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European Journal of Pharmacology 486 (2004) 121–131
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Involvement of Na^+/H^+ exchanger in hypoxia/re-oxygenation-induced neonatal rat cardiomyocyte apoptosis

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Received 4 September 2003; received in revised form 4 December 2003; accepted 12 December 2003

Abstract

Although increased Na^+/H^+ exchanger type-1 (NHE-1) activity has been implicated in the pathogenesis of myocardial infarction, the role of NHE-1 in induction of apoptosis, and the potential mechanisms involved have not been fully characterized. This study tested the hypothesis that NHE-1 activity is involved in hypoxia (H)/re-oxygenation (Re)-induced cardiomyocyte apoptosis by increasing mitochondrial Ca^{2+} ($[Ca^{2+}]m$). Primary cultured neonatal rat cardiomyocytes were subjected to 4.5 h of H followed by 12 h of Re. Relative to H alone, the level of X-rhod-1 acetoxymethyl (AM)-labeled $[Ca^{2+}]m$ was increased, and the frequency of cell death (propidium iodide (PI) staining) and apoptotic cells (terminal deoxynucleotidyl transferase (TdT)-mediated-UTP nick end labeling [TUNEL]), confirmed by Annexin-V, were augmented at the end of Re, along with appearance of cytosolic cytochrome *c*, activation of caspase-3, and increased ratio of Bax and Bcl-2. Addition of cariporide (20 μmol/l), a well-known NHE-1 inhibitor, to cultured cells before H significantly reduced $[Ca^{2+}]m$, the number of PI and TUNEL positive cells relative to the levels at end of Re, but did not completely eliminate these changes compared to Sham control. There was a strong trend for attenuation in increased levels of $[Ca^{2+}]m$, and the number of PI and TUNEL positive cells when same dose of cariporide was added only at Re, but the difference in these variables did not reach significance. In contrast, the levels of $[Ca^{2+}]m$ and the number of PI and TUNEL positive cells were significantly reduced to a level comparable to Sham control when cariporide (20 μmol/l) was administered before H and during Re, respectively, associated with a reduction in cytosolic cytochrome *c*, caspase-3 activity and ratio of Bax and Bcl-2. In conclusion, these data suggest that NHE-1 is involved in induction of cardiomyocyte apoptosis during both H and Re through a $[Ca^{2+}]m$-dependent manner, thereby resulting in activation of cytochrome *c*-caspase-3 signaling pathways.

Keywords: Apoptosis; Ca^{2+}; Cytochrome *c*; Caspase-3; Hypoxia/re-oxygenation; Na^+/H^+ exchanger

1. Introduction

Intracellular Ca^{2+} overload has long been implicated in the pathogenesis of myocardial injury during ischemia and reperfusion (An et al., 2001; Segawa et al., 2000). It is generally accepted that intracellular Ca^{2+} overload occurs primarily through Na^+/Ca^{2+} exchanger as a result of intracellular sodium accumulation by stimulated Na^+/H^+ exchanger when cells become acidotic (Xiao and Allen, 2000; Piper et al., 1996). Accordingly, previous studies have shown that inhibition of Na^+/H^+ exchange activity reduces ischemia/reperfusion-induced myocardial infarction by lowering intracellular Ca^{2+} (Gumina et al., 2000; Hurtado and Pierce, 2000). In the multiple subtypes of Na^+/H^+ exchanger distinguished, Na^+/H^+ exchanger type-1 (NHE-1) is the predominate isoform in cardiomyocytes (Karmazyn et al., 1999). NHE-1 activity is mainly stimulated by a transmembrane hydrogen ion gradient. Under normal physiological conditions, NHE-1 mechanism works as an antiporter to extrude intracellular hydrogen ion in exchange for extracellular sodium ion, and therefore, participates in maintaining intracellular pH and regulating intracellular sodium level. With intracellular hydrogen accumulation due to an acidosis during ischemia, however, NHE-1 is activated by increasing the influx of sodium ions, resulting in intracellular sodium accumulation (Karmazyn et al., 1999). As ischemic condition

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doi:10.1016/j.ejphar.2003.12.016

continues, NHE-1 may become inactive when the gradient of transmembrane hydrogen ion declines (An et al., 2001; Hurtado and Pierce, 2000). At reperfusion, however, rapid washout of extracellular hydrogen ion after restoration of blood supply may reactivate NHE-1 and further result in accumulation of intracellular sodium (Hurtado and Pierce, 2001). The consequence of excessive accumulation of intracellular sodium ion is stimulation of Ca^{2+} ion influx through reversal of NHE-1, eventually causing intracellular Ca^{2+} overload. On the basis of this sequence of events, inhibition of NHE-1 activation with NHE-1 inhibitors administered either before ischemia or during reperfusion has been reported to protect the heart from necrosis by reducing intracellular Ca^{2+} overload (Hurtado and Pierce, 2001; Maddaford and Pierce, 1997).

There has been a renewed interest in intracellular Ca^{2+} as a trigger for myocardial apoptosis in addition to necrosis. Excessive accumulation of cytosolic Ca^{2+} is taken up by energized mitochondria during ischemia and reperfusion, resulting in an increase in mitochondrial matrix Ca^{2+} level (Pacher et al., 2001; Budd, 1998). The mitochondrial Ca^{2+} overload may, in turn, stimulate the opening of the mitochondrial permeability transition pore and the release of pro-apoptotic factors such as cytochrome *c* and/or apoptosis inducer factor, thereby initiating a receptor-independent apoptotic death cascade (Zhao and Vinten-Johansen, 2002; Molkentin, 2001). A previous study has shown that inhibition of NHE-1 activity before ischemia significantly reduces myocardial apoptosis in isolated rat heart (Chakrabarti et al., 1997). However, it is not clear whether this attenuation in apoptosis is based on reduction in mitochondrial Ca^{2+} uptake and inhibition in mitochondria-mediated death pathway. Therefore, in the current study, we used primary cultured neonatal rat cardiomyocytes to determine the time course of NHE-1 involvement in induction of apoptosis during hypoxia (H) and re-oxygenation (Re) with the NHE-1 inhibitor, cariporide, and confirm signaling pathways initiated by activated NHE-1.

