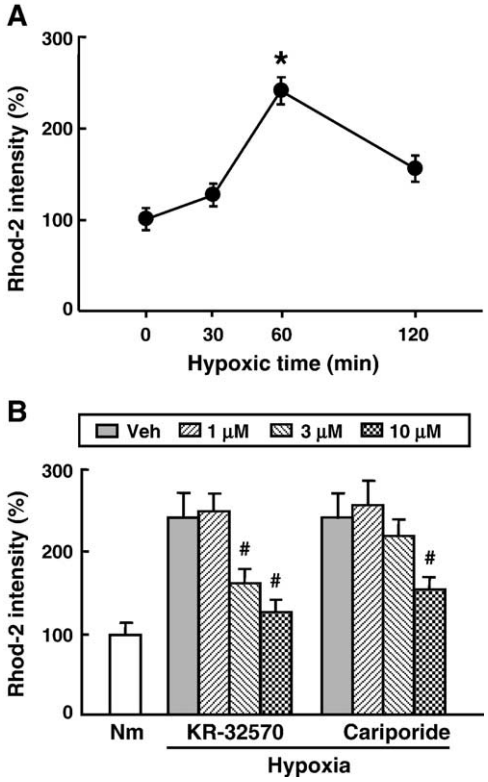

Fig. 4. Effect of KR-32570 on hypoxia-induced mitochondrial Ca^{2+} overload. (A) Time course of mitochondrial Ca^{2+} change during hypoxia by measuring fluorescence intensity of rhod-2 in H9c2 cells. (B) Quantitative analysis of rhod-2 intensity after 1 h of hypoxia. H9c2 cells were treated with KR-32570 (1, 3 and 10 μM) and cariporide (1, 3 and 10 μM) 1 h before and during hypoxia. The data shown are mean±S.D. ($n=5$). Veh, Vehicle; Nm, Normoxia. *$P<0.05$ vs. time 0 (control). #$P<0.05$ vs.Veh-hypoxia.

3.3. Effect of KR-32570 on mitochondrial Ca^{2+} overload

As shown in Fig. 4A, rhod-2 fluorescence was remarkably augmented by hypoxia. The mitochondrial Ca^{2+} during hypoxia began to increase at 30 min after hypoxia and further increased as maximum level (about 2.5 fold) after 1 h of hypoxia, and then recovered toward basal levels during the next hour. This following decrease with hypoxic time may be attributed to the mechanisms for mitochondrial Ca^{2+} efflux through Na^+/Ca^{2+} exchanger and Ca^{2+}/H^+ exchanger system (Gunter et al., 1994). We, thereafter, observed the effect of KR-32570 and cariporide on mitochondrial Ca^{2+} at 1 h of hypoxia. The mitochondrial Ca^{2+} increased at 1 h of hypoxia was decreased by treatment

with 3 and 10 μM KR-32570 and 10 μM cariporide (Fig. 4B). KR-32570 and cariporide alone did not affect mitochondrial Ca^{2+} in the normoxia (data not shown).

3.4. The role of cytosolic Ca^{2+} and mitochondrial Ca^{2+} on hypoxia-induced cell death

To determine whether increased mitochondrial Ca^{2+} is necessary for apoptotic and/or necrotic cell death, we employed ruthenium red, an inhibitor of mitochondrial Ca^{2+} uniporter (Griffiths, 2000). As shown in Fig. 5A, hypoxia-induced LDH release after 10 h of hypoxia (69.7±2.1%) was increased (81.1±3.7%) by treatment with 30 μM ruthenium red. Also, the number of TUNEL-positive cells was 39.6±0.8% in vehicle-treated cells exposed to hypoxia for 8 h, and this number was significantly decreased by treatment with ruthenium red (4.2±0.3%, Fig. 5B). To investigate the time course effect of ruthenium red on cytosolic Ca^{2+}after hypoxic insult, we measured cytosolic Ca^{2+} level using the fluo-3. As shown in Fig. 5C, fluo-3 fluorescence augmented at 30 min of hypoxia was prolonged by treatment with 30 μM ruthenium red. To evaluate whether 30 μM ruthenium red inhibited mitochondrial Ca^{2+} uptake in H9c2 cells exposed to hypoxia, we measured mitochondrial Ca^{2+} level using the rhod-2. As shown in Fig. 5D, rhod-2 fluorescence augmented at 1 h of hypoxia was inhibited by treatment with 30 μM ruthenium red.

