The Cardioprotective Effect of Brief Acidic Reperfusion after Ischemia in Perfused Rat Hearts is not Mimicked by Inhibition of the Na⁺/H⁺ Exchanger NHE1

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Key Words

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Abstract

Background: Ischemic postconditioning (PostC), i.e. brief ischemia-reperfusion cycles before full reperfusion, is protective against cardiac ischemia/reperfusion (I/R) injury. Inhibition of the Na⁺/H⁺ exchanger NHE1 and delayed intracellular pH-normalization have been proposed to underlie protection by PostC. **Methods and Results:** We used Langendorff perfused rat hearts exposed to 35 min global ischemia to show that 15 min acidic (pH 6.5) treatment at onset of reperfusion decreased infarct size and functional deterioration at least to the same extent as PostC. In contrast, NHE1 inhibition by EIPA was detrimental. To evaluate HL-1 atrial cardiomyocytes as a cellular model for PostC, we exposed the cells to simulated ischemia/reperfusion (I/R) mimicking that in perfused hearts. Necrosis and apoptosis induced by I/R were unaffected by 15 min of pH 6.0 at onset of reperfusion. I/R increased the activity of c-Jun N-terminal Kinase 1/2 (JNK1/2) and Akt, but not

of p38 MAPK, with no further effect of acidic reperfusion or EIPA. **Conclusion:** In rat hearts, 15 min acidic reperfusion improves myocardial performance at least as much as does PostC, whereas NHE1 inhibition is detrimental. In contrast, in HL-1 cardiomyocytes, acidic reperfusion or NHE1 inhibition affect neither survival nor JNK1/2-, Akt-, and p38 MAPK activity after I/R, pointing to different mechanisms of damage and protection in these systems.

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Introduction

Acute coronary artery occlusion resulting in myocardial ischemia and infarction is a major cause of death world-wide. Reperfusion is a prerequisite for recovery, yet injury initiated during the reperfusion phase accounts for a large fraction of the cardiomyocyte damage and death following myocardial ischemia [1, 2]. Postconditioning (PostC), i.e. rapid, repeated cycles of ischemia and reperfusion before full reperfusion, exerts

a considerable, protective effect against reperfusion injury [3-5] under certain conditions and has been shown to reduce myocardial infarct size *in vivo* and *in vitro* [3]. Despite substantial advances in recent years, the mechanisms through which PostC exerts its protective effects are still incompletely understood [3, 6].

Cardiac pH homeostasis is severely compromised by ischemia: intracellular pH (pH_i) can decline by about 0.5 pH units within the first 5 min, and extracellular pH (pH_o) can reach pH 6-6.5 [7]. Reduced pH_i is protective to the heart through a number of different mechanisms, including inhibition of mitochondrial permeability transition pore (mPTP) formation, gap junction conductivity, several Ca^{2+} -channels, $\text{Na}^+/\text{Ca}^{2+}$ exchange, activity of calpain and caspases, and other death effectors, and through reduced hypercontracture [e.g. [7, 8]. Upon reperfusion, cardiac pH_i is rapidly normalized through wash out of protons and the activity of acid extruding transport proteins, allowing the opening of the mPTP, and leading to Na^+ and Ca^{2+} overload, hypercontracture, necrosis, and apoptosis [7, 9]. Consequently, it has been proposed that a major mechanism through which PostC exerts its protective effect may be a delay in normalization of pH [8, 10-12]. In accordance with this view, staged reperfusion delayed the recovery of coronary venous blood pH (and thus presumably cardiomyocyte pH_i) in dogs exposed to coronary artery occlusion, and the protective effect of PostC was dependent on this delay [13]. Moreover, PostC delayed pH_i recovery in isolated rat hearts in a manner correlating with the protective effect of the PostC treatment [11, 14], and in isolated rat and rabbit hearts, brief acidosis upon reperfusion was as protective as PostC [10, 11].

