Orally administered NHE1 inhibitor cariporide reduces acute responses to coronary occlusion and reperfusion

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Humphreys, Rachael A., James V. Haist, Subrata Chakrabarti, Qingping Feng, J. Malcolm O. Arnold, and Morris Karmazyn. Orally administered NHE1 inhibitor cariporide reduces acute responses to coronary occlusion and reperfusion. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H749–H757, 1999.—Na+/H+ exchange (NHE) mediates myocardial ischemic and reperfusion injury. We examined the effects of dietary administration of the potent and selective NHE1 inhibitor on acute responses to coronary artery ligation and reperfusion in the anesthetized rat. Male Sprague-Dawley rats received control rat chow or an identical diet containing 3 parts per million of cariporide for 1 wk before 225 min of occlusion of the left main coronary artery or 45 min of occlusion followed by 180 min of reperfusion. Hearts were excised and divided into left ventricle, right ventricle, and interventricular septum for analysis of NHE1 mRNA expression and apoptosis by staining with terminal deoxynucleotidyl transferase-mediated nick end labeling. Ischemia and reperfusion were associated with a threefold elevation in NHE1 mRNA expression in control animals that was significantly reduced in cariporide-fed rats. Cariporide reduced mortality from 26% of animals to 0%. The incidence of all arrhythmias was significantly reduced, including ventricular fibrillation (from 42 to 0%) and ventricular tachycardia (from 81 to 15%), as well as the number of ventricular premature beats (from 70 ± 12 to 17 ± 6). Cariporide moderately reduced apoptosis only in the reperfused left ventricle to values not significantly greater than those in sham-operated animals, and this was associated with a significantly higher ratio of Bcl-2 to Bax. This study suggests that NHE inhibition with dietary cariporide represents an effective management of acute postinfarction responses.

sodium-hydrogen exchange; HOE-642; infarction; arrhythmias; sodium-hydrogen exchanger subtype 1 messenger ribonucleic acid expression; apoptosis

THE MYOCARDIAL Na+/H+ exchanger (NHE) represents one of the major mechanisms for intracellular pH regulation in response to ischemia-induced acidosis (15). Extensive studies over the past number of years have provided evidence that, in addition to its role in intracellular pH regulation, the exchanger likely contributes to cardiac injury associated with myocardial ischemia and reperfusion (reviewed in Refs. 6 and 11). This concept is based on extensive evidence that pharmacological inhibition of NHE results in cardiac protection that is generally independent of experimental model, animal species, or the type of NHE inhibitor used (6, 11). Moreover, protection appears to be associated with beneficial effects with respect to a wide array of parameters ranging from inhibition of necrosis and antiarrhythmic effects to an antiapoptotic influence (1, 6, 11).

One NHE inhibitor that is of particular interest is 4-isopropyl-3-methylsulfonylbenzoylguanidine methanesulfonate (HOE-642), which has been assigned the nonproprietary name cariporide. In contrast to amiloride analogs, which are nonspecific NHE inhibitors (12), cariporide appears to be a selective inhibitor of the NHE1 isoform, which is the primary, if not sole, cardiac NHE subtype, thereby rendering it particularly attractive for therapeutic interventions for cardiac disorders while minimizing the potential for side effects (19). It is of substantial interest that cariporide is currently in clinical trials to assess its potential influence in high-risk cardiac patients with acute coronary syndromes (GUARDIAN study). Recently, another NHE1-selective inhibitor, EMD-85131, has been demonstrated to potentially inhibit infarct size in dogs even when administered after coronary artery occlusion (7). The vast majority of studies demonstrating beneficial effects of NHE inhibitors including cariporide have utilized either in vitro approaches or, when whole animal models were used, direct infusion of the active compound. Because of the potential clinical value of cariporide in the treatment of heart disease, the present study was undertaken to assess whether a 1-wk dietary administration of cariporide alters immediate postligation responses in anesthetized rats subjected to coronary artery occlusion with or without reperfusion. We assessed the effect of this approach in terms of expression of the NHE1 gene and the incidence of apoptosis. Of particular importance was the detailed assessment of mortality and ventricular arrhythmias, because both are critical events occurring in patients with acute myocardial infarction.

