Cariporide (HOE642), a Selective Na\(^+\)-H\(^+\) Exchange Inhibitor, Inhibits the Mitochondrial Death Pathway

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Background—The Na\(^+-\)H\(^+\) exchanger figures prominently in cardiac ischemia-reperfusion injury. Several experimental and clinical studies have demonstrated a cardioprotective effect of Na\(^+-\)H\(^+\) exchanger inhibition; however, the precise mechanisms have not been established.

Methods and Results—We examined the effects of cariporide (HOE642, 10 \(\mu\)mol/L) on cell death induced by oxidative stress (H\(_2\)O\(_2\), 100 \(\mu\)mol/L) in cultured neonatal rat cardiomyocytes. Cariporide significantly suppressed markers of cell death, such as TUNEL positivity and caspase-3 cleavage, at 8 or 16 hours after H\(_2\)O\(_2\). The early phase of cell death, reported by increases in phosphatidylserine exposure and propidium iodide uptake, was also inhibited by cariporide. To explore the mechanisms of cell protection, we examined the effects of cariporide on increases in intracellular Na\(^+\) and Ca\(^{2+}\) induced by oxidative stress. Cariporide remarkably suppressed cytosolic Na\(^+\) and Ca\(^{2+}\) accumulation. Next, we investigated the effects of cariporide on mitochondria-associated death process. Mitochondrial Ca\(^{2+}\) overload induced by H\(_2\)O\(_2\) was remarkably suppressed by cariporide. Mitochondrial membrane potential is a critical step of the death pathway; cariporide prevented mitochondrial membrane potential loss induced by H\(_2\)O\(_2\).

Conclusions—Cariporide protects cardiomyocytes against oxidant-induced cell death by preserving intracellular ion homeostasis and mitochondrial integrity. (Circulation. 2003;108:2275-2281.)

Key Words: ion channels ■ apoptosis ■ prevention ■ calcium ■ sodium

The cardiac sarcolemmal Na\(^+-\)H\(^+\) exchanger (NHE) regulates intracellular pH via proton extrusion driven by the transmembrane Na\(^+\) gradient. Inhibition of NHE produces cardioprotective effects in various animal models of myocardial ischemia-reperfusion with consistent improvement in functional recovery, metabolic status, and attenuation of arrhythmias.\(^1,2\) Furthermore, a clinical study revealed that NHE inhibition during reperfusion therapy preserved cardiac function in patients with acute myocardial infarction.\(^3\) NHE is relatively quiescent under basal conditions but becomes activated during ischemia in response to intracellular acidosis. Inhibition of intracellular Na\(^+\) ([Na\(^+\)]\(_i\)) accumulation induced by increased Na\(^+-\)H\(^+\) exchange and prevention of excessive Ca\(^{2+}\) influx via Na\(^+-\)Ca\(^{2+}\) exchanger working in reverse mode have been proposed as the mechanisms of cardioprotection by NHE inhibition.\(^2,4\) However, the precise mechanisms of cardioprotection by NHE inhibition remain unclear.

Mitochondria play key roles in cell death. The mitochondrial death pathway features the sequential loss of mitochondrial membrane potential (\(\Delta\Psi\_m\)), the release of toxic proteins into the cytoplasm, and caspase activation. We have recently reported that \(\Delta\Psi\_m\) loss in response to oxidative stress is accompanied by the opening of the mitochondrial permeability transition pore (PTP) and is irreversible,\(^5\) indicating \(\Delta\Psi\_m\) loss is a critical step to cell death. Mitochondrial Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{mito}\)) overload is a major trigger of PTP opening.\(^6,7\) We had previously shown that activation of mitochondrial ATP-sensitive potassium (mitoK\(_{ATP}\)) channels prevents \(\Delta\Psi\_m\) loss and inhibits apoptosis,\(^8\) presumably by inhibition of [Ca\(^{2+}\)]\(_{mito}\) overload.\(^9\) Based on these observations, we hypothesized that NHE inhibition protects cardiomyocytes against cell death by inhibiting [Ca\(^{2+}\)]\(_{mito}\) overload and preserving \(\Delta\Psi\_m\). We investigated the precise mechanisms of cardioprotection by NHE inhibition using cariporide, a specific blocker of the NHE-1 isoform, in cultured cardiomyocytes.