The major ion transporters contributing to normalization of pH_i in the heart after an acid load are the Na^+/H^+ exchanger NHE1 and $\text{Na}^+-\text{HCO}_3^-$ cotransporters (NBCs, [15]). Inhibition of NHE1 has been shown to exert protective effects in a large number of *in vivo* and *in vitro* experimental models of cardiac I/R [see [7]. It has thus been speculated that the protective effect of PostC could be related to that of inhibiting NHE1 [8, 12]. However, this has never been directly addressed, although a role for cGMP/protein kinase G (PKG) mediated NHE1 inhibition was recently proposed [12]. Adding further complexity, NHE1 activity is anti-apoptotic in a number of settings [16, 17]. These include, under some conditions, cardiac I/R, ostensibly because of a shift in the balance between the pro-survival and pro-death effects of NHE1 [18, 19]. It is also notable that clinical

trials have largely failed to demonstrate a protective effect of NHE1 inhibitors [20, 21], whereas PostC generally appears to be protective also in the clinical setting, although the number of such studies is still limited [22].

A central mechanism through which pH_i – and hence pH_i regulatory ion transporters – may influence survival after I/R is via modulation of cellular signaling pathways regulating death-survival balance under these conditions. Well-established such mechanisms are the inhibition of mPTP formation by acidic pH_i [23], and altered activity of stress-activated protein kinases such as p38 MAPK, JNK1/2, and protein kinase B (PKB)/Akt [24, 25]. Notably, p38 MAPK and JNK have previously been shown to exhibit marked pH_i -sensitivity, and Akt to be activated through NHE1-mediated recruitment of ezrin-radixin-moesin (ERM) proteins [26-29].

Studies in cell culture model systems have been widely used to supplement and support ischemia-reperfusion studies in intact heart and whole animal experiments. Many such studies have been carried out in one of the very few available immortalized cardiomyocyte lines with reasonable resemblance to adult cardiomyocytes, the HL-1 mouse atrial cardiomyocyte line [30]. However, while HL-1 cardiomyocytes bear many resemblances to adult cardiomyocytes [30], they also exhibit distinct differences from these cells with respect to e.g. mitochondrial organization and metabolism [31-33]. Also the contractile apparatus is not as organized as in the adult cardiomyocyte. This together could strongly impact on their response to I/R- and postconditioning protocols. To our knowledge, no studies have yet evaluated their usefulness as a model for PostC.

The aim of the present study was to determine whether the protective effect of PostC can be mimicked by brief acidic reperfusion or by inhibition of NHE1, in intact perfused hearts compared to HL-1 cultured cardiomyocytes, and to assess the possible contribution of pH- and NHE1-sensitive regulation of stress kinases in reperfusion. We show that 15 min acidic treatment at the onset of reperfusion was at least as protective as PostC against I/R damage in intact rat hearts, whereas NHE1 inhibition was detrimental. In contrast, in HL-1 cardiomyocytes, necrotic or apoptotic death induced by I/R in the presence of HCO_3^- was not attenuated by a 15 min acidic period at the onset of reperfusion. Finally, I/R in HL-1 cardiomyocytes increased the activity of the stress-activated kinases JNK1/2 and Akt. However, JNK1/2 and Akt activity was not regulated by acidic pH and NHE1 inhibition.

Materials and Methods

Reagents

Unless otherwise stated, reagents were analytical grade and obtained from Sigma-Aldrich. 5' N-ethyl-N-isopropyl-amiloride (EIPA) was from Invitrogen and was dissolved in ddH₂O. Stock solutions of paraformaldehyde (20% wt/vol in ddH₂O) were prepared fresh regularly and stored at 4°C. Antibodies against caspase-3 (detecting full-length caspase-3 (35 kDa) and the 17/19 kDa fragment resulting from cleavage at Asp¹⁷⁵), poly (ADP-ribose) polymerase isoform 1 (PARP1) (detecting full length PARP1 (116 kDa) as well as the 89 kDa cleaved fragment), total JNK1/2, JNK1/2 phosphorylated at Thr¹⁸³ and Tyr¹⁸⁵, p38 MAPK, p38 MAPK phosphorylated at Thr¹⁸⁰ and Tyr¹⁸², total Akt and Akt phosphorylated at Ser⁴⁷³ were from Cell Signaling Inc. 2,3,5-triphenyltetrazolium chloride (TTC) was from Sigma.