2. Materials and methods

2.1. Isolation of neonatal rat cardiomyocytes and hypoxia/re-oxygenation experiments

Primary cultures of neonatal rat cardiomyocytes were prepared as described previously (Sun et al., 2000). Briefly, the hearts from 1–3-day-old Wistar rat were minced and dissociated with 0.08% trypsin. The dispersed cells were then plated to a field density of 2×10^5 cells/cm^2 on 60 mm culture dishes with double minimum essential medium (DMEM) supplemented with 10% fetal bovine serum. After 24 h of plating in a 5% CO_2 incubator at 37 °C, the culture medium was changed to DMEM with 10% fetal bovine serum containing cytosine arabinoside (Ara C, 10 μM) to eliminate non-cardiomyocytes. A hypoxic condition was produced by incubating the cells in a flow-through hypoxia chamber with 5% CO_2 and 95% N_2 at a flow rate of 25 l/min for 20 min. The chamber was sealed and the range of PO_2 was maintained between 20 and 25 mm Hg. After hypoxia, the chamber was re-opened and the culture medium was rapidly replaced with fresh DMEM for initiating re-oxygenation.

2.2. Experimental protocols

At the end of 24 h culture with 2% fetal bovine serum-DMEM, cells were randomly divided into six groups: (1) Sham Control: cells were seeded on the plate for a total of 16.5 h of incubation in normoxia; (2) hypoxia: cells underwent 4.5 h hypoxia only; (3) hypoxia/re-oxygenation: cells were re-oxygenated for 12 h by replacing hypoxic culture medium; (4) cariporide-hypoxia (C-H): cariporide (20 μmol/l) was added to the medium 20 min before hypoxia and washed out at re-oxygenation to confirm effect of cariporide on cell death only during hypoxia; (5) cariporide-re-oxygenation (C-Re): cariporide (20 μmol/l) was added to the newly replaced medium at beginning of re-oxygenation after hypoxia to confirm effect of cariporide on cell death only at re-oxygenation; (6) cariporide-hypoxia/re-oxygenation (C-H/Re): cariporide (20 μmol/l) was added to the medium before hypoxia and during re-oxygenation, respectively. The concentration of cariporide used was determined based on preliminary studies and previous reports showing effective inhibition of NHE activity at a dose range of 10 to 30 μmol/l (Hurtado and Pierce, 2000; Holmes et al., 2002; Loh et al., 2002; Maddaford and Pierce, 1997; An et al., 2001).

2.3. Identification and quantification of cell death by flow cytometry

Trypsinized cells from different groups were harvested and re-suspended in 75% cold ethanol after centrifugation. Cells (1×10^5 cells per sample) were vitally stained for 30 min with 0.5 ml staining solution consisting of propidium iodide (50 μg/ml, Molecular Probes, Eugene, OR), RNase A (10 μg/ml) and sodium citrate (1 mg/ml) in the presence of 0.1% Triton X-100 for a total count of cell death. The stained cells were subjected to flow cytometric analysis with a FACSCalibur (Becton Dickinson, San Jose, CA). Cell death was quantified as percentage of the sub-G1 peak, an indicator of cell death, in a total of 10,000 collected counts using single statistical histogram software.

2.4. Confirmation in type of cell death by using fluorescein isothiocyanate (FITC)-conjugated Annexin-V–propidium iodide (PI) dual staining

Cells treated with Triton X-100 become permeable to PI. Therefore, the PI positive cells counted by flow cytometer indicate a total number of dead cells (Kainulainen et al., 2002). In order to differentiate types of cell death after hypoxia and re-oxygenation, cells (1×10^5 cells) on the same

slide were dual stained with 20 μl FITC-conjugated Annexin-V and 1 μl PI (100 μg/ml) according to the manufacturer's instructions (Vybrant Apoptosis Assay Kit 2, Molecular Probes). Morphology of cell nuclei after staining was viewed under a florescent microscope. Cells binding FITC-conjugated An-nexin-V and excluding PI were classified as apoptotic and live cells because of their intact membranes. In contrast, necrotic cells were stained by both PI and FITC-conjugated Annexin-V because of the compromised integrity of their cellular membranes (Narayan et al., 2001; Eray et al., 2001). At least 400 cells were counted in each field.

2.5. Detection of apoptotic cells by terminal deoxynucleotidyl transferase (TdT)-mediated-UTP nick end labeling (TUNEL) assay

Identification of apoptotic cells was performed using an in situ cell death detection kit (Boehringer Mannheim, Ridgefield, CT). The cells seeded on the culture slides were fixed with 4% paraformaldehyde solution in PBS at pH 7.4 for 60 min at room temperature. After rinsing with PBS, the slides were suspended in a solution with 0.1 % Triton X-100 and 0.1% sodium citrate for 2 min to increase permeability of the cell membrane. The slides were incubated with 50 μl TUNEL reaction mixture containing TdT, which catalyzes polymerization of nucleotides of single to free 3′ -OH DNA ends, for 60 min at 37 °C. Converter-AP (50 μl) was then added to the slides for 30 min followed by incubation in 100 μl substrate solution for an additional 10–15 min. The slides were mounted with glass cover slips and analyzed under light microscope. Cell death was expressed as a percentage of total cells counted.

2.6. Agarose gel electrophoresis for DNA fragmentation

The presence of fragmented DNA was investigated with a DNA isolation Kit (Puregene Gentra Systems, Minneapolis, MN). In brief, cells (3×10^6 per sample) were lysed in 0.6 ml of lysis buffer and digested with RNase A (20 μg/ml) for 1 h at 37 °C. After precipitation by isopropanol, DNA pellets were washed with 75% ethanol, air-dried, and suspended in 20 μl TE buffer (10 mmol Tris–HCI, pH 8.0 and 1 mmol EDTA) at pH 8.0. The fragmented DNA was then electrophoretically fractioned on a 1.5% agarose gel for 1 h at 85 V and stained with ethidium bromide (0.5 μg/ml). DNA ladders, an indicator of nucleosomal DNA fragmentation from apoptotic cells, was examined and photographed by an Ultra Violet Products Gel Documentation System (FOTODYNE, Hartland, WI).

2.7. Measurement of mitochondrial Ca^{2+} ($[Ca^{2+}]m$) concentration

$[Ca^{2+}]$m concentration was determined according to the manufacturer's instructions (Molecular Probes). In brief, cells (1×10^6 per sample) were initially washed using HEPES buffer (mmol/l: NaCl 130, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, HEPES 10, Glucose 11 and $CaCl_2$ 0.2) at pH 7.4 and then stained using 5 μmol/l X-rhod 1-acetoxymethyl (AM) esters, a selective fluorescent indicator for mitochondrial Ca^{2+}, for 30 min at room temperature. Pluronic F-127 (0.02%) was added to the reaction buffer to dissociate the AM ester from the fluorescent ion. To complete de-esterification of intracellular X-rhod 1 AM esters, cells were washed using HEPES buffer after staining and stabilized for an additional 30 min. Fluorescence measurements were determined using a fluorescent plate reader (PerSeptive Biosystems, Framingham, MA) at $\lambda_{ex} = 580$ nm and $\lambda_{em} = 645$ nm. $[Ca^{2+}]$m was calculated using the equation: $[Ca^{2+}]m = K_d \times [(R - R_{min})/(R_{max} - R)]$ and expressed in nmol/l, where K_d (700 nmol/l) is a dissociation constant, R is the measured fluorescence intensity (corrected for background fluorescence), R_{min} is the fluorescence at zero Ca^{2+} and derived by exposing cells to 25 μmol/l digitonin in a Ca^{2+}-free HEPES buffer containing 10 μmol/l EGTA, and R_{max} is the fluorescence under saturating $[Ca^{2+}]$ (2.5 mmol/l) and derived by exposing the cells to HEPES buffer containing 2.5 mmol/l $CaCI_2$ without EGTA.