Methods
All procedures were performed in accordance with the Johns Hopkins University animal care guidelines, which conform to the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

Primary Culture of Neonatal Rat Cardiac Ventricular Myocytes
Cardiac ventricular myocytes were prepared from 1- to 2-day-old Sprague-Dawley rats (Zivic Laboratories, Inc, Pittsburgh, Pa) and cultured as previously described.\(^6\)
Experimental Protocol
Neonatal rat cardiomyocytes were randomly assigned to 1 of the following 3 experimental groups: (1) control group, (2) incubation with 100 μmol/L hydrogen peroxide (H₂O₂), or (3) pretreatment with 10 μmol/L cariporide for 20 minutes followed by 100 μmol/L H₂O₂. Cariporide was a gift from Aventis Pharma (Frankfurt, Germany).

Terminal Deoxynucleotidyl Transferase–Mediated TUNEL Staining
TUNEL staining was performed according to the manufacturer’s protocol (Roche) at 16 hours after 100 μmol/L H₂O₂ application. Fluorescein labels incorporated in nucleotide polymers were detected by laser-scanning confocal microscopy.

Caspase-3 Activity Assay
Caspase-3 activity was measured as described previously¹⁰ by detection of the cleavage of a colorimetric caspase-3 substrate, N-acetyl-Asp-Glu-Val-Asp-p-nitroaniline, using an assay kit, ApoAlert CPP32 (Clontech), at 8 and 16 hours of 100 μmol/L H₂O₂ stimulation.

Loading of Cells With Fluorescent Indicators
For quantification of cellular viability, cells were double-stained with annexin V and propidium iodide (PI) according to manufacturer’s instructions (Roche). To monitor ΔΨᵹ, cells were loaded with 100 nmol/L tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes) at 37°C for 20 minutes. CoroNa Red (1 μmol/L) was used to examine [Na⁺] level. To monitor intracellular [Ca²⁺] ([Ca²⁺]ᵹ) levels, cells were loaded with 1 μmol/L fluo-4 and 2 μmol/L thio-2 AM, respectively (Molecular Probes) at 37°C for 30 minutes.

Confocal Imaging
Cells plated on glass-bottom dishes were loaded with fluorescent probes as described above. After loading with each dye, cells were placed in phenol red–free DMEM supplemented with 25 mmol/L Hepes (pH 7.4). Cells were illuminated (488- and 568-nm line of a krypton/argon laser), and images were taken by confocal microscopy (UltraVIEW, Perkin-Elmer). Time-lapse confocal imaging was carried out with various intervals between frames using a ×20 objective lens.

Image Analysis
Fluorescence intensity was assessed at mitochondria-rich lesions randomly selected around the nucleus in each scan. Quantitative image analysis was performed using image analysis software (ImageJ; http://rsb.info.nih.gov/ij/).

FACS Analysis
For FACS analysis of ΔΨᵹ, TMRE-loaded cells were harvested by trypsinization at the end of experimental protocols and analyzed using FACSScan (20,000 cells per sample) (Becton Dickinson). The fluorescence intensity of TMRE was monitored at 582 nm (FL-2 channel). FACS data were analyzed using WinMDI software (http://facs.scripps.edu).

Statistical Analysis
Data are presented as mean±SEM. Multiple comparisons among groups were carried out by one-way ANOVA with Fisher’s least-significant difference as the post-hoc test. A level of P<0.05 was accepted as statistically significant.

Results
Effects of Cariporide on Cell Death
First, we examined the long-term effects of cariporide on cell death. We performed TUNEL staining 16 hours after H₂O₂ application. Representative images and quantitative data are shown in Figures 1A and 1B, respectively (n=4 for each group). In the control group, 11.7±1.4% of the cells showed TUNEL-positive nuclei, whereas exposure to H₂O₂ increased the number of TUNEL-positive nuclei to 33.9±0.8% (P<0.001). This increase of TUNEL positivity was suppressed by cariporide (18.6±0.7%) (P<0.001 versus H₂O₂). Activation of caspase-3 is a critical step in the process of cell death. Figure 1C shows relative caspase-3 activity in each group at 8 and 16 hours after H₂O₂ application. Caspase-3 activity was increased in H₂O₂ compared with control at each time point (P<0.01), and cariporide suppressed these increases (P<0.05 versus H₂O₂ at each time point) (n=3 for each data point).