Studies on isolated perfused rat hearts

Animals. Male Sprague-Dawley rats (Taconic, Denmark, body weight 250-450 g) were used. The study protocol was approved by the Danish Committee on Research Animals, and all procedures were carried out in accordance with the Danish National Committee for Animal Studies guidelines.

Langendorff isolated perfused heart preparation. The isolated perfused heart experiments were carried out essentially as previously described [34]. The rats were anesthetized by subcutaneous injection of a mixture of midazolam (2.5 mg/kg), fluanisone (2.5 mg/kg), and fentanyl citrate (0.08 mg/kg), and heparin (1000 i.e. per kg) was injected intravenously to prevent coagulation. The trachea was intubated, allowing the lungs to be artificially ventilated prior to opening of the chest cavity. The heart was dissected free, the aorta cannulated, and retrograde coronary perfusion rapidly initiated, using a modified Krebs-Henseleit (KH) solution (in mM: 119.9 NaCl, 3.3 KCl, 25 NaHCO₃, 11 glucose, 1.2 MgSO₄, 1.2 KH₂PO₄, 1.7 CaCl₂; pre-warmed to 37°C and gassed with 5% CO₂/95% O₂ to maintain pH at 7.4). The hearts were mounted in a Langendorff perfusion apparatus (Hugo Sachs-Harvard Apparatus GmbH, Germany) and perfused at a constant pressure of 80 mm Hg. The left atrial appendix was removed and a latex balloon coupled to a pressure transducer was inserted into the left ventricle via the mitral valve to allow monitoring of left ventricular pressure and heart rate.

Experimental protocols. The rats were randomly assigned to one of 4 different treatment protocols (Fig. 1). In all cases, the perfused hearts were stabilized for 40 min, followed by 35 min of global no-flow ischemia and 120 min of reperfusion. The 4 groups received the group-specific treatments immediately after ischemia, followed for all groups by 105 min reperfusion (i.e. a total reperfusion period of 120 min) in KH solution at pH 7.4. The treatment groups were: (i) 15 min 0.1% DMSO (vehicle ctrl); (ii) 15 min KH solution pH 6.5 (NaHCO₃ concentration 1 mM, replaced by NaCl); (iii) PostC, i.e. 6 cycles of 10 s reperfusion interspersed by 10 s of no-flow ischemia (6 x 10 s R/I); and (iv) 15 min in KH solution containing 3 µM of the NHE1 inhibitor EIPA. There were no significant differences

Fig. 1. Experimental protocols for the isolated perfused rat heart experiments. The basal setup comprised 40 min of stabilization (Eq), 35 min of global no-flow ischemia (isch.), and 120 min of reperfusion (rep.). The treatment groups differed only during the first 15 min of reperfusion. Ctrl: No treatment (DMSO vehicle); PostC: 6 x 10 s R/I at the onset of reperfusion; pH 6.5: Acidic reperfusion (KH solution adjusted with HCO₃⁻) during the first 15 min of reperfusion and; EIPA: Treatment with 3 µM of the NHE1-inhibitor EIPA in the KH-solution during the first 15 min of reperfusion.

between any of the treatment groups between the basal values of heart rate, aortic flow, left ventricular end diastolic pressure (LVEDP) and left ventricular developed pressure (LVDP).