METHODS

Experimental protocol. Male Sprague-Dawley rats (250–350 g) were initially randomized to receive either the control rat chow or an identical diet containing 3 parts per million of cariporide ad libitum. After 1 wk of feeding, the animals were weighed and then anesthetized with pentobarbital sodium (50 mg/kg ip) and buprenorphine (0.03 mg/kg sc). Animals were then intubated and ventilated (10 ml/kg, 70 breaths/min) with air using a rodent respirator (model 683, Harvard Apparatus). A standard lead I electrocardiogram (ECG) was recorded (model 79E, Grass recorder), and a thermistor probe was inserted into the rectum to measure core temperature, which was maintained at 37–38°C with the aid of a heating pad. An incision was made in the skin on the left side of the
certain and the pectoral muscles were retracted to expose the chest. An incision was then made through the fourth intercostal space, the ribs were retracted, and the pericardium was incised to allow access to the heart. A 6-0 braided silk suture attached to a 10-mm micropoint reverse cutting needle was then placed under the left main coronary artery ~2 mm from its origin. The ends of the suture were threaded through a length of polyethylene tubing, forming a snare, which could then be used to ligate and reperfuse the coronary artery as required. In sham-operated animals the ligature was put in place but was not tied. Animals were subjected to either a prolonged 225-min ischemic period or a 45-min period of reperfusion. In all groups negative pressure was applied 45 min after initial placement of the ligature (immediately after ligature removal for the reperfusion group), and the chest wall was sutured closed in three layers (ribs, muscle, and skin). The animals were maintained for a further 180 min, after which they were killed by decapitation and the hearts excised for subsequent determination of NHE1 expression and apoptosis. In addition, blood was collected for determination of plasma cariopride levels. For the latter, plasma was sent on dry ice to Hoechst-Marion-Roussau laboratories (Frankfurt, Germany) for analysis by HPLC.

Arrhythmia measurement and assessment. The ECG was obtained with platinum needle electrodes inserted subcutaneously for a 10-min stabilization period before coronary ligation, after which the ECG was determined for a further 55-min period. This represented 55 min of ischemia in nonreperfused hearts or 45 min of ischemia plus 10 min of reperfusion in hearts subjected to reflow. Initial experiments revealed that virtually all ischemia-induced arrhythmias occurred during a 10- to 30-min postligation period. Moreover, arrhythmias were almost completely absent during reperfusion (see DISCUSSION). Ischemia-induced ventricular arrhythmias were analyzed according to the Lambeth Convention guidelines for the analysis of experimental arrhythmias (22). All ventricular premature beats were recorded. The percentage of incidence and duration of ventricular tachycardia and ventricular fibrillation, when present, were also noted. Ventricular tachycardia was defined as a run of four or more beats at a rate faster than 180 beats/min. Ventricular fibrillation was defined as a signal for which individual QRS complexes could no longer be distinguished from one another and for which a rate could no longer be measured. Ventricular fibrillation was considered irreversible if it did not revert within 3 min of onset. Mortality was also recorded.

RNA isolation. Hearts for NHE expression studies were divided into left ventricle, right ventricle, and interventricular septum and stored in liquid nitrogen. RNA was subsequently isolated using TRIzol reagent (Canadian Life Technologies, Burlington, ON, Canada). Tissues were homogenized, and RNA was extracted with chloroform followed by centrifugation to separate the solution into aqueous and organic phases. RNA was recovered from the aqueous phase by precipitation with isopropyl alcohol and suspended in diethyl pyrocarbonate-treated water. Quantification of RNA was performed by determining the absorbency at 260 and 280 nm.