2.8. Preparation of cytosolic extracts for detection of cytochrome c release from mitochondria

To quantify cytochrome *c* release, Western blot analysis of cytochrome *c* in the mitochondrial and the cytosolic fractions was performed. Cells (1×10^6 per sample) for lysate preparation were harvested at the end of the experiment and washed twice in an ice-cold PBS buffer. After centrifugation, cell pellets were re-suspended in 0.5 ml cytosol extraction buffer A containing (mmol/l): sucrose 250, HEPES 20, KCl 10, $MgCl_2$ 1.5, EGTA 1, EDTA 1 and dithiothreitol 1 at pH 7.5. After 30-min incubation on ice, cells were homogenized for 15 strokes with a Dounce homogenizer. Cell homogenates were initially centrifuged at $1000 \times g$ for 5 min at 4 °C and the supernatant was further centrifuged at $40{,}000 \times g$ for another 30 min at 4 °C. The supernatant was saved as a cytosolic fraction, while the precipitate was suspended in buffer A containing 0.5% (v/v) Nonidet P-40, and saved as a mitochondrial fraction. Cytosolic and mitochondrial fractions were analyzed by Western blot with a primary mouse anti-cytochrome *c* monoclonal antibody (BD PharMingen Tech) and a second anti-mouse IgG 2b (Amersham). The density of each band was measured by NIH image analysis software and data were shown as percent change.

2.9. Measurement of caspase-3 activity

Caspase-3 activity was evaluated by using caspase-3 colorimetric activity assay kit (Chemicon International

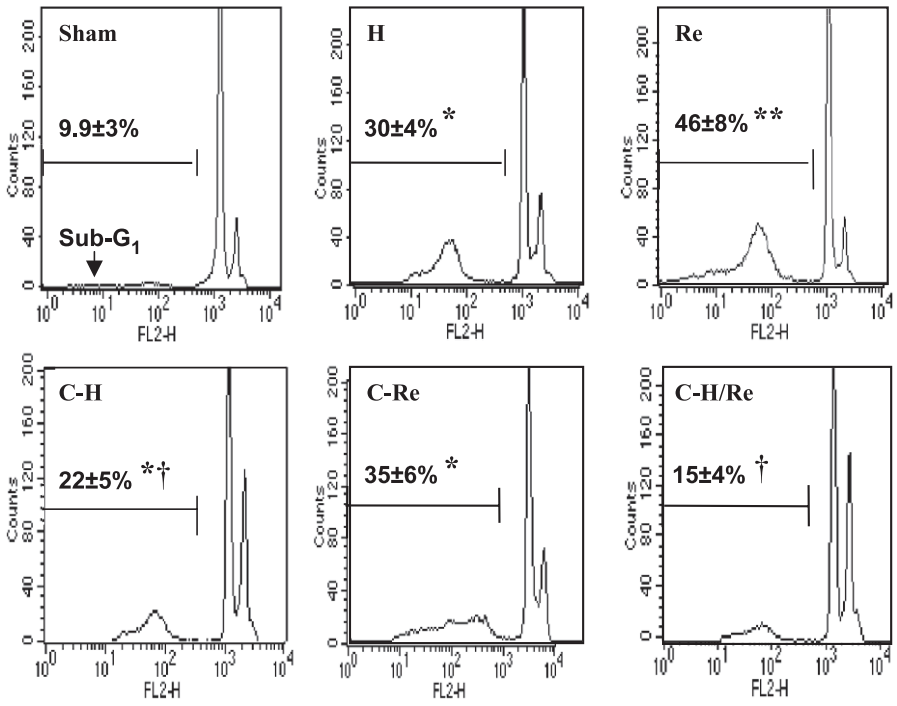

Fig. 1. Representative flow cytometric analysis of neonatal rat cardiomyocyte death after hypoxia and re-oxygenation. The percentage of total cell death was determined by propidium iodide (PI) staining as shown in the histograms. Sham: 16.5 h of cell culture; H: 4.5 h hypoxia only; Re: 12 h of re-oxygenation after hypoxia; C-H: addition of cariporide before hypoxia; C-Re: addition of cariporide during re-oxygenation; C-H/Re: addition of cariporide before hypoxia and during re-oxygenation. The peak of sub-G_1 fraction is an indicator of the total number of dead cells. Each value represents the group mean ± S.E.M. of at least six independent experiments. *$P<0.05$ vs. Sham control; **$P<0.05$ vs. hypoxia; $^{\dagger}P<0.05$ vs. hypoxia and re-oxygenation.

Molecular, Temecula, CA). In brief, cells (2×10^{-6} per sample) were lysed using 100 μl lysis buffer for 10 min on ice and then centrifuged at 10,000 × *g* for 5 min. Protein concentration in the supernatant (cytosolic extraction) was determined. After 1 h of supernatant incubation with caspase-3 substrate AC-DEVD-ρNA at 37 °C on a 96-well-plate, caspase-3 activity was determined using a SpectroMax plate reader (Molecular Devices, Sunnyvale, CA) at 405 nm.

2.9.1. Western blotting analysis of Bcl-2 and Bax

Cells (3×10^6 per sample) were lysed in 0.2 ml of lysis buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) in 1× PBS). The cell lysate was then centrifuged at 10,000 × *g* and the protein concentration in supernatants was determined by DC Protein Assay (Bio Rad). All samples were mixed with loading buffer and subjected to 12% SDS-polyacrylamide gel electrophoresis. Each lane was loaded with 40 μg of protein. All samples were then transferred on nitrocellulose membranes that were blocked with 6% milk in 1× Tris buffered saline-Tween-20 (TBS-T) buffer for 1 h at room temperature. Membranes were subsequently exposed to rabbit polyclonal anti-rat Bcl-2 and rabbit polyclonal anti-rat Bax (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 concentration in 6% milk in TBS-T for 1 h, respectively. Bound antibody was detected by horseradish peroxidase conjugated anti-IgG. Finally, enhanced chemiluminescence detection reagents were employed to visualize the peroxidase reaction product