Exclusion criteria. Hearts were excluded from analysis if they fulfilled one of the following criteria: (i) Inability to maintain a constant aortic pressure of 80 mm Hg at a flow rate of 10-20 ml/min; (ii) sustained arrhythmia during the pre-ischemia stabilization phase; (iii) LVDP at the end of the stabilization phase of below 70 or above 150 mmHg; (iv) ventricular fibrillation at the onset of reperfusion which did not revert spontaneously within 10 min and could not be reverted by gentle mechanical stimulation. 5 hearts were excluded from analysis due to enduring arrhythmia after ischemia, and 8 hearts were excluded due to too high flow rate (> 20 ml/min) in the stabilization period.

Assessment of heart functional parameters. The volume of the latex balloon was adjusted to achieve an LVEDP of 0–8 mm Hg during the stabilization phase. All data were acquired at a sampling rate of 1 kHz using a 16-channel PowerLab system (ADInstruments, UK) and analyzed using CHART 5 Pro software (ADInstruments, UK). LVDP was calculated as the difference between end-systolic and end-diastolic left ventricular pressure.

Determination of myocardial infarct size. The extent of infarct size was determined by 2,3,5-triphenyltetrazolium chloride (TTC) staining [35]. At the end of the 120 min of reperfusion the hearts were quickly detached, frozen at -80°C for 15 min, and cut into five 2 mm thick transverse sections. The slices were thawed, and incubated for 5 min at 37°C in TTC (1% in phosphate buffer pH 7.4, 37°C). The stained tissue slices were fixed in 4% formalin for 24 h, washed three times in ddH₂O, weighed, and both sides of each slice were scanned at a resolution of 1200 dpi using a CANON Lide70 flatbed scanner. The area of infarct and the total area of the slice were quantified

using Image-Tool 2.0 software (NIH, USA). The person conducting the measurements was blinded to the experimental groups. Total infarct size determined as the infarcted (white) area as a percentage of total muscle mass (representing total area, AAR) was calculated as the weighted mean of individual slice values, i.e.: $(AI_1/AAR_1) \times (W_1/W_{total}) + (AI_2/AAR_2) \times (W_2/W_{total}) + \dots + (AI_5/AAR_5) \times (W_5/W_{total})$, where AI_n is the area of infarct for the slice (n), W_n is the weight of section n and W_{total} is the total weight of all five slices.

Studies on HL-1 cardiomyocytes

Cell culture. HL-1 mouse cardiomyocytes were a kind gift from Dr. W. Claycomb (Louisiana State University Health Sciences Center, New Orleans, LA), and were cultured as previously described [18, 30]. Briefly, non-contracting HL-1 cardiomyocytes were maintained in Claycomb medium supplemented with 0.1 mM norepinephrine and 10% FBS (all JRH Biosciences, Lenexa, Kansas), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all Invitrogen) in 5% CO₂ at 37°C and 95% humidity. The cells were passaged every 3 days by trypsinization (trypsin/EDTA, Invitrogen) and transferred to culture flasks coated with gelatin/fibronectin (Becton Dickinson). All experiments were performed on confluent cells.

Simulated ischemia-reperfusion protocol, HL-1 cells. I/R was simulated essentially as previously described [18]. Confluent HL-1 cells were placed in a closed incubator/workspace system (XVIVO G300C, BioSpherix, Redfield, NY) preset to 37°C, 95% humidity, 21 (normoxia/reperfusion) or 0.5% (simulated ischemia, replacement with N₂) O₂, and 5% CO₂ (HCO₃⁻-buffered solutions). The HCO₃⁻ buffered Ringer solution (HR) contained (in mM) 1.25 CaCl₂, 1 MgCl₂, 137 NaCl, 6 KCl, 10 glucose, and 0.9 NaH₂PO₄, and 25 or 1 mM of NaCl was replaced by NaHCO₃ for pH 7.4 and pH 6.0 solutions, respectively. Simulated ischemia was carried out in Ischemia Ringer solution, containing (in mM) 1.25 CaCl₂, 1 MgCl₂, 119 NaCl, 1 NaHCO₃, 8 KCl, 20 Na-lactate, 0.9 NaH₂PO₄, pH 6.0. HL-1 cells were exposed to simulated ischemia for 5 h, followed by 2 h of simulated reperfusion +/- acidic reperfusion during the first 15 min of the reperfusion period, or to normoxia.