Reverse transcription-polymerase chain reaction. First-strand cDNA synthesis was performed using the SuperScript II system (Canadian Life Technologies). RNA (10 µg) was added to oligo(dT) primers (Canadian Life Technologies), denatured at 65°C for 10 min, and quenched on ice for 10 min. RT was carried out by the addition of 200 units of Moloney murine leukemia virus-reverse transcriptase and 1 mmol/l dNTP and 10 mmol/l dithiothreitol at 42°C for 50 min in a total reaction volume of 20 µl. The reaction was terminated by a 15-min incubation at 70°C. The resulting RT products were stored at ~20°C.

The amplification was carried out using the following cDNA sequences. For NHE1, primer 1 (5’-TACGTTACCT-GCTTCTGCTCT-3’) and primer 2 (5’-GGATGGAGG-GATCCTCTCCCTC-3’), with a predicted product size of 649 bp, were used (4). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified simultaneously for each sample in a separate set of tubes using the same quantity of RT product. For GAPDH, primer 1 (5’-TATGCGCCGCTTGTACCA-3’) and primer 2 (5’-CCACCT-TCTTATGTCATCA-3’), with a predicted product size of 746 bp, were used. PCR reactions were performed in a total volume of 50 µl containing 1× PCR buffer, 2.5 mmol/l MgCl2, 250 µmol/l dNTP mix, 0.5 µmol/l of each amplification primer, 2.5 units Taq polymerase (Canadian Life Technologies), and 4 µl of the RT product. The amplification was carried out as follows: 45 s at 94°C (denaturation), 45 s at 60°C (annealing), and 1 min at 72°C (extension) for 30 cycles. In initial studies we observed that this thermal cycling reaction was optimal for both NHE and GAPDH, with both reactions remaining within the linear (logarithmic) phase of amplification over a range of template concentrations.

Electrophoresis. The amplification products were analyzed on 3% agarose gels stained with ethidium bromide in 1× Tris-acetate-EDTA buffer. Ten microliters of each PCR product were loaded in each lane and electrophoresed at 110 V for 90 min. At the end of the electrophoresis the gel was visualized under ultraviolet light. Positive amplifications for NHE1 were demonstrated as single bands at 649 bp. GAPDH amplification was demonstrated as a single band at 746 bp.

Southern blotting. The specificity of the amplification was confirmed by Southern blotting following the transfer of the PCR products from gel onto nylon membranes after denaturation and neutralization. Hybridization with biotinylated amplification product-specific oligoprobe was carried out using the NHE1 oligoprobe 5’-TAGACCTGGTGGCCTG-TGAAGA-3’ (4). A similar amplification product-specific oligoprobe (5’-GGATGGAGG-GATCCTCTCCCT-3’) was used for GAPDH. The detection was carried out by incubation of nylon membranes in the dark at 37°C for 2 h with thiglyceraldehyde blue tetrazolium-5-bromo-4-chloro-3 indolylphosphate system (Oncor, Gaithersburg, MD) for visualizing the specific blue bands. Semiquantification of the RT-PCR products was carried out by densitometry using a Hewlett-Packard 4C scanner and Mocha software (andel Scientific). The densitometric values were expressed as arbitrary units per microgram of total RNA added to the RT reaction.