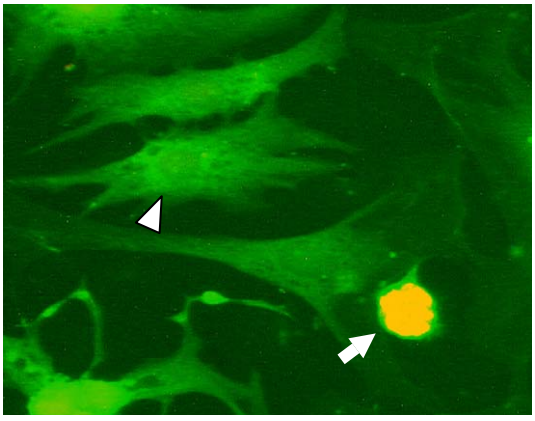

Fig. 2. The dual staining of neonatal rat cardiomyocytes with fluorescein isothiocyanate (FITC)-conjugated Annexin-V (green) and PI (red) after 4.5 h hypoxia followed by 12 h of re-oxygenation. Cardiomyocytes positive for green fluorescence only are apoptotic (arrowheads). Cardiomyocytes positive for green and red fluorescence (arrow) are necrotic. Normal cardiomyocytes show weak Annexin-V staining, but exclude PI staining. The image is captured and digitized from different fields of at least six independent experiments. Magnification × 400.

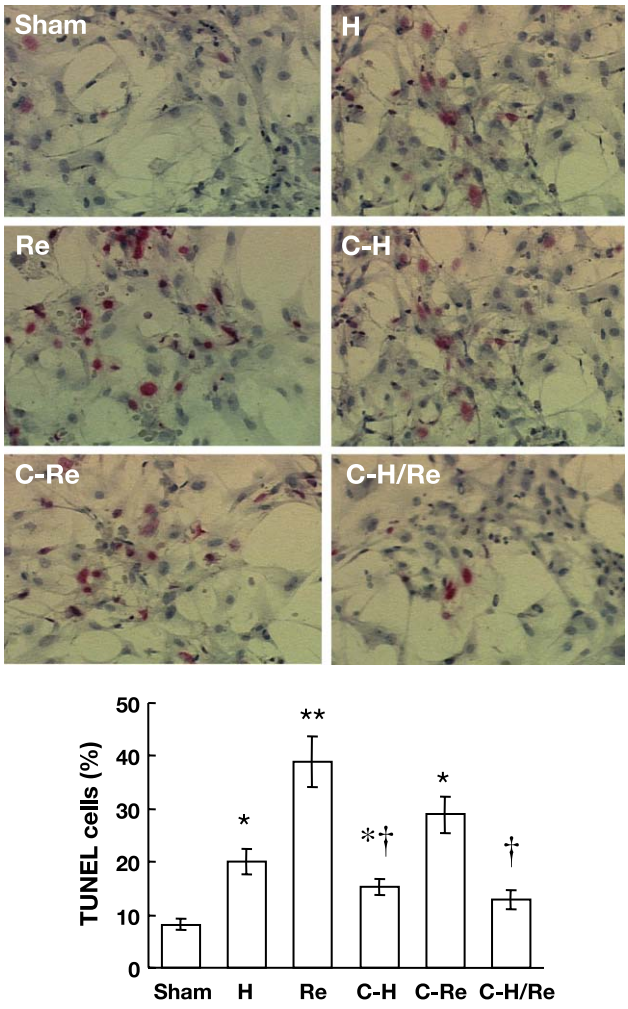

Fig. 3. Representative illustration of TUNEL staining in neonatal rat cardiomyocytes after hypoxia and re-oxygenation (upper panel). Nuclei with red staining indicate TUNEL positive cells. Quantitative results of TUNEL staining for different groups are shown in the lower panel. Sham: 16.5 h of cell culture; H: 4.5 h hypoxia only; Re: 12 h of re-oxygenation after hypoxia; C-H: addition of cariporide before hypoxia; C-Re: addition of cariporide during re-oxygenation; C-H/Re: addition of cariporide before hypoxia and during re-oxygenation. Values represent group mean ± S.E.M. *$P<0.05$ vs. Sham control; **$P<0.05$ vs. hypoxia; †$P<0.05$ vs. hypoxia and re-oxygenation.

(Amersham). The Bcl-2 protein was detected as a 28-kDa band and the Bax protein was detected as a 23-kDa band. The density of each band was measured by NIH image analysis software and data were shown as percent change.

2.9.2. Statistical analysis

All experiments were repeated at least four times. Within each experiment, either duplicate or triplicate plates were analyzed for each parameter observed. All values are expressed as means ± S.E.M. For determination of decrease in apoptotic cells and down-regulation of proteins, comparisons between groups were assessed by using one way analysis of variance followed by post hoc test. A p value <0.05 was considered significant.

3. Results

3.1. Confirmation of NHE-1 in participation of hypoxia/re-oxygenation-induced cell death

As shown in Fig. 1, hypoxia alone significantly increased the frequency of cell death, as evidenced by an elevated peak of the sub-G_1 fraction compared with the Sham control. Cell death was further increased from 30 ± 4% to 48 ± 8% after re-oxygenation. Administration of cariporide before hypoxia significantly reduced the number of nonviable cells relative to untreated hypoxia/re-oxygenation, but did not completely eliminate cell death compared to the Sham control. In addition, cariporide treatment only at re-oxygenation tended to reduce the number of nonviable cells, but the decrease did not reach significance relative to the untreated hypoxia/re-oxygenation. However, administration of cariporide before hypoxia and again during re-oxygenation significantly decreased the number of dead cells to a level comparable to the Sham control. These data suggest that NHE-1 was activated during hypoxia, and further enhanced during re-oxygenation. To differentiate type of cell death from PI positive cells, dual staining with Annexin-V and PI on the same culture slide was used. As shown in Fig. 2, 40 ± 5% of cells were Annexin-V positive and 2 ± 0.5% of cells were both Annexin-V and PI positive after hypoxia and re-oxygenation. Morphology of apoptotic cells was clearly distinguishable by their condensed or fragmented chromatin.