SDS gel electrophoresis and immunoblotting. Immunoblotting was carried out essentially as previously described [18]. Briefly, HL-1 cells grown to confluence in gelatin/fibronectin-coated Petri dishes were washed twice in ice-cold PBS (in mM: 137 NaCl, 2.7 KCl, 8.1 Na₂HPO₄, 1.7 KH₂HPO₄), lysed in lysis buffer (1% SDS, 10 mM Tris pH 7.4, 1 µM NaVO₄ and Complete Mini protease-inhibitor cocktail (Roche, Denmark)), homogenized through a 27-gauge syringe, and cleared by centrifugation (5 min, 20,000 g). Protein content was determined (DC Protein Assay, Bio-Rad, Copenhagen, Denmark), lysates with equal amounts of protein were prepared in LDS NuPAGE sample buffer with 50 mM DTT, resolved by SDS-PAGE on 10% NuPage Bis-Tris gels in NOVEX electrophoresis chambers and transferred to nitrocellulose membranes (Invitrogen). After Ponceau staining to confirm equal loading, membranes were incubated overnight at 4°C in blocking buffer (120 mM NaCl, 10 mM Tris pH 7.5, 0.1% Tween 20, 5% nonfat dry milk), for 2 h at room temperature with primary

antibody diluted in blocking buffer, washed in TBST (120 mM NaCl, 10 mM Tris pH 7.5, and 0.1% Tween 20), incubated with alkaline phosphatase-conjugated secondary antibody, washed, and immunolabeling detected by BCIP/NBT (KPL, Gaithersburg, MD). Densitometric analysis of the blots was performed using UN-SCAN-IT software (Silk Scientific, Orem, UT).

Estimates of necrotic and apoptotic cell death. Necrotic cell death in HL-1 mouse cardiomyocytes was estimated as release of the cytosolic housekeeping enzyme glucose 6-phosphate dehydrogenase (G6PD) to the surrounding medium due to compromised sarcolemmal integrity. The Vybrant Cytotoxicity Assay Kit (Molecular Probes) was used, following the manufacturer's directions. The activity of G6PD released from necrotic cells reduces NADP⁺ to NADPH followed by reduction of resazurin to resorufin. Resorufin emits at 587 nm after excitation at 563 nm. The signal is proportional to the amount of released G6PD and correlating to the number of necrotic cells in the sample. For these experiments, HL-1 cardiomyocytes were grown to confluence in black gelatine/fibronectin coated 96 well plates. The experiments were performed in HCO₃⁻ buffered Ringer solutions. In these experiments, changes in Ringer composition were made by addition of modified Ringer solutions, and lactate during simulated ischemia omitted, as this was necessary to avoid aspiration steps. Fluorescence was detected on a FLOUstar OPTIMA microplate reader (BMG Labtechnologies) ex/em 544/590 nm. All estimates of necrotic cell death are relative to samples of fully lysed HL-1 cardiomyocytes.

Induction of apoptotic cell death pathways was assessed by monitoring cleavage of caspase-3 and PARP1. This was done by western blotting as above, using antibodies recognizing both full-length and cleaved forms of these proteins (35 and 17 kDa procaspase-3 and caspase-3; 116 and 89 kDa for full-length and cleaved PARP) (both Cell Signaling Technology). Apoptotic cell death in HL-1 mouse cardiomyocytes was also measured as chromatin condensation. Cells were grown in small gelatin/fibronectin coated Petri dishes (~10 cm²) to an approximated monolayer. The experiments were performed in HCO₃⁻-buffered Ringer solution. After stimulation the DNA-binding component Hoechst 33258 (Sigma-Aldrich) was added to a final concentration of 25 µg/ml for the last 20 min of each protocol setup. Hoechst 33258 in a DNA-binding complex emits fluorescence at 465 nm after excitation at 356 nm and was detected using the 20x/0.6 NA objective of a Leica DM-IRB/E microscope (Leica, Heidelberg, Germany). Cells in five randomly chosen areas of each sample were counted. Apoptotic cells with condensed chromatin appear brighter compared to cells with non-condensed chromatin.