Detection of apoptosis. A 3-mm transverse section, 2 mm from the site of ligation through the center of the myocardium, was obtained and separated into left ventricular, right ventricular, and interventricular septal regions. The tissues were fixed in 10% buffered formaldehyde and then embedded in paraffin. Paraffin sections (4–6 µm) were prepared and then placed on positively charged slides for in situ apoptosis detection using terminal deoxynucleotidyl transferase (TdT)-mediated deoxyribonucleotide triphosphate-digoxigenin nick end labeling (TUNEL) technique. Slides were incubated with 20 µg/ml protease K for 15 min at room temperature and then washed in deionized water. Endogenous peroxidase was inactivated with 3% H2O2 and methanol for 5 min at room temperature. The subsequent staining procedure was carried out according to the manufacturer’s instructions (ApopTag, Oncor). Briefly, sections were rinsed and preincubated with equilibration buffer for 10 min, and digoxigenin-labeled dUTP and dATP in TdT buffer were added to cover the section.
which was then incubated in a humidified chamber at 37°C for 60 min. The reaction was stopped by immersing the slides into a stop/wash buffer. After sections were washed in PBS (0.01 mol/l, pH 7.4), they were incubated in anti-digoxigenin peroxidase for 30 min at room temperature. The slides were stained for 6 min with 1 mg/ml 3,3'-diaminobenzidine (DAB) and 0.3% H2O2 in PBS and counterstained with Harris hematoxylin. Negative control slides were processed at the same time at which the TdT enzyme was excluded. The slides were examined by light microscopy at ×400 magnification, and the number of apoptotic cells per microscopic field was counted and expressed as the number of cells per 100 high-power fields. Adjacent hematoxylin-eosin stained sections were examined to confirm that apoptotic cells represented cardiac myocytes.

Immunostaining for Bax and Bc-2. To assess the presence of Bax and Bc-2, we utilized immunohistochemical techniques as described in detail previously (13). Formalin-fixed, paraffin-embedded tissue sections (5 µm) were mounted on poly-L-lysine-coated slides. Samples were deparaffinized by three changes of 2 min each with xylene followed by dehydration with 100, 95, and 70% ethyl alcohol (two changes, 2 min for each alcohol concentration). After dehydration, samples were incubated for 5 min in PBS followed by antigen retrieval (boiling by microwaving for 10 min) in 10 mM citrate buffer, pH 6.0. Samples were cooled for 20 min in citrate buffer, washed twice in PBS for 5 min, and then blocked for 45 min in Tris-Na-K (TNK) solution (100 mmol/l Tris, 550 mmol/l NaCl, and 10 mmol/l KCl, pH 7.6–7.8) containing 2% BSA, 0.1% Triton X-100, and 3% normal goat serum. Rabbit polyclonal anti-mouse/rat Bc-2 or anti-mouse/rat Bax primary antibody (1:700 dilution, Pharmingen, Mississauga, ON, Canada) was added to the blocking solution, and the samples were incubated overnight at room temperature in a humidified chamber. Slides were then washed three times for 5 min each with PBS, endogenous peroxidases were blocked with 3% H2O2 in methanol for 5 min, and samples were then washed three times with PBS. A Vectastain elite ABC kit (Vector Laboratories, Burlington, ON, Canada) was used for staining that was carried out according to kit instructions. Color was developed by incubating slides for 5 min in TNK solution containing 1 mg/ml DAB and 0.3% H2O2. Counterstaining was done with Harris hematoxylin.

Slides were evaluated at a ×400 magnification. The intensity of DAB staining was graded by one of us (J. V. Haist) using similar criteria described previously (13) and using the following scale: 0, no staining; 1, light staining; 2, moderate staining; and 3, intense staining. In each field of view, the approximate percentage of the field at each intensity was recorded, and the average for 50 fields at each intensity was calculated for each experiment. A histochemical score was determined for each slide by calculating the sum of the stain intensity multiplied by the percentage of the field for all four intensity grades and then dividing by 100 (17). For negative control slides the primary antibody was left out. No staining was observed in these samples. Normal rat pancreas was used for positive control staining. Bax staining was observed in acinar but not islet cells, in agreement with Krajewski and co-workers (13). Bc-2 staining was evident in both acinar and islet cells but not in the ducts, in agreement with Hockenbery et al. (8).

Statistical analysis. Data for mortality and percent incidence of arrhythmic events were analyzed using chi-square analysis. Duration of arrhythmias and incidence of ventricular premature beats were analyzed using Mann-Whitney's rank sum test. Analysis of NHE1 expression and the incidence of apoptosis was done using Kruskal-Wallis one-way ANOVA. A value of P < 0.05 was considered to indicate a significant difference.