3.2. Apoptotic cell death by TUNEL staining

Low level of TUNEL positive cells was detected in cultured neonatal rat cardiomyocytes after 16.5 h of incubation in the Sham control as shown in Fig. 3. Hypoxia

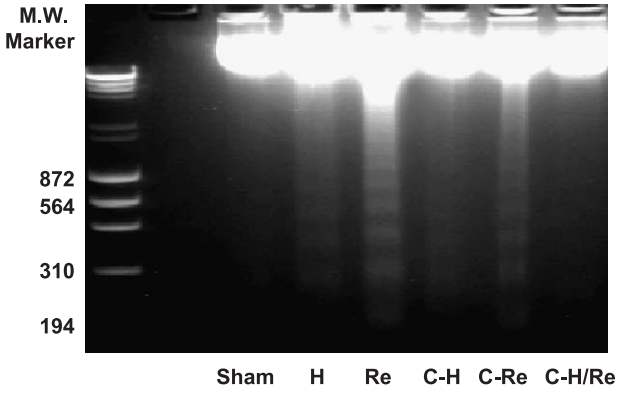

Fig. 4. Detection of DNA fragmentation of neonatal rat cardiomyocytes using agarose gel electrophoresis after hypoxia and re-oxygenation. Sham: 16.5 h of cell culture; H: 4.5 h hypoxia only; Re: 12 h of re-oxygenation after hypoxia; C-H: addition of cariporide before hypoxia; C-Re: addition of cariporide during re-oxygenation; C-H/Re: addition of cariporide before hypoxia and during re-oxygenation. This picture indicates a representative of at least four independent experiments.

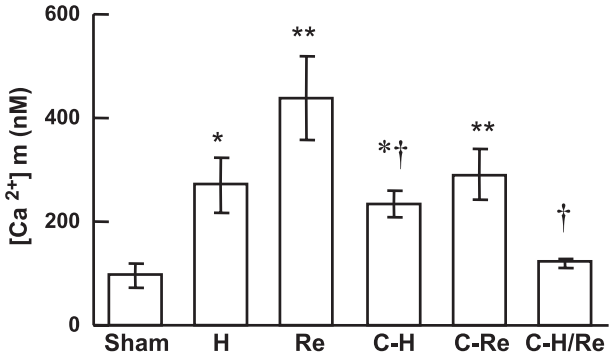

Fig. 5. Mitochondrial calcium ([Ca^{2+}]m) concentration measured by X-rhod-1 AM fluorescence showing increased [Ca^{2+}]m uptake after hypoxia and re-oxygenation. Sham: 16.5 h of cell culture; H: 4.5 h hypoxia only; Re: 12 h of re-oxygenation after hypoxia; C-H: addition of cariporide before hypoxia; C-Re: addition of cariporide during re-oxygenation; C-H/Re: addition of cariporide before hypoxia and during re-oxygenation. Values represent group mean ± S.E.M. *$P<0.05$ vs. Sham control; **$P<0.05$ vs. hypoxia; $^{\dagger}P<0.05$ vs. hypoxia and re-oxygenation.

alone significantly increased the number of TUNEL positive cells compared with the Sham control, but apoptotic cell death was significantly increased during re-oxygenation. Consistent with the number of dead cells identified by PI staining, cariporide administered either before hypoxia or before re-oxygenation tended to reduce the number of TUNEL positive cells, but no significant difference was found compared with the Sham or hypoxia/re-oxygenation groups. However, the addition of cariporide both before hypoxia and during re-oxygenation significantly reduced the number of total TUNEL positive cells. These data supported that cell death detected by PI staining in flow cytometry was primarily through an apoptotic death pathway in this preparation, and NHE-1 may play a role in induction of apoptotic cell death.

3.3. DNA fragmentation

Fragmented DNA by agarose gel electrophoresis is illustrated in Fig. 4. No clear DNA laddering was found in the Sham control. Hypoxia alone induced visible DNA laddering, but the intensity of DNA laddering was further enhanced by re-oxygenation. Cells treated with cariporide either before hypoxia or during re-oxygenation did not totally eliminate the appearance of DNA laddering, consistent with the observation from TUNEL staining. No DNA laddering was detected when cells were treated with cariporide both before hypoxia and before re-oxygenation. In addition, no "smear" background in the DNA laddering pattern was found in any group, which has been suggested

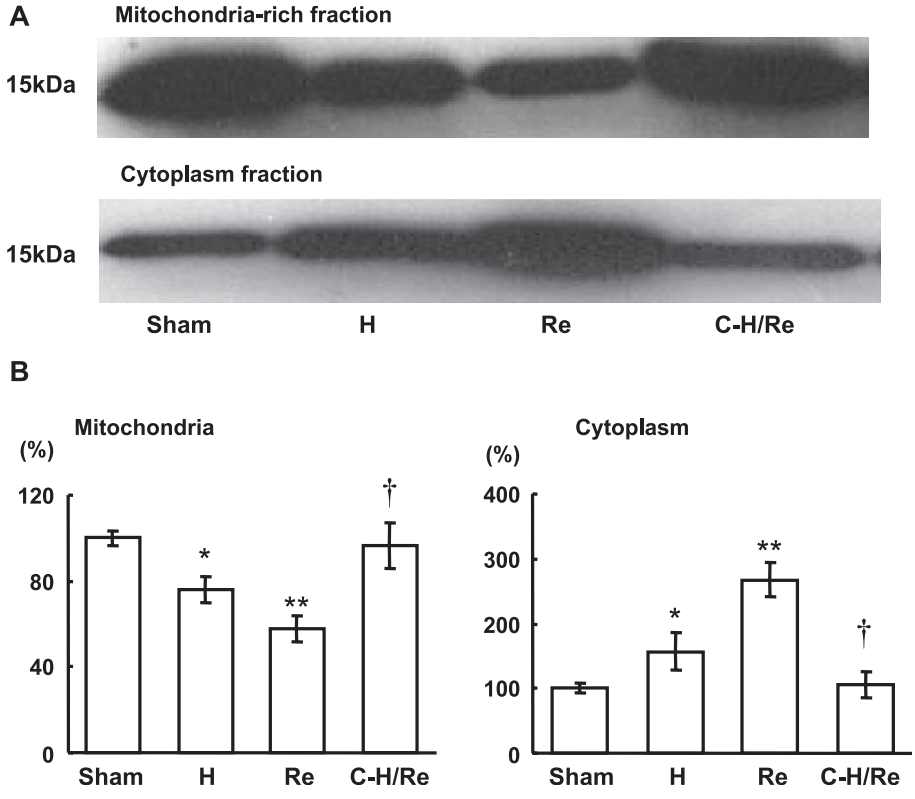

Fig. 6. Representative results of Western blot analysis for cytochrome *c* in mitochondrial and cytosolic fractions in neonatal rat cardiomyocytes after hypoxia and re-oxygenation (A). Sham: 16.5 h of cell culture; H: 4.5 h hypoxia only; Re: 12 h of re-oxygenation after hypoxia; C-H/Re: addition of cariporide before hypoxia and during re-oxygenation. Quantitative data of Western blot analysis for cytochrome *c* in mitochondria and cytoplasm (B). Values represent group mean ± S.E.M. *$P<0.05$ vs. Sham control; **$P<0.05$ vs. hypoxia. $^{\dagger}P<0.05$ vs. hypoxia and re-oxygenation.