Effects of Cariporide on Early Phase of Cell Death
To assess the effects of cariporide in the early phase of cell death, we investigated the alterations of annexin V and PI fluorescence in each group. Time-lapse confocal microscopy at 5-minute intervals began immediately after the application of 100 μmol/L H₂O₂. Annexin V fluorescence started to
increase approximately 30 minutes after H$_2$O$_2$ application and gradually increased to a plateau at approximately 100 minutes (Figure 2A). Cariporide remarkably suppressed this increase of annexin V fluorescence (Figure 2A). PI fluorescence was increased after 70 minutes of latency and reached a plateau at approximately 120 minutes by H$_2$O$_2$ stimulation (Figure 2B). This increase of PI fluorescence was also abrogated by cariporide (Figure 2B). After 150 minutes of scanning, cells were permeabilized with saponin (Sigma) to calculate the percentage of PI-positive cells in each group. At the end of scanning, 92.1±3.0% of cells were PI positive by H$_2$O$_2$ stimulation. In contrast, only 19.9±9.5% of cells were PI positive in cariporide-treated cells (n=3, P<0.001). Because annexin V is an indicator of apoptosis and PI of necrosis, these results suggest that cariporide reduces both types of cell death.

**Effects of Cariporide on Cytosolic Na$^+$ and Ca$^{2+}$ Overload**

Preservation of intracellular ion homeostasis is a natural potential mechanism of cardioprotection by NHE inhibition. We examined the effects of cariporide on [Na$^+$], and [Ca$^{2+}$], accumulation using coroNa-red and fluo-4. Time-lapse confocal microscopy began with the addition of H$_2$O$_2$. Fluorescence did not change during 60 minutes of scanning in controls. CoroNa-red fluorescence increased gradually approximately 10 minutes after H$_2$O$_2$ application. Cariporide mitigated the increase in coroNa-red fluorescence induced by H$_2$O$_2$ (Figures 3A and 3B). Fluo-4 fluorescence also increased approximately 20 minutes after H$_2$O$_2$ application and reached a plateau at approximately 40 minutes. Cariporide also attenuated the increase in fluo-4 fluorescence induced by H$_2$O$_2$ (Figures 4A and 4B).

**Effects of Cariporide on Mitochondrial Ca$^{2+}$ Overload**

Ca$^{2+}$ overload in mitochondria is one of the critical triggers of cell death and is known to open the PTP. We examined the effects of cariporide on mitochondrial Ca$^{2+}$ level using the mitochondrial Ca$^{2+}$-sensitive dye rhod-2. Rhod-2 fluorescence was remarkably augmented by H$_2$O$_2$ (Figures 5A and 5B). Cariporide completely inhibited the Ca$^{2+}$ increase observed in H$_2$O$_2$-stimulated cells (Figures 5A and 5B).

**Effects of Cariporide on $\Delta$Ψ$_m$**

Dissipation of $\Delta$Ψ$_m$ is a critical event early in the process of cell death. To examine whether preservation of $\Delta$Ψ$_m$ is associated with cardioprotective effects by cariporide, we assessed the change of TMRE fluorescence by H$_2$O$_2$ in each group using FACS analysis. Incubation with H$_2$O$_2$ for 1 hour decreased TMRE fluorescence and shifted the distribution curve leftward, indicating the depolarization of $\Delta$Ψ$_m$ (Figure 6A, top). The decrease of TMRE fluorescence was remarkably suppressed by cariporide, indicating the preservation of $\Delta$Ψ$_m$ (Figure 6A, bottom). Summarized data from FACS analysis are shown in Figures 6B and 6C. We evaluated the percentage of cells that exhibited a high level of TMRE fluorescence (defined as $>1\times10^3$ in this analysis). Cariporide preserved $\Delta$Ψ$_m$ in a concentration-dependent manner (n=3 for each group) (Figure 6B). Inhibition of mitoK$_{ATP}$ channels by 5-hydroxydecanoate (5HD) or glibenclamide did not cancel $\Delta$Ψ$_m$ preservation by cariporide (n=3 for each group) (Figure 6C). Thus, the effects of cariporide are not attribut-
able to indirect mitoK<sub>ATP</sub> channel activation. To examine time-dependent changes of ∆Ψ<sub>m</sub>, confocal microscopy was performed using cells loaded with TMRE. Time-lapse scanning began immediately after application of H<sub>2</sub>O<sub>2</sub>. Figure 7A shows sequential images in each group, and Figure 7B shows the average of TMRE fluorescence intensity from 10 randomly selected cells in each group. TMRE fluorescence did not change during 60 minutes of scanning in control. Cells exposed to H<sub>2</sub>O<sub>2</sub> progressively lost TMRE fluorescence intensity, indicating irreversible dissipation of ∆Ψ<sub>m</sub>. Cariporide remarkably preserved TMRE fluorescence.