RESULTS

Animal presurgical data. The pooled mean body weights for control and cariporide-fed animals were 295 ± 6 (n = 58) and 286 ± 5 g (n = 44), respectively (P > 0.05). The mean plasma concentration of cariporide after 1 wk of feeding was 41 ± 8 ng/ml.

NHE1 mRNA expression. We assessed NHE1 expression at the end of the ischemic or reperfusion periods in left ventricle as well as the interventricular septum and right ventricle. These data are summarized in Fig. 1, which shows the ratios of NHE1 to GAPDH expression, with the latter being completely unaffected by any treatment (data not shown). Compared with sham animals, both ischemia and ischemia-reperfusion were associated with an approximately threefold elevation in NHE1 mRNA expression in animals fed a control diet. Cariporide feeding alone had no effect on NHE1 expression in animals not subjected to coronary ligation (data not shown). However, in cariporide-fed rats subjected to ligation, the elevation in left ventricular

**Fig. 1.** Na+/H+ exchanger (NHE1) mRNA expression in left ventricle (A), right ventricle (B), and interventricular septum (C) of hearts subjected to various treatments. Values represent densitometric measurement of RT-PCR products after Southern hybridization. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values are means ± SE; n = 10 for each treatment group. *P < 0.05 vs. sham; **P < 0.05 vs. respective values obtained in absence of cariporide.
NHE1 expression was significantly less than that observed in animals fed the control diet. As also summarized in Fig. 1, B and C, no effects were observed in either the interventricular septum or right ventricles in these animals. Examples of individual Southern blots of RT-PCR reaction products for various treatments are provided in Fig. 2. As noted earlier, there was no effect of any treatment on the mRNA expression of the GAPDH housekeeping gene (Fig. 2).

Mortality. The total incidence of mortality in all animals is summarized in Fig. 3A. In control animals a mortality rate of 26% (11 of 43 animals) was observed, whereas no deaths occurred in animals fed the cariporide diet. Ventricular fibrillation was readily identified as the cause of death in five animals subjected to ECG measurement at the time, whereas six animals died during the 3-h recovery period.

Incidence of arrhythmias. Virtually no arrhythmias occurred during the reperfusion, most likely reflecting the prolonged ischemic duration (see Discussion). Moreover, arrhythmias were generally restricted to the period of 10–25 min after coronary artery ligation. The incidence of arrhythmias during ligation was pooled for all animals irrespective of subsequent reperfusion. The data for ventricular fibrillation are summarized in Fig. 3B, which shows that 42% demonstrated this type of arrhythmia. The average duration of fibrillation was 23 ± 9 s. Although the data are not shown, sham animals did not demonstrate any arrhythmias irrespective of dietary regimen. In contrast, fibrillation was completely absent in animals receiving the cariporide diet (Fig. 3). Figure 4 demonstrates the incidence of ectopic activity (Fig. 4A) as well as an analysis of ventricular tachycardia in terms of incidence (Fig. 4B) and duration (Fig. 4C). The number of ventricular premature beats averaged 70 ± 12 for control diet-fed animals, which was significantly decreased to 17 ± 6 by cariporide. Moreover, 35 of 43 control animals (81%) demonstrated ventricular tachycardia, which was significantly reduced to 15% (5 of 38) by dietary cariporide, as was the duration of ventricular tachycardia, from 40 ± 9 to 4 ± 1 s (P < 0.05). Examples of ECG recordings from two control rats showing the types of arrhythmias observed in this study are provided in Fig. 5.