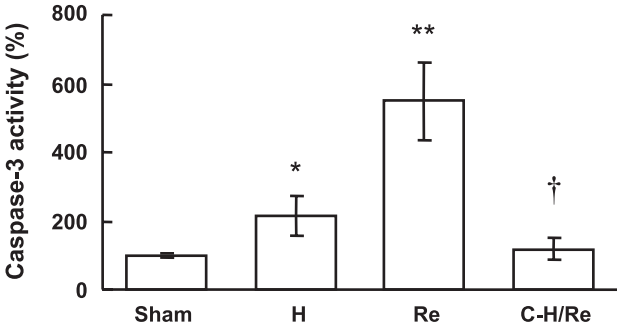

Fig. 7. Caspase-3 activity (% Sham) determined by caspase-3 Elisa kit in cytosolic extraction from neonatal rat cardiomyocytes after hypoxia and re-oxygenation. Sham: 16.5 h of cell culture; H: 4.5 h hypoxia only; Re: 12 h of re-oxygenation after hypoxia; C-H/Re: addition of cariporide before hypoxia and during re-oxygenation. Values represent group mean ± S.E.M. *$P<0.05$ vs. Sham control; **$P<0.05$ vs. hypoxia. $^{\dagger}P<0.05$ vs. hypoxia and re-oxygenation.

to indicate random DNA fragmentation in necrotic cell death.

3.4. Change in $[Ca^{2+}]m$ after hypoxia and re-oxygenation

Consistent with the decrease in apoptotic cell death after hypoxia and re-oxygenation, the concentration of $[Ca^{2+}]m$ during hypoxia alone was significantly elevated compared to the Sham control as shown in Fig. 5. However, this increase in $[Ca^{2+}]m$ was further enhanced during re-oxygenation. Addition of cariporide into the culture media either before hypoxia or during re-oxygenation did not significantly alter the increase in $[Ca^{2+}]m$ relative to the Sham control or re-oxygenation groups. However, treatment of myocytes with cariporide both before hypoxia and during re-oxygenation significantly reduced the concentration of $[Ca^{2+}]m$ relative to that observed with hypoxia/re-oxygenation, consistent with the concept that the elevation in $[Ca^{2+}]m$ may participate in the development of apoptotic cell death.

3.5. Detection of cytochrome c release from mitochondria

To further confirm potential signaling pathways in the induction of apoptotic cell death after $[Ca^{2+}]m$ overload and possible protective mechanisms of NHE-1 inhibition during hypoxia and re-oxygenation, changes in cytochrome *c* release, and activity of caspase-3 and Bcl-2 family (see below) were quantified. As shown in Fig. 6, hypoxia alone significantly increased cytochrome *c* release from mitochondria into the cytoplasm relative to the Sham control as evidenced by a reduction in the concen-

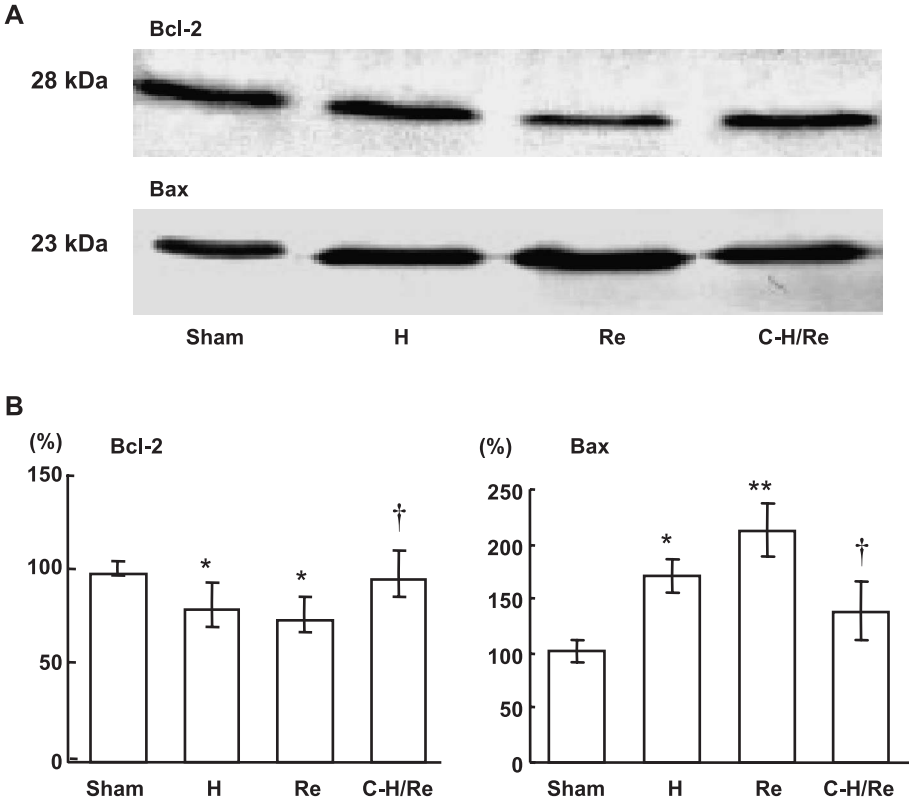

Fig. 8. Expression of Bcl-2 and Bax proteins determined by Western blot analysis in neonatal rat cardiomyocytes after hypoxia and re-oxygenation was shown in Panel A. Sham: 16.5 h of cell culture; H: 4.5 h hypoxia only; Re: 12 h of re-oxygenation after hypoxia; C-H/Re: addition of cariporide before hypoxia and during re-oxygenation. Quantitative data of Western blot analysis for expression in these two proteins were shown in Panel B. Values represent group mean ± S.E.M. *$P<0.05$ vs. Sham control; **$P<0.05$ vs. hypoxia. $^{\dagger}P<0.05$ vs. hypoxia and re-oxygenation.

tration of cytochrome *c* in mitochondria. Release of cytochrome *c* was further augmented during re-oxygenation relative to hypoxia alone. Treatment with cariporide during both hypoxia and re-oxygenation clearly reduced and preserved contents of cytochrome *c* in cytoplasm and mitochondria, respectively, release to levels in the Sham control.

3.6. Caspase-3 activity

Caspase-3 activity was analyzed from the cytosolic fraction of myocytes after hypoxia and re-oxygenation. As shown in Fig. 7, hypoxia alone significantly increased caspase-3 activity compared to the Sham control. However, increase in caspase-3 activity during hypoxia was further enhanced during re-oxygenation. Treatment with cariporide both during hypoxia and re-oxygenation significantly reduced caspase-3 activity to a level comparable to the Sham control.