Statistical analysis

Data are shown as representative individual experiments or as mean with standard error of the mean (s.e.m.) error bars. Unless otherwise indicated, significance was tested using one-way ANOVA test followed by Tukey Kramer post-hoc Multiple Comparison test. For bootstrap analysis of Langendorff data, mean differences (AUC_{0-120 min}) and 95% confidence intervals were computed via bootstrapping 10,000 resamples in pairwise comparisons. A priori four pairwise comparisons were selected

Fig. 2. PostC and acidic reperfusion in isolated perfused rat hearts is protective against I/R-induced deterioration of heart functional parameters whereas EIPA is detrimental. Hearts were stabilized for 40 min (Eq), followed by 35 min of global no-flow ischemia (isch.), and 120 min of reperfusion (rep.). Ctrl (●): No treatment (DMSO vehicle); PostC (○): 6 x 10 s R/I at the onset of reperfusion; pH 6.5 (▲): Acidic reperfusion during the first 15 min of reperfusion and; EIPA (△): Treatment with 3 μ M of the NHE1-inhibitor EIPA during the first 15 min of reperfusion. Shown are the effects of the different treatments on a: LVEDP, and c: LVDP. Each point represents the mean + SEM of the preceding 5 min of recording. n=4-5 per group. One-way Anova was performed on the final datapoint (i.e. 115-120 min of reperfusion, n=3-4). ** p <0.01, Rep. pH 6.5 compared to control conditions; $\square\square$ p <0.01, PostC compared to control conditions, p =0.087, Rep. pH 6.5 compared to control conditions. b, d): Assessment of difference between chosen treatment groups over the whole reperfusion period was performed by bootstrap analysis.

for bootstrap analysis: Control against all treatments and acidic reperfusion against PostC. The Bootstrap simulations were programmed in SAS® rel. 9.1.3.

Results

PostC and brief acidic reperfusion both exert protective effects on heart functional parameters and infarct size, whereas NHE1 inhibition is detrimental

The treatment groups are outlined in Fig. 1. In control

hearts, 35 min of no-flow ischemia followed by 120 min of reperfusion strongly attenuated cardiac function and the ability to obtain proper relaxation in the diastolic interval, addressed as left ventricular end-diastolic pressure (LVEDP) was markedly reduced reaching a maximum of about 70 mm Hg, recovering to about 40 mm Hg after 120 min of reperfusion (Fig. 2a). The ability to obtain appropriate contractile force was addressed from left ventricular developed pressure (LVDP) and was about 90 mm Hg prior to ischemia, recovering to about 40 mm Hg at the end of the reperfusion period (Fig. 2c);

Fig. 3. Effect of PostC and acidic reperfusion on infarct size after I/R. Hearts were stabilized for 40 min (Eq), followed by 35 min of global no-flow ischemia (isch.), and 120 min of reperfusion (rep.). Ctrl: No treatment (DMSO vehicle); PostC: 6 x 10 s R/I at the onset of reperfusion; pH 6.5: Acidic reperfusion during the first 15 min of reperfusion and; EIPA: Treatment with 3 μ M of the NHE1-inhibitor EIPA during the first 15 min of reperfusion. After this protocol, hearts were stained with TTC to demarcate infarcted and viable areas. a. Representative images of slices of rat hearts from the different treatment groups are shown. Red color represents viable tissue while white color represents the infarct: tissue damaged by necrotic cell death. b. Quantification of images as in A. Quantification was carried out blinded, using ImageJ 1.42 NIH software, as described in Experimental Procedures. Data are from 3-4 independent experiments for each group. One-way ANOVA was performed.

and the flow rate, which was about 12 ml/min prior to ischemia, initially recovered upon reperfusion but then slowly declined (data not shown), reflecting extensive myocardial damage. In congruence with the functional data, TTC staining showed that about 40% of total muscle mass of the control hearts was infarcted at the end of the reperfusion period (Fig. 3). The data are summarized for all treatment groups in Table 1.

