Effective protection by NHE-1 inhibition in ischemic and reperfused heart under preconditioning blockade

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Effective protection by NHE-1 inhibition in ischemic and reperfused heart under preconditioning blockade. Am J Physiol Heart Circ Physiol 284: H798–H803, 2003; 10.1152/ajpheart.00659.2002.—We compared the protective effects of ischemic preconditioning (IPC) and the Na\(^+\)/H\(^+\) exchanger-1 (NHE-1) inhibitor cariporide in isolated rat hearts subjected to global ischemia (45 or 90 min) and 30-min reperfusion and determined the protective effects of cariporide under IPC blockade with the mitochondrial ATP-sensitive K\(^+\) channel blocker 5-hydroxydecanoate (5-HD). With 45-min ischemia, both IPC and cariporide equally increased maximum recovery of left ventricular developed pressure twofold (P < 0.05), although recovery was significantly greater with cariporide for the first 15 min of reperfusion. 5-HD almost completely blocked the protective effects of IPC on recovery but had no influence on the salutary effects of cariporide. With 90-min ischemic control, recovery was only 3% of preischemia and was unaffected by IPC, although cariporide increased recovery to ~30% (P < 0.05). This was associated with a 37% preservation of viable cardiac cells, whereas no structurally intact cells were found in either IPC or control hearts. Our study shows that NHE-1 inhibition is a more effective cardioprotective strategy than IPC in this model, possibly because of enhanced myocyte salvage, and because protection by NHE-1 inhibition is completely unaffected by IPC blockade with 5-HD.

Na\(^+\)/H\(^+\) exchange (NHE) inhibition has been extensively demonstrated to exert substantial cardioprotective action against ischemic and reperfusion injury. The mechanism of such protection likely involves a blunting of NHE-dependent sodium influx, thereby attenuating subsequent calcium elevation through Na\(^+\)/Ca\(^2+\) exchange (reviewed in Refs. 1, 12, and 13). Thus the protective effect of NHE inhibitors, especially NHE-1 isoform-selective inhibitors, is related to the ability of these agents to ultimately inhibit calcium overload in the ischemic and reperfused myocardium. Another effective cardioprotective strategy is ischemic preconditioning (IPC), in which a brief period or brief multiple periods of ischemia bestow protection against a subsequent prolonged ischemic insult (18). The mechanisms underlying IPC-mediated protection are not known with certainty but involve activation of various cell signaling cascades. Recent evidence (4, 5, 9) suggests an important role for the mitochondrial ATP-sensitive K\(^+\) (mitoK\(_{ATP}\)) channel in this phenomenon. The implication of mitoK\(_{ATP}\) channel involvement in IPC comes primarily from studies that used its putative inhibitor 5-hydroxydecanoate (5-HD), which is a nonspecific agent. Thus, although 5-HD is effective in blocking IPC, its mechanism of action should be interpreted cautiously.

Preconditioned ischemic myocardium demonstrates reduced proton accumulation (3, 6, 19, 22), although this is unlikely to be caused by NHE-dependent mechanisms (6). Indeed, most studies (2, 16, 21) have shown that NHE inhibition when combined with IPC produces additive protective effects, suggesting different mechanisms of action for each cardioprotective strategy. Although both IPC and NHE inhibition are effective cardioprotective strategies, an important consideration is the demonstration of comparative effectiveness. A recent study (11), which used a canine coronary artery occlusion model, demonstrated that NHE-1 inhibition is superior to IPC when ischemia is prolonged. The present study was done to expand on these observations by using the isolated heart of the rat and to compare the efficacy of IPC and the NHE-1-specific inhibitor cariporide. In addition, we examined the interaction between IPC and cariporide to assess whether cariporide is effective when IPC is prevented by 5-HD. Finally, we determined the cardiac ultrastructural integrity after various treatment protocols.

METHODS

Animals. Male Sprague-Dawley rats weighing 275–300 g were purchased from Charles River (St. Constant, Quebec, Canada). The animals were maintained in the Health Sciences Animal Care Facility of the University of Western Ontario in accordance with the guidelines of the Canadian Council on Animal Care (Ottawa, Ontario, Canada).