3.5. Effect of KR-32570 on hypoxia-induced reduction in $\Delta\Psi_m$

To examine whether preservation of $\Delta\Psi_m$ is associated with cardioprotective effects by KR-32570, we assessed the change of rhodamine 123 fluorescence (Emaus et al., 1986) by hypoxia using FACS analysis. Fig. 6A shows the time-dependent changes in rhodamine 123 fluorescence. The $\Delta\Psi_m$ reduction was remarkably reduced above 64.2±4.8% as early as after 2 h of hypoxia and reached a maximum level after 4 h of hypoxia compared to normoxic control. The $\Delta\Psi_m$ reduction during hypoxia 4 h (32.1±1.6%) was completely abolished by 3 and 10 μM KR-32570 (62.6±7.5% and 95.2±7.2%, respectively) and 10 μM cariporide (93.4±10.8%).

3.6. Effect of KR-32570 on cytochrome c release from mitochondria

As shown in Fig. 7, hypoxia significantly increased cytochrome *c* release from mitochondria into the cytoplasm relative to the normoxic control as evidenced by a reduction in the concentration of cytochrome *c* in mitochondria. The treatment

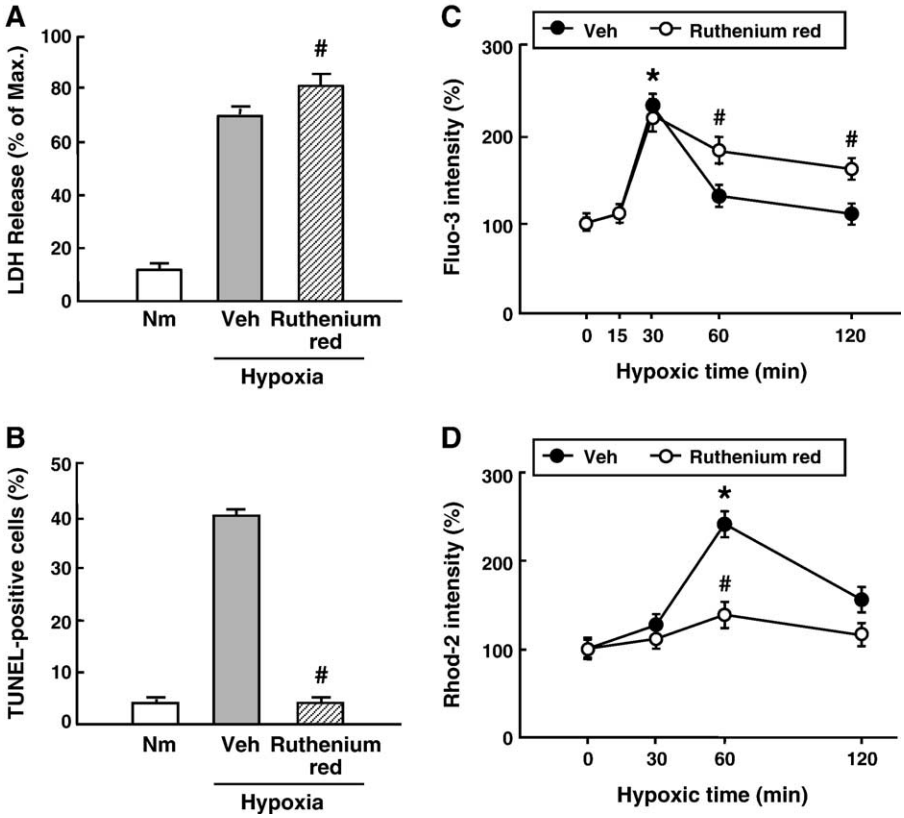

Fig. 5. The role of cytosolic Ca^{2+} and mitochondrial Ca^{2+} on hypoxia-induced cell death. (A) Effect of ruthenium red on hypoxia-induced necrotic cell death. LDH release was measured 10 h after hypoxic insult. H9c2 cells were treated with 30 μM ruthenium red 1 h before and during hypoxia. (B) Quantitative analysis of TUNEL-positive cells. Cell death (%) was calculated by dividing the number of TUNEL-stained cells by total number of cells 8 h after hypoxic insult. H9c2 cells were treated with 30 μM ruthenium red 1 h before and during hypoxia. (C) Time course of cytosolic Ca^{2+} change during hypoxia by measuring fluorescence intensity of fluo-3 in H9c2 cells. H9c2 cells were treated with 30 μM ruthenium red 1 h before and during hypoxia. (D) Time course of mitochondrial Ca^{2+} change during hypoxia by measuring fluorescence intensity of rhod-2 in H9c2 cells. H9c2 cells were treated with 30 μM ruthenium red 1 h before and during hypoxia. The data shown are mean± S.D. ($n=5$). Veh, Vehicle; Nm, Normoxia. *$P<0.05$ vs. time 0 (control). $^{\#}P<0.05$ vs.Veh-hypoxia.