3.7. Expression of Bcl-2 and Bax

Expression of Bcl-2 and Bax proteins was analyzed by Western blot assay. As shown in Fig. 8, hypoxia alone significantly down-regulated Bcl-2 and up-regulated Bax relative to the Sham control. Re-oxygenation further up-regulated the expression in Bax, but did not alter Bcl-2 expression compared with hypoxia alone. Treatment with cariporide during both hypoxia and re-oxygenation significantly prevented the decrease in Bcl-2 and reversed Bax expression to levels similar to Sham control.

4. Discussion

Primary cultured neonatal rat cardiomyocytes have been previously used to detect a role of activated NHE-1 in H_2O_2- and metabolic inhibition/recovery-induced intracellular Ca^{2+} overload (Sabri et al., 1998; Rothstein et al., 2002; Karwatowska-Prokopczuk et al., 1998). In this study, we used cariporide, a specific inhibitor of the NHE-1 isoform, to detect the role of NHE-1 in induction of cardiomyocytes apoptosis and signaling pathway involved after hypoxia and re-oxygenation. Cardiomyocyte apoptosis detected by TUNEL staining and DNA fragmentation was apparent after hypoxia alone but was further exaggerated after re-oxygenation. Reduction of apoptosis by cariporide when it was co-added before hypoxia and during re-oxygenation was associated with a significant reduction in mitochondrial Ca^{2+} concentration, attenuation of the release of mitochondrial cytochrome *c* and caspase-3 activity and increase in the anti-apoptotic protein Bcl-2 and a decrease in the pro-apoptotic protein Bax. These results suggest that stimulation of NHE-1 induces mitochondrial Ca^{2+} overload and elicits activation of cytochrome *c*-caspase-3 apoptotic signaling pathways.

The cells treated with Triton X-100 become permeable to PI (Eray et al., 2001) and therefore, the PI staining alone was used to detect the total number of dead cells in the present study. However, it has been reported that the PI staining can also distinguish necrotic cells from apoptotic and living cells in absence of the DNA fragmentation. In this connection, the dual staining of PI and Annexin-V is commonly used to reflect if cells die by apoptosis or necrosis (Narayan et al., 2001). The PI is excluded by the intact plasma membrane of apoptotic and living cells, and enters the cytoplasm in necrotic cells. Annexin-V predominantly binds to negatively charged phospholipids (i.e., phosphatidylserine) on the surface of cell membrane during early stage of apoptosis when the loss of plasma membrane asymmetry occurs (Narayan et al., 2001). Based on characteristics of these assays, the dead cells analyzed by flow cytometry in the present study contained primarily apoptotic cells after hypoxia/re-oxygenation as revealed by dual staining with PI and Annexin-V.

The role of NHE in cellular injury in response to ischemia/reperfusion or hypoxia/re-oxygenation has been widely explored in recent years. It has been proposed that the potential endogenous stimulators underlying activation of NHE in these pathological processes may include a change in the balance between intra- and extracellular pH, reactive oxygen species, thrombin, endothelin and catecholamines (Avkiran and Snabaitis, 1999). Administration of NHE inhibitors before or after the onset of ischemia has consistently shown a reduction in infarct size (Gumina et al., 2000, 2001; Hattori et al., 2001) and the inflammatory response (Redlin et al., 2001; Hurtado and Pierce, 2001) as well as an improvement in cardiac and endothelial function (Gumina et al., 2001; Myers and Karmazyn, 1996). However, protection by NHE inhibitors only at reperfusion demonstrated inconsistent results (Maddaford and Pierce, 1997; Hurtado and Pierce, 2000, 2001; Van Emous et al., 1998; An et al., 2001). Differences among these studies would appear to be related to a dose-dependent effect, and whether the duration of inhibitors used conferred optimal inhibition of the NHE-1. Recent clinical observations and animal studies have reported that significant protection can be achieved only when higher doses of NHE inhibitors were given at reperfusion during primary angioplasty (Buerke et al., 1999) and coronary artery bypass graft surgery (Muraki et al., 2003). In the present study, we used a fixed concentration of cariporide to demonstrate the time course of cell injury initiated by NHE stimulation. It was shown that presence of cariporide in the culture medium only before the end of hypoxia was effective at significantly reducing the number of TUNEL positive cells, but it did not completely eliminate hypoxia/re-oxygenation-induced apoptotic cell death relative to the Sham control. To confirm whether cardiomyocytes die during re-oxygenation by NHE-1, cariporide was added only at re-oxygenation after culture medium was newly replaced at the end of hypoxia. Although no statistically significant reduction in

TUNEL positive cells was detected when cariporide was added at this time point, there was a strong trend in reduction of cell death as detected by flow cytometric analysis and TUNEL staining, suggesting that cells die partially during re-oxygenation. Co-addition of cariporide both before hypoxia and at re-oxygenation completely inhibited cell death elicited by hypoxia/re-oxygenation, consistent with previous reports showing that reduction in necrosis in in vivo model can be accomplished by inhibiting Na^+/H^+ exchanger either during ischemia or at reperfusion (Gumina et al., 1999; Maddaford and Pierce, 1997; Hurtado and Pierce, 2000, 2001).

In identification of cell membrane receptor-independent death pathway, it has become clear that mitochondria play a predominant role in development or amplification of the pathological processes leading to apoptotic cell death (Gogvadze et al., 2001; Chen et al., 2002; Pacher et al., 2001). Among many triggers for this signaling cascade as previously reported, the uptake of Ca^{2+} from cytoplasm into the mitochondria of cardiomyocyte is a fundamental step in initiating apoptotic cell death (Pan et al., 2001; Budd, 1998; Gunter et al., 2000). The intramitochondrial Ca^{2+} rises with increased intracellular Ca^{2+}, which starts when cytosolic Na^+/Ca^{2+} exchanger activity is increased after stimulation by the NHE (Hurtado and Pierce, 2000; Myers and Karmazyn, 1996). Massive Ca^{2+} overload in the mitochondria causes a Ca^{2+}-dependent increase in mitochondrial inner membrane permeability with a subsequent release of intermembrane proteins to the cytoplasm, including cytochrome *c* or apoptosis inducing factor (Pacher et al., 2001; Gogvadze et al., 2001; Lemasters et al., 1998; Mathiasen et al., 2002). The released cytochrome *c* forms an intermediary complex with Apaf-1 and pro-caspase-9 to indirectly activate downstream "execution" caspases (i.e., caspase-3) which then initiates apoptosis. In this regard, previous studies have shown that the selective blockade of mitochondrial Ca^{2+} uptake reduces cytochrome *c* release and caspase activity, and further attenuates apoptotic cell death (Griffiths et al., 1998; Zhu et al., 2000). In the present study, an elevated level of mitochondrial Ca^{2+} during hypoxia alone or re-oxygenation was associated with a significant increase in cytochrome *c* release in cytoplasm and caspase-3 activity, consistent with these previous reports. Blockade of NHE-1 activity with cariporide during hypoxia and re-oxygenation was correlated with an inhibition in mitochondrial Ca^{2+} overload and cytochrome *c* release as well as caspase-3 activity. These data, therefore, suggest that a reduction in apoptotic cell death by blocking NHE-1 is primarily archived by inhibiting a mitochondria-mediated