Heart perfusion. The rats were euthanized by decapitation, and the hearts were immediately removed, placed in cold...
Krebs-Henseleit buffer (see composition below) to inhibit any further contractions, then mounted by the aorta on a stainless steel cannula, and arranged for retrograde perfusion with the use of the Langendorff method, as described previously (15). The hearts were perfused at a flow rate of 10 ml/min with the use of a peristaltic pump with Krebs-Henseleit buffer composed of the following (in mmol/l): 120 NaCl, 4.63 KCl, 1.17 KH2PO4, 1.25 CaCl2, 1.2 MgCl2, 20 NaHCO3, and 8 glucose. The buffer was initially equilibrated and then continuously gassed with a 95% O2-5% CO2 mixture. The pH of the buffer was 7.4, and temperature was maintained at 37°C by enclosing the entire system in a series of water-jacketed coils. Coronary pressure was measured via a side arm of the perfusion cannula, which was connected to a pressure transducer (model P23 XL, Spectramed). A latex water-filled balloon fixed to a pressure transducer was inserted through the mitral valve into the left ventricle (LV) for the determination of LV pressure developed pressure (LVEDP). End-diastolic pressure (LVEDP) was adjusted to 5 mmHg before the start of the experiment by adjusting the volume in the intraventricular balloon with the aid of a micrometer syringe. The determinations of ventricular performance were obtained online with a Biopac data analysis system (Biolynx Scientific Equipment).

Experimental protocol. Hearts were equilibrated for 30 min, after which zero-flow ischemia was initiated for 45 or 90 min with a further 30-min reperfusion. For IPC, most hearts were subjected to a 5-min ischemic period, followed by a 10-min reperfusion before prolonged ischemia was initiated. The 90-min ischemic hearts were also subjected to two 5-min cycles separated by 10-min reperfusion to ensure that the IPC-induced protection was optimal. Cariporide (5 μM; Aventis Pharma; Frankfurt, Germany) or 5-HD (100 μM; Sigma/RBI; St. Louis, MO) was added 20 min before ischemia (5 min before IPC). Drugs were present during both the ischemic and reperfusion periods. To determine whether NHE-1 inhibition protects under IPC blockade, studies were also carried out in which both cariporide and 5-HD were added before the IPC protocol.

Electron microscopy. Three hearts from each experimental group subjected to 90 min of ischemia and reperfusion were evaluated to assess the degree of ultrastructural abnormalities. The hearts were immediately removed at the end of the reperfusion period, fixed to an aortic cannula, mounted on a Langendorff system, and perfused at a constant perfusion pressure of 80 cmH2O with 150 ml fixative containing 0.08 mol/l sodium cacodylate, 2% glutaraldehyde, and 1% paraformaldehyde; pH 7.4. Sections measuring 1 cm × 1 mm × 1 mm were then cut from the LV. The subsequent processing and sectioning were performed with a Lynx automatic tissue processor at the Department of Pathology, University Hospital Campus of the London (Canada) Health Sciences Center. Tissues were postfixed with 1% osmium tetroxide and dehydrated with graded ethanol and acetone rinses. The final ethanol-acetone solution was replaced by en bloc stain consisting of a 3:7 ratio of uranyl nitrate-saturated lead acetate solution for 1 h. The tissues were infiltrated and embedded in Epon-Araldite resin and polymerized overnight at 37°C. Thin sections (60–90 nm) were cut with a diamond microtome knife and stained with uranyl acetate and lead citrate. Samples were viewed on an electron microscope (model 109, Carl Zeiss) by one of the authors (M. Karmazyn) in a “blinded” fashion (without knowledge of the treatment protocol to which it was subject-
ed). Approximately 200 cells were examined from each tissue sample with the assistance of an experienced electron microscopy technician.

Statistical analysis. Data were analyzed using ANOVA with \( P < 0.05 \) indicating differences between treatment groups. Data are presented as means ± SE of not <5 experiments per group.

RESULTS

We first examined the ideal IPC protocol producing maximum cardioprotection in hearts subjected to 45 min of ischemia. These studies revealed identical protection with either one or two 5-min ischemic periods, and, accordingly, the former protocol was utilized for most of the studies reported. As shown in Fig. 1, both IPC and cariporide produced identical degrees of maximum functional recovery in hearts subjected to 45 min of ischemia. However, a major difference between these protective approaches was the accelerated recovery seen in those hearts treated with cariporide with maximal recovery evident after 10 min of reperfusion, whereas 25 min of reperfusion was required in preconditioned hearts. There was no significant elevation on LVEDP in reperfused hearts after 45 min of ischemia (not shown).

With 90 min of ischemia, virtually no recovery of function was observed in control hearts after reperfusion nor were there any beneficial effects of IPC (Fig. 2). However, as shown in Fig. 2, cariporide significantly enhanced recovery of LVEDP compared with either control or IPC hearts. No beneficial effects of either IPC or cariporide were observed in terms of LVEDP, although values tended to be lower throughout the reperfusion period in the IPC group. Increasing the number of preconditioning cycles to two failed to improve recovery in hearts subjected to 90 min of ischemia (not shown).