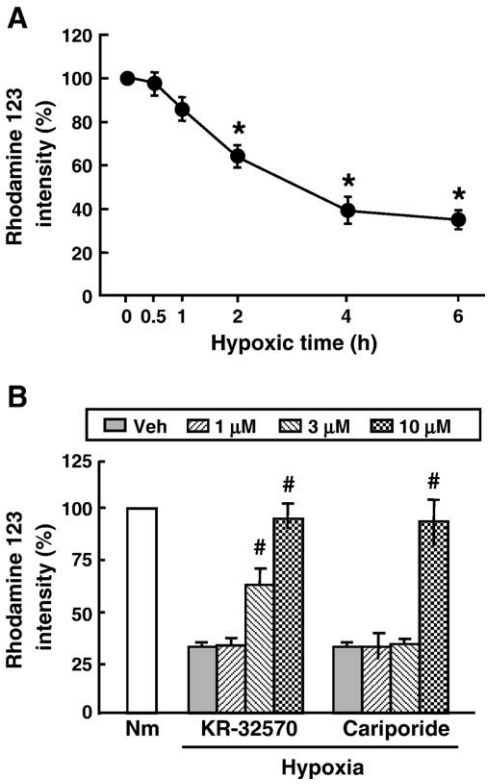

Fig. 6. Effect of KR-32570 on hypoxia-induced reduction in $\Delta\psi_m$. (A) Time course of hypoxia-induced $\Delta\psi_m$ depolarization. $\Delta\psi_m$ was determined by FACScan analysis of rhodamine 123 intensity in H9c2 cells. (B) Effect of KR-32570 and cariporide on hypoxia-induced decrease in $\Delta\psi_m$. Rhodamine 123 intensity was measured 4 h after hypoxic insult. H9c2 cells were treated with KR-32570 (1, 3 and 10 μM) and cariporide (1, 3 and 10 μM) 1 h before and during hypoxia. The data shown are mean±S.D. (n=5). Veh, Vehicle; Nm, Normoxia. *P<0.05 vs. time 0 (control). #P<0.05 vs.Veh-hypoxia.

with KR-32570 (3 and 10 μM) and cariporide (10 μM) 1 h before and during hypoxia clearly reduced cytochrome *c* release from mitochondria, release to levels in the normoxic control.

4. Discussion

In the present study, we have demonstrated that a novel NHE-1 inhibitor KR-32570 can prevent both the necrotic and the apoptotic cell death induced by hypoxia, with greater potency than cariporide in heart-derived H9c2 cells.

We have previously demonstrated that a new compound KR-32570 is highly selective NHE-1 inhibitor and that it elicits cardioprotective effect against ischemic injury in perfused rat heart (Lee et al., 2005a,b). In the present in vitro study, we have found that KR-32570 produced anti-necrotic cell death and to a greater extent, anti-apoptotic effect against hypoxia-induced H9c2 cell death. Comparing the potencies between protective effects of KR-32570 and cariporide, KR-32570 appears to be equipotent to cariporide or somewhat a little more potent.

It has been shown that the cardioprotective effects of cariporide and other NHE-1 inhibitors are produced by prevention of cytosolic Ca^{2+} overload (Hendrikx et al., 1994). Cytosolic Ca^{2+} accumulation beyond the physiological level leads to activation of Ca^{2+}-dependent proteases and phospholipases, which disrupts organelle membranes and cytoskeletal components, culminating in necrotic cell death. Indeed, a cytosolic Ca^{2+} chelator BAPTA has been demonstrated to reduce necrotic cell death in various cardiomyocyte injury models (Korge et al., 2001). In this context, our results that KR-32570 inhibited both the cytosolic Ca^{2+} accumulation and the necrotic cell death during hypoxia (Figs. 1 and 3) implicate that anti-necrotic effect of KR-32570 is contributed to cytosolic Ca^{2+} lowering effect associated with NHE-1 inhibition.