Incidence of apoptosis. We next quantified the number of apoptotic nuclei in three regions of the heart after occlusion with or without subsequent reperfusion. These data are summarized in Fig. 6. In the left ventricle the incidence of apoptosis was significantly elevated by coronary artery ligation as well as by both ligation and reperfusion. Cariporide failed to attenuate apoptosis in the left ventricle after sustained ischemia, such that values were identical to those seen in animals fed the control diet. However, in the reperfused left ventricle, an ~50% decrease in the number of apoptotic nuclei was evident such that the values obtained in this group were not significantly higher than those seen in the sham group. Very few apoptotic nuclei (note differences in scale in Fig. 6) were found in either the right ventricle or interventricular septum, with no significant differences among any of the treatment groups. Examples of positively TUNEL-stained nuclei in sham hearts and hearts subjected to occlusion and reperfusion from control or cariporide-fed animals are provided in Fig. 7.
Bcl-2 and Bax expression. To further characterize apoptosis, we next used a histochemical technique to semiquantify the expression of Bcl-2 and Bax in hearts subjected to various treatments. As summarized in Table 1, ligation with or without reperfusion produced a modest elevation in Bcl-2 expression, although none of these effects were significant compared with those in sham animals. A trend toward elevated Bax expression was similarly observed in response to occlusion, although a significant increase was seen only in reperfused hearts. The elevation in Bax expression in these hearts was completely prevented by dietary cariporide, resulting in a significantly increased ratio of Bcl-2 to Bax that was not observed with any other treatment group. Examples of Bcl-2 and Bax immunohistochemical staining are provided in Fig. 8.

DISCUSSION

The present study demonstrates that in vivo coronary artery occlusion and reperfusion increases NHE1 expression in the infarcted left ventricle of the rat, which is associated with increased apoptosis and a high incidence of ventricular arrhythmias. Moreover, we demonstrated that dietary administration of the NHE inhibitor cariporide attenuates these early postinfarction responses. The mean plasma levels of cariporide (41 ± 8 ng/ml or ~0.1 µM) is a concentration that has been shown to inhibit NHE1 by ~68% in rat cardiac myocytes in which intracellular acidosis was produced by ammonium chloride pulsing (20). There is now good evidence that inhibition of NHE produces cardioprotective effects in the ischemic and reperfused myocardium in a wide range of experimental models and animal species (reviewed in Refs. 6 and 11). The protective effect of NHE inhibitors likely reflects diminished intracellular Ca^{2+} loading, because blocking the NHE will result in reduced Na^{+} influx via the antiporter. As a result, more effective Ca^{2+} removal through the Na^{+}/Ca^{2+} exchanger would occur (18). An important advance in the development of potentially useful cardioprotective agents that target NHE has been the development of compounds less related to the amiloride structure with generally greater potency and specificity against NHE, namely, HOE-694 and HOE-642 (19). Of these two compounds, HOE-642 is of particular interest because it is a specific inhibitor of the primary, if not sole, isofrom NHE1, which is found in the mammalian myocardium (19). Moreover, HOE-642, which has been assigned the nonproprietary name cariporide, has been found to exert substantial protection against various forms of myocardial injury (9, 10, 16, 19, 20). In addition, this drug is currently under clinical evaluation in a multicentered international study to evaluate its potential effects in patients with acute coronary syndromes (GUARDIAN study).

The present study was carried out to assess whether dietary preadministration of cariporide alters the early responses to occlusion and may be of importance with respect to potential development of this drug, or other NHE inhibitors, for oral use in cardiac therapeutics. In addition, we examined in detail the effects of this treatment on the development of arrhythmias, including ventricular fibrillation, as well as mortality after coronary ligation. Moreover, early changes in NHE1 mRNA expression were determined in view of evidence that in vitro ischemia increases expression of the antiporter in isolated rat hearts (2). Finally, we determined whether apoptosis is affected by dietary administration of the NHE inhibitor. A 1-wk feeding regimen was determined based on initial plasma sampling for cariporide concentrations that showed that plasma levels retained steady-state values during this period.