Examples of electron micrographs of hearts from various treatment groups are shown in Fig. 3. Ultrastructural assessment of post-90-min control ischemic-reperfused hearts revealed massive cellular injury, including sarcolemmal and sarcomere disruption, as well as clearly evident mitochondrial swelling, which was observed in 100% of cells examined. An example of such injury is shown in Fig. 3B. No cell salvage was observed in IPC hearts (Fig. 3C) with results virtually identical to those seen in control ischemic-reperfused hearts.
fused hearts (Fig. 3B). However, cariporide-treated hearts showed greatly reduced cell damage with relatively intact sarcomeres and cell membrane, although mitochondrial swelling in these cells was evident (Fig. 3D). Moreover, in 37% of cells studied, no gross morphological abnormalities were identified with these cells exhibiting nearly normal ultrastructural characteristics.

The next series of experiments was designed to address two fundamental questions concerning IPC interaction with NHE-1 inhibition. First, we sought to determine potential additive effects of IPC and cariporide. Second, we assessed whether NHE-1 inhibition exerts salutary effects under conditions where IPC is prevented with 5-HD. The results of these experiments are summarized in Figs. 4 and 5 for reperfusion recovery and ischemic contracture, respectively. Because IPC failed to improve recovery in 90-min ischemic hearts, these studies were therefore done only in hearts subjected to 45 min of ischemia.

Figure 4 illustrates the recovery in LVDP and LVEDP values 15 and 30 min after reperfusion following 45 min of ischemia. Both IPC and cariporide significantly enhanced recovery of function and reduced LVEDP values. As expected, 5-HD abolished the protective effect of IPC. However, under this condition of IPC blockage, cariporide exerted identical protection as with cariporide alone, indicating that NHE-1 inhibition exerts full protective effects when IPC is completely blocked. Interestingly, IPC plus cariporide produced an additive trend with 100% recovery of function under these conditions, although these values were not significantly higher than either treatment alone.

We further analyzed contracture development during the ischemic period, before reperfusion in terms of
peak elevation in LVEDP, as well as time to reach peak LVEDP values (Fig. 5). Ischemic contracture was generally unaffected by IPC, although under all treatment conditions where cariporide was present, LVEDP was significantly attenuated. No additive effect with IPC was observed nor was there any modulating effect of 5-HD. No significant effects were seen on time to peak LVEDP values. Figure 6 illustrates representative recordings of LV pressure values under various experimental conditions.

**DISCUSSION**

Extensive evidence in the literature has demonstrated that both IPC and NHE-1 inhibition exert potent myocardial protection against ischemia and reperfusion (1, 11–13, 15, 16, 18–24). The mechanisms for the beneficial effects mediated by IPC are not known with complete certainty. Recent evidence has strongly implicated a role for KATP channel activation; however, how this translates to cardioprotection is still unknown (see below). Protection via NHE-1 inhibition occurs through a different process primarily involving the attenuation of intracellular calcium elevation secondary to a reduction in NHE-dependent sodium influx (1, 12, 13).

Despite the evidence indicating that protection produced by IPC and NHE-1 inhibition occurs via distinct, and likely unrelated, mechanisms, controversy still exists concerning interaction between these two modes of protective interventions. For example, attenuation of intracellular acidosis during ischemia appears to be a hallmark of IPC (3, 6, 22), and it has been suggested that activation of NHE-1 may contribute to this phenomenon (19). These investigators reported increased sodium influx in preconditioned hearts and also demonstrated the ability of an amiloride analog that inhibits NHE to prevent the preconditioning phenomenon (19). However, amiloride analogs are nonspecific inhibitors because they can modulate many ion regulatory processes (14). Moreover, the finding of increased sodium influx in the preconditioned heart contradicts other studies (22), as does the prevention of IPC-induced protection by NHE inhibition. Indeed, with respect to the latter, studies (2, 21) have shown that NHE-1 inhibition not only fails to reduce IPC-induced protection but actually produces additive protective effects. We found a similar trend in the present study, where a combination of IPC plus cariporide, a NHE-1-selective inhibitor that is devoid of effects on ion channels or other transporters, produced a 100% recovery of contractile function, which was greater, albeit slightly, than either intervention alone.