Mitochondria are known to play a central role in cytosolic Ca^{2+} dynamics (Rizzuto et al., 2000) and to be key determinants of cell death. Mitochondria can take up cytosolic Ca^{2+} rapidly via the ruthenium red-sensitive Ca^{2+} uniporter. This uptake is driven by the negative potential difference across the mitochondrial membrane, which is maintained by proton extrusion from the electron transport chain (Parekh, 2003), allowing mitochondria to function as buffers against cytosolic Ca^{2+} overload (Parekh, 2003). During myocardial hypoxia, cytosolic Ca^{2+} is elevated as a result of NHE-1 and subsequent NCX activation, and this increased cytosolic Ca^{2+} is taken up by mitochondria (Smaili et al., 2003). Because electron transport is inhibited during hypoxia, increased mitochondrial Ca^{2+} leads to a fall in of $\Delta\Psi_m$, and permeability transition pore opening (Orrenius et al., 2003). Increased mitochondrial membrane permeability can cause release of cytochrome *c*, triggering a sequential apoptotic cascade such as activation of Apaf-1 and caspase-3. Consistent with this concept, our data showed that mitochondrial Ca^{2+} increased maximally after 1 h of hypoxia (Fig. 4) and that $\Delta\Psi_m$ began to decrease significantly after 2 h of hypoxia with a maximum decrease at 4 h (Fig. 6). Our results that 30 μM ruthenium red inhibited mitochondrial Ca^{2+} overload during hypoxia and decreased the number of TUNEL-positive cells (Fig. 5), further support the pro-apoptotic role of mitochondrial Ca^{2+} during hypoxia (Teshima et al., 2003). Our results further showed that treatment with ruthenium red during hypoxia prolonged the maintenance of the elevated level of cytosolic Ca^{2+} over 2 h of hypoxia. Taken together, it is

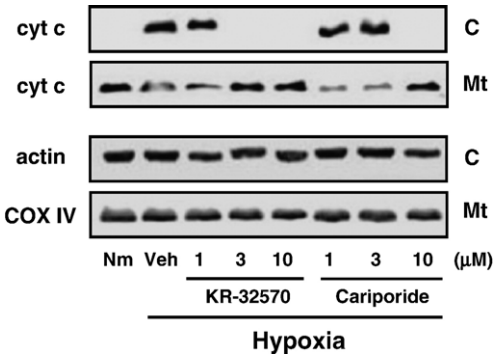

Fig. 7. Western blot analysis of cytochrome *c* in H9c2 cells after hypoxic insult. Western blots for cytochrome *c* detected in the cytosolic (C) and mitochondrial (Mt) fractions 6 h after hypoxic insult. H9c2 cells were treated with KR-32570 (1, 3 and 10 μM) and cariporide (1, 3 and 10 μM) 1 h before and during hypoxia. An equal loading of proteins was confirmed by probing with monoclonal antibody to actin, a marker for cytosol, and cytochrome oxidase subunit IV (COX IV), a marker for mitochondria, respectively. The data shown are representative Western blots from four separate experiments.

supposed that cytosolic Ca^{2+} may be associated with necrotic cell death and mitochondrial Ca^{2+} may be primarily associated with apoptotic cell death, although further study remains to be investigated to demonstrate this hypothesis.

Treatment with KR-32570 significantly inhibited the hypoxia-induced increase in both cytosolic Ca^{2+} and mitochondrial Ca^{2+}. In good agreement with our results, 10 μM cariporide has been previously demonstrated to attenuate the H_2O_2-induced increase in cytosolic and mitochondrial Ca^{2+} in rat cardiomyocytes (Teshima et al., 2003). KR-32570 also significantly recovered the large reduction in $\Delta\Psi_m$ and cytochrome *c* release induced by hypoxia. Anti-apoptotic effect of KR-32570 was in good correlation with inhibitory effect of mitochondrial Ca^{2+} overload and cytochrome *c* release as well as mitochondrial depolarization. Taken together, it is suggested that the reduction in apoptotic cell death by KR-32570 is primarily achieved by inhibition of the mitochondria-mediated death pathway.

In summary, we have demonstrated that a novel NHE-1 selective inhibitor KR-32570 produces a marked cardioprotection from hypoxia-induced H9c2 cell death, at least in part, through inhibition of intracellular Ca^{2+} overload and mitochondrial death pathway.

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