Potential role of NHE in early postinfarction responses. The present report shows that coronary artery occlusion in this model is associated with a stimulation of NHE1 expression in the left ventricular myocardium with no significant effect on either right ventricle or interventricular septum. It is well known that NHE activity is stimulated by myocardial ischemic and reperfusion conditions, and to our knowledge this is the first demonstration that coronary artery occlusion can also stimulate NHE1 expression. As noted above, this finding is in agreement with an earlier report that global...
ischemia in the isolated rat heart increases NHE1 mRNA (2). The relevance of increased NHE1 expression to early postocclusion responses is unclear, although it is logical to suggest that increased NHE expression will result in subsequent increased protein synthesis of the exchanger, suggesting that the myocardial chronic response to infarction may involve altered NHE1 protein expression. Accordingly, because cariporide blunted the increased mRNA expression in the infarcted myocardium, the long-term consequence of this effect would be important to assess. The reason for the somewhat unexpected effect of cariporide in attenuating the elevation in NHE1 mRNA expression is unclear at this time, although it may be a consequence of diminished injury due to inhibition of NHE activity, resulting in modulated production of factor(s), yet to be determined, that are important in the regulation of NHE1 gene expression. Alternatively, it could be suggested that NHE1 expression is regulated by NHE activity.

A major thrust of the present study was to assess arrhythmias in detail, because most sudden cardiac deaths, which represent 50% of all deaths after infarction, can be attributed to ventricular fibrillation. In our model, the occurrence of arrhythmias was restricted only to the ligation period, with no arrhythmias observed during reperfusion. The lack of reperfusion-associated arrhythmias is in agreement with the concept that the incidence of these arrhythmias is inversely related to the preceding duration of ischemia, which may in turn reflect the degree of cell injury. Our results show that dietary cariporide is effective in preventing arrhythmias produced by coronary artery occlusion. These findings are in agreement with a previous report (19) demonstrating these effects in rats administered either intravenous or oral cariporide, particularly in terms of abolition of ventricular fibrillation. The mechanism underlying the ability of dietary cariporide to prevent ischemia-induced arrhythmias may reflect the fact that NHE inhibitors can attenuate electrolyte disturbances in the ischemic myocardium, including attenuation of both Ca$^{2+}$ and Na$^+$ elevation (18). Moreover, NHE inhibition can block the direct detrimental effects of lysophosphatidylcholine as well as H$_2$O$_2$, two products whose levels are increased in the ischemic myocardium and that can contribute to development of arrhythmias (9, 10).

Our study further demonstrates that dietary cariporide modestly inhibits the development of apoptosis; however, the effect of cariporide occurred only with respect to apoptosis associated with 180 min of reperfusion following 45 min of ischemia, with no effect against apoptosis produced by prolonged (225 min) ischemia alone. We utilized durations of occlusion and reperfusion identical to those recently reported by Fliss and Gattinger (5) using a similar model. Our results agree with that study in terms of the similar level of apoptosis observed during either ligation or reperfusion. The selective salutary effect of cariporide seen in our study agrees with the concept proposed by these investigators of potentially different mechanisms of inducing apoptosis in the sustained-ligated versus occluded-reperfused myocardium, under which conditions neutrophil activation may play an important role. Moreover, it appears...
that various properties of apoptotic nuclei appear to be
different between those found in the infarcted versus
reperfused myocardium (5). It is interesting that neutro-
phil activation is a pH-dependent phenomenon, and,
indeed, neutrophil-induced cardiac injury can be attenu-
ated by NHE inhibition (3). Although it is intriguing to
speculate that inhibition of neutrophil activation con-
tributed to the reduction in apoptosis in the reperfused
myocardium, it should be added that cariporide also
inhibits apoptosis in the ischemic and reperfused iso-
lated rat heart devoid of neutrophils, suggesting that
the antiapoptotic influence of the drug likely reflects
multifaceted mechanisms (1). The inability of caripo-
ride to affect apoptosis associated with ligation indi-
cates that apoptosis and ischemia-induced arrhyth-
mias are unlikely to be related in this model. To further
examine the effect of cariporide on apoptosis, we car-
rried out histochemical assessment of the protoonco-
genesis Bcl-2 and Bax, which are important modulators
of apoptosis by virtue of their abilities to inhibit or
stimulate the process, respectively (14, 23). Because
Bcl-2 promotes cell survival, whereas Bax accelerates
cell death, the ability of cariporide to increase the ratio