Our study also demonstrates that in general the protective effect of cariporide appeared to be superior to that produced by IPC. The relative efficacies of NHE-1 inhibition with cariporide were determined under conditions of two durations of ischemia. With 45 min of ischemia, the ultimate maximum degrees of functional recoveries were identical, although with cariporide recovery occurred significantly faster than that seen with IPC alone. Moreover, with prolonged more severe ischemia, IPC completely failed to improve recovery of function, whereas a significant recovery

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**Fig. 5.** Peak LVEDP pressures seen during ischemia before reperfusion (A) and time-to-peak LVEDP (B) under various treatments. Each point represents the means ± SE of 8 experiments for each group. *$P < 0.05$ from representative values obtained from untreated hearts.
was seen in cariporide-treated hearts. These results are in agreement with those reported by Gumina and colleagues (11), who showed a lack of effect of IPC on infract size in canine hearts subjected to 90 min of left anterior descending coronary artery occlusion, whereas the NHE-1 inhibitor BIIB-513 produced a significant infract size reduction. In support of this finding, we further observed that enhanced recovery in function observed in our study was associated with substantial salvage of cardiac myocytes: indeed, 37% of cells examined demonstrated virtually no evident ultrastructural changes, and in those cells that did demonstrate injury, the degree of injury appeared to be markedly less than that observed either in the control group or in hearts subjected to IPC.

As noted previously, there is evidence that activation of mitoKATP channels exerts cardioprotective effects and plays an important role in mediating the protective effect of myocardial IPC (4). The exact mechanism for the involvement of this channel is not known with certainty (7, 10), but it has been suggested that this may be due to improved mitochondrial bioenergetics, possibly secondary to mitochondrial volume changes (7). Others have proposed that mitoKATP channel activation induces a partial 5-HD-sensitive mitochondrial membrane depolarization, which results in diminished mitochondrial calcium overload (17). As already alluded to, the contribution of mitoKATP channels to IPC is suggested primarily from studies showing that 5-HD can prevent the IPC response (4, 5), a finding clearly observed in our studies. However, it should be noted that the precise involvement, or indeed the existence of mitoKATP channels, has as yet not been confirmed. Indeed, in a recent study (23), others have proposed that it is the sacoemmal KATP channel that is of primary importance in mediating IPC, at least in the mouse myocardium.

We also clearly demonstrate that blocking IPC with 5-HD exerts no effect on the beneficial effects of cariporide in that the NHE-1 inhibitor exerted identical effects in terms of its ability to enhance the magnitude of recovery of function after 45 min. These results strongly suggest that NHE-1 inhibition and IPC exert protection via distinct mechanisms. Of further importance, the results suggest that under pathological conditions, which could result in defective endogenous IPC processes, NHE-1 inhibition would be expected to still exert optimum cardioprotective effects. That such a phenomenon can occur has been shown by Ghosh and colleagues (8), who reported the inability to precondition isolated right atrial tissue from diabetic (both insulin dependent and noninsulin dependent) patients, which the authors attributed to dysfunctional mitoKATP channels in hearts from these individuals.

Our results demonstrating protection with cariporide under mitoKATP channel blockade differ from a recent study by Miura and colleagues (15), who showed that the protective effects of either cariporide or ethylisopropylamiloride in infarcted or stunned rabbit hearts are prevented by 5-HD. The basis for the diverse results are difficult to explain but may be related to species differences or experimental design. In terms of the latter, Miura et al. (15) did not use preconditioned hearts but instead determined the direct effects of 5-HD on cardioprotection produced by NHE-1 inhibition. However, we have been unable to demonstrate any effect of 5-HD on the cardioprotective effects of cariporide in nonpreconditioned hearts subjected to either 45 or 90 min of ischemia (data not shown).

In conclusion, our study demonstrates the protective effects of IPC and NHE-1 inhibition in the ischemic and reperfused rat heart, although the beneficial effect of IPC is lost with more prolonged ischemia. The ability of the NHE-1 inhibitor cariporide to protect under this condition reflects its ability to salvage cardiac myocytes. Our study also demonstrates that NHE-1 inhibition is able to fully protect the myocardium under conditions of IPC blockade with mitoKATP channel inhibition, suggesting distinct mechanisms for the two modes of protection.

Fig. 6. Representative recordings of isolated hearts subjected to 45 min of ischemia (between arrows) showing developed pressures after various treatments. A: control ischemia; B: IPC (○); C: cariporide; D: IPC + 5-HD; E: IPC + 5-HD + cariporide; F: IPC + cariporide. Horizontal bar, 45 min; vertical bar, 60 mmHg.
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