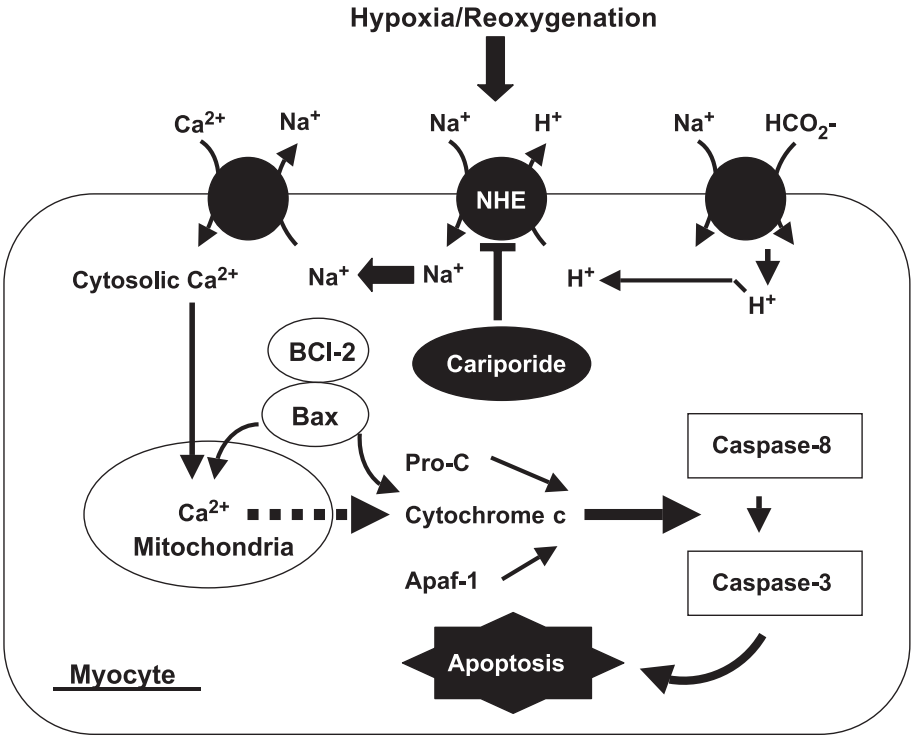

Fig. 9. Summary of proposed mechanisms involved in prevention of apoptotic cell death by Na^+/H^+ exchanger inhibitor, cariporide. Stimulation of Na^+/H^+ exchanger (NHE) during hypoxia and re-oxygenation increases the cytosolic Ca^{2+} and further results in mitochondrial Ca^{2+} overload by concentration-dependent manner. Release of cytochrome *c* from mitochondria after Ca^{2+} overload forms a complex with pro-caspase-9 (Pro-c) and Apaf-1, further activates caspase-3 and induces apoptosis. A balance in expression between anti-apoptotic protein, Bcl-2 and pro-apoptotic protein, Bax may co-determine mitochondrial function by interfering Ca^{2+} overload and cytochrome *c* release. Cariporide reduces apoptosis by inhibiting NHE-mediated elevation in intracellular Ca^{2+} after hypoxia and re-oxygenation.

death pathway. These results are also consistent with a previous study that showed a reduction in necrosis in rat model of ischemia and reperfusion by NHE inhibitor is correlated with attenuation in mitochondrial Ca^{2+} overload and improvement in mitochondrial respiratory function (Yamamoto et al., 2002). However, alternative mechanism by which inhibition of NHE-1 reduces cell death is proposed from a recent study showing that co-incubation of cariporide with mouse cardiomyocytes during simulated ischemia significantly decreased the mitochondrial matrix acidification, associated with a delayed ATP exhaustion (Ruiz-Meana et al., 2003).

Among the members of Bcl-2 family investigated, the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 have been shown to be involved in modulation of mitochondria-mediated apoptotic cell death (Kirshenbaum and de Moissac, 1997; Misao et al., 1996). Bax is normally present in the cytoplasm of cells, but it can be recruited to the mitochondria during induction of apoptosis. Translocation of Bax to the mitochondria alters the ratio of pro- to anti-apoptotic proteins in the Bcl-2 family members, and most likely predisposes the mitochondria to release cytochrome *c* (Nutt et al., 2002a,b; Pan et al., 2001). Bcl-2 protein is nonuniformly distributed in intracellular membranes such as in the outer and inner mitochondrial membrane, nuclear envelop and endoplasmic reticulum. It has been shown that overexpression of Bcl-2 inhibits Bax translocation from the cytoplasm to the mitochondria, Bax-mediated mitochondrial Ca^{2+} mobilization and the release of cytochrome *c* (Zhu et al., 2001; Kruman et al., 1998; Yang et al., 1997; Kluck et al., 1997). Therefore, the balancing effect of Bcl-2 and Bax on mitochondrial Ca^{2+} homeostasis could play a role in determining the fate of cells to survive or undergo apoptosis. Although we did not directly measure the translocation of Bax from the cytoplasm to mitochondria and subsequent Bax-enhanced cytochrome *c* release during hypoxia/re-oxygenation, an increase in expression of Bcl-2 and a decrease in expression of Bax proteins after NHE-1 blockade may attenuate mitochondria-mediated apoptosis by stabilizing the permeability transition pore, and therefore inhibit cytochrome *c* release as demonstrated in the present study.

As summarized in Fig. 9, the present study indicates that selective inhibition of NHE-1 during hypoxia and re-oxygenation could directly protect cardiomyocytes against mitochondrial Ca^{2+} overload and cytochrome *c*-caspase-3 signaling pathways, and therefore, potentially inhibit the triggering of apoptotic cell death. A balance between anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax seems to be important in maintenance of cell survival following increased activity of the NHE-1 at hypoxia and re-oxygenation. These data also support that for the best protection by attenuating NHE-induced cell injury, NHE inhibitors may need to be administered after the onset of ischemia and the effective treatment regimen should cover, at least, the early phase of reperfusion.

Acknowledgements

The authors are grateful for the technical contributions of Sara Katzmark and Susan Schmarkey in performing this study, and for the assistance of Laurie Berley in preparing the manuscript. Cariporide was kindly provided by Aventis Pharma, Frankfurt am Main, Germany. This work was supported by a grant from the National Institute of Health (HL64886) and funds from the Carlyle Fraser Heart Center of Emory University.

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