Fig. 6. Quantitative assessment of apoptosis in left ventricle (A),
right ventricle (B), and interventricular septum (C) after various
treatments. Values are means ± SE and indicate terminal deoxy-
nucleotidyl transferase-mediated nick end labeling (TUNEL)-
positive nuclei as described in METHODS.*P < 0.05 vs. sham treat-
ment group. Note differences in scale in A vs. B and C.

Fig. 7. Micrographs demonstrating positively TUNEL-stained nuclei
(arrows) in left ventricles from a sham rat heart (A) or hearts
subjected to occlusion and reperfusion without (B) or with (C)
cariporide.
of Bcl-2 to Bax, primarily by preventing Bax upregulation, may explain, at least in part, the beneficial effects of this drug on the occluded-reperfused myocardium. However, on the basis of the data, cause-and-effect relationships between these proteins and the incidence of apoptosis cannot be firmly established.

Potential clinical relevance. In conclusion, our study demonstrates that dietary administration of the NHE inhibitor cariporide attenuates various early events associated with coronary artery occlusion and reperfusion. Studies in the rat myocardium must be interpreted cautiously; however, the potent ability of dietary cariporide to prevent arrhythmias, particularly in terms of abolishing ventricular fibrillation and, indeed, mortality postocclusion, is encouraging for the development of potentially effective agents for patients at risk of myocardial infarction. The relevance of increased NHE expression or the reduction in apoptosis in the acutely infarcted myocardium requires further studies. However, apoptosis has recently been shown to be the earliest and most predominant form of cell death in patients with myocardial infarction (21). Of particular relevance to the present study, that report (21) also demonstrated that apoptosis is further accelerated by reperfusion. As such, the selective attenuation of the latter by cariporide may be of further clinical importance.

### Table 1. Histochemical semiquantification of Bcl-2 and Bax expression in rat heart

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Bcl-2 (mean ± SD)</th>
<th>Bax (mean ± SD)</th>
<th>Bcl-2/Bax</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>5</td>
<td>1.18 ± 0.12</td>
<td>0.72 ± 0.19</td>
<td>1.49 ± 0.21</td>
</tr>
<tr>
<td>Sham + cariporide</td>
<td>5</td>
<td>1.24 ± 0.17</td>
<td>0.77 ± 0.11</td>
<td>1.42 ± 0.18</td>
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<tr>
<td>Ligation</td>
<td>7</td>
<td>1.65 ± 0.19</td>
<td>1.17 ± 0.09</td>
<td>1.41 ± 0.13</td>
</tr>
<tr>
<td>Ligation + cariporide</td>
<td>7</td>
<td>1.40 ± 0.18</td>
<td>0.98 ± 0.12</td>
<td>1.42 ± 0.17</td>
</tr>
<tr>
<td>Ligation-reperfusion</td>
<td>5</td>
<td>1.58 ± 0.12</td>
<td>1.37 ± 0.16*</td>
<td>1.15 ± 0.14</td>
</tr>
<tr>
<td>Ligation-reperfusion + cariporide</td>
<td>5</td>
<td>1.71 ± 0.19</td>
<td>0.61 ± 0.05†</td>
<td>2.81 ± 0.03‡</td>
</tr>
</tbody>
</table>

Values for Bcl-2 and Bax depict histochemical scores obtained as described in METHODS; n = no. of hearts examined in group. Bcl-2/Bax, ratio of Bcl-2 to Bax. *P < 0.05 vs. both sham groups; †P < 0.05 vs. ligation-reperfusion group not treated with cariporide; ‡P < 0.05 vs. both sham groups and ligation-reperfusion group not treated with cariporide (ANOVA).
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