**Discussion**

The major findings in the present study are as follows. First, cariporide suppressed markers of cell death, such as increased TUNEL positivity, caspase-3 activity, phosphatidylserine exposure, and PI positivity induced by oxidative stress. Second, [Na<sup>+</sup>], and [Ca<sup>2+</sup>], accumulation induced by oxidative stress were reduced by cariporide. Third, [Ca<sup>2+</sup>]<sub>mito</sub> overload was prevented by cariporide. Fourth, cariporide prevented oxidative stress–induced ∆Ψ<sub>m</sub> loss in a dose-dependent manner. Fifth, ∆Ψ<sub>m</sub> preservation by cariporide was not abolished by 5HD or glibenclamide.

Figure 8 shows a proposed mechanism of cardioprotection by cariporide. During ischemia, NHE becomes activated to induce [Na<sup>+</sup>], overload in response to intracellular acidosis. In addition, inhibition of Na<sup>+</sup>/K<sup>+</sup> ATPase in ischemia prompts the sarcolemmal Na<sup>+</sup>/Ca<sup>2+</sup> exchanger to work in reverse mode. In the present study, stimulation by H<sub>2</sub>O<sub>2</sub> increased [Na<sup>+</sup>], indicating that not only ischemia but also oxidative stress itself can activate NHE. These alterations of intracellular ion dynamics inhibit Ca<sup>2+</sup> efflux and enhance Ca<sup>2+</sup> influx, thus producing [Ca<sup>2+</sup>], overload. Consistent with this theory, we observed [Ca<sup>2+</sup>], increase in response to H<sub>2</sub>O<sub>2</sub>, although we lack direct evidence that [Ca<sup>2+</sup>], increase is obligatorily linked to the [Na<sup>+</sup>], increase. Cariporide suppressed increases in both [Na<sup>+</sup>], and [Ca<sup>2+</sup>], in the present study. These results are consistent with previous reports that cariporide preserved [Ca<sup>2+</sup>], transients in an ischemia-reperfusion rat heart model and that cariporide suppressed intracellular acidosis-induced [Na<sup>+</sup>], increase. Although the present results are striking, we cannot exclude the possibility that the dramatic effect of cariporide observed in the present study may be modified in a beating heart, in which excitation-contraction coupling may be more robust than it is in cultured cells.

Reduction of functional cardiomyocytes by apoptosis and necrosis contributes to ischemia-reperfusion injury in the...
The previous reports showed that NHE inhibitors can attenuate apoptosis in animal models, such as ischemia/reperfusion in isolated heart and in vivo coronary artery occlusion. In the present study, we demonstrated that cariporide reduced TUNEL positivity, caspase-3 activity, and annexin V/PI fluorescence in cardiomyocytes stimulated by H2O2, indicating that cariporide can inhibit both types of cell death, apoptosis and necrosis. Despite nearly complete suppression of intracellular Ca2⁺ overload, the partial effects of cariporide on TUNEL positivity and caspase-3 activity may suggest a contribution of Ca2⁺-independent death pathways, such as Fas, tumor necrosis factor-α, and Ca2⁺-independent cytochrome c release. Time-lapse confocal microscopy using annexin V and PI demonstrated that cariporide prevents the early phase of cell death. Mitochondria are key determinants of cell death. We recently reported that cardiomyocytes exposed to oxidative stress show Ca2⁺-dependent morphological changes in mitochondria, such as swelling and loss of cristae before the opening of PTP. Increases in [Ca2⁺]i should lead to [Ca2⁺]mito accumulation, which triggers PTP opening, accompanied by catastrophic ΔΨm loss. Our findings that cariporide remarkably inhibited the [Ca2⁺]mito increase and loss of ΔΨm induced by oxidative stress indicate that cariporide may prevent cell death by preserving mitochondrial integrity.

![Figure 5](image1.png)

**Figure 5.** Time-lapse analysis of mitochondrial Ca2⁺ level. A, Representative sequential images of rhod-2 fluorescence, an indicator of mitochondrial Ca2⁺ level, in each group. B, Quantitative data of rhod-2 fluorescence. Data are mean±SEM from 3 independent experiments. Cariporide abrogated the increase of rhod-2 fluorescence in response to H2O2.

![Figure 6](image2.png)

**Figure 6.** Effects of cariporide on ΔΨm. A, Representative histograms obtained by FACS analysis of cells loaded with TMRE are shown. Top, Gray area shows H2O2 group and black line shows control (CON). Bottom, Representative histogram from H2O2 plus cariporide. B and C, Quantitative results of FACS analysis. B, Cariporide preserved ΔΨm in a concentration-dependent manner. C, Effects of 5HD or glibenclamide on ΔΨm preservation by cariporide. Neither 5HD nor glibenclamide canceled the effects of cariporide. Data are expressed as percentage of cells with high TMRE fluorescence (defined as above 1×10² of fluorescence intensity). Data are mean±SEM (n=3 per group). *P<0.05 vs H2O2.
Despite the proposed mechanisms in Figure 8, we cannot exclude the possible role of NHE isoform located in the mitochondrial inner membrane in the observed cardioprotective effects by cariporide. Cariporide may directly work on mitochondria and may reduce \([\text{Ca}^{2+}]_{\text{mito}}\) overload by inhibiting mitochondrial NHE. Indeed, another NHE inhibitor, SM-20550, was reported to inhibit \([\text{Na}^+]_{\text{mito}}\) and \([\text{H}^+]_{\text{mito}}\) transport in mitochondria, and it preserved mitochondrial respiratory function.

MitoK\(_{\text{ATP}}\) channels have cardioprotective effects against ischemia/reperfusion injury. The mitoK\(_{\text{ATP}}\) channel opener, diazoxide, prevents apoptosis induced by oxidative stress in cultured cardiomyocytes. Inhibition of mitochondrial \([\text{Ca}^{2+}]_{\text{mito}}\) overload and subsequent preservation of \(\Delta\Psi_{\text{m}}\) are considered the mechanisms of cardioprotection. A recent study demonstrated that 5HD, a mitoK\(_{\text{ATP}}\) channel blocker, did not abolish infarct size limitation by a NHE inhibitor. In contrast, another group showed that 5HD and glibenclamide, but not a sarcolemmal K\(_{\text{ATP}}\) channel blocker (HMR1098), abolished the cardioprotection by cariporide. We find that neither 5HD nor glibenclamide inhibits the effect of cariporide. These results suggest that the cardioprotective effects by cariporide may not be mediated by activation of mitoK\(_{\text{ATP}}\) channels. Consistent with the present results, a recent report showed that the cardioprotective effects of cariporide operate through a different pathway from 5HD-inhibitable ischemic preconditioning.

NHE inhibition is one of the most promising therapeutic strategies for ischemia-reperfusion injury. The classic NHE inhibitor, amiloride, was used in the first report of cardioprotective effects by NHE inhibition. Thereafter, NHE inhibitors have attracted much attention, resulting in the development of specific and selective compounds (eg, cariporide). Based on experimental and clinical evidence of cardioprotection by NHE inhibitors, several multicentered international clinical trials have been initiated. In the Evaluation of the Safety and Cardioprotective Effects of Eniporide in Acute Myocardial Infarction (ESCAMI) trial, one of the newly developed compounds did not show any salutary effect. However, cariporide significantly reduced combined incidence of death and myocardial infarction in patients with high-risk coronary artery bypass grafting surgery in subgroup analysis in the Guard During Ischemia Against Necrosis (GUARDIAN) trial. Based on these results, the Na\(^+/\text{H}^+\) Exchanger Inhibition to Prevent Coronary Events in Acute Cardiac Conditions (EXPEDITION) trial was recently initiated using a modified dosing regimen of cariporide. The results of EXPEDITION, although eagerly awaited, are not yet available.

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References
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