The effects of PostC and acidic reperfusion were tested using a PostC protocol of 6 x 10 s/10 s R/I at the onset of reperfusion, or exposure of the hearts to KH solution of pH 6.5 for the first 15 min of reperfusion, respectively. As seen in Fig. 2a, the LVEDP during reperfusion was significantly reduced by the two treatments, both when assessed for the duration of the reperfusion period (bootstrap analysis, Fig. 2b) and when analyzed for the 120 min time point (ANOVA). Interestingly the bootstrap analysis showed a near significant difference between the PostC and the acidic reperfusion treatment groups ($p<0.077$). This points to a more pronounced effect of acidic reperfusion compared to that of PostC and was further supported by the fact that LVEDP in the pH 6.5-treated and not in the PostC hearts was significantly lower than that of control hearts already within the first 5 min of the acidic treatment phase, and remained so throughout the 15 min. In the 5-10 and 10-15 min periods, it was also significantly different from that of PostC hearts (ANOVA). Thus, the protective effect is pronounced, and greater than that of PostC, within a few minutes of acidic exposure. The effects on LVDP were less marked but there was a non-significant tendency for improvement at least after acidic reperfusion (Fig 2c-d). Also there were no significant changes in the

	Infarct size (% of total muscle mass)	LVDP (mmHg)	LVEDP (mmHg)
Control	40.9 ± 1.9	35.9 ± 2.1	46.7 ± 2.7
PostC	24.3 ± 5.1	42.6 ± 10.3	25.2 ± 2.2 ** [□]
Rep. pH 6.5	20.7 ± 10.4	70.7 ± 17.6 (A) p=0.087 (B) p=0.097	17.4 ± 3.2 ** ^{□□}
EIPA	48.5 ± 4.3	26.1 ± 0.9 ^{□□}	53.3 ± 6.9

Table 1. Heart functional parameters and infarct size. Summarized data from the isolated perfused heart experiments. Infarct size is expressed as a percentage of total muscle mass. Significance was tested by one-way ANOVA, for functionality data on the datapoint representing 115-120 min of reperfusion (A), or via bootstrap analysis (B). ** $p<0.01$, compared to control conditions by (A); $\square p<0.05$, $\square\square p<0.01$, compared to control conditions by (B); $p=0.087$ and $p=0.097$ compared to control conditions. $n=3-4$.

flow rate, but a tendency towards a slightly lower initial recovery in the acidic group was observed (data not

Fig. 4. Effect of simulated ischemia and neutral or acidic reperfusion on necrotic and apoptotic cell death mechanisms in HL-1 cardiomyocytes. Cells were exposed to 5 h of simulated ischemia followed by 15 min of acidic (pH_o 6.0) or pH-neutral (pH_o 7.4) reperfusion before another 1 h 45 min of pH-neutral reperfusion. a: Necrotic cell death was measured as glucose 6-phosphate dehydrogenase (G6PD) release as described in Experimental Procedures. Data are shown as fractions of G6PD release relative to fully lysed cells and are means + SEM of 8 experiments. Student's t-test was performed. ** $p < 0.01$ *** $p < 0.001$, indicates significant difference from that in parallel normoxic controls. b: Cells were treated with or without 5 μ M EIPA to inhibit NHE1 during reperfusion. Subsequently, cells were lysed, and death effector activity was determined by Western blotting as cleavage of b1: procaspase and b2: full length PARP. *Top*: representative Western blots. *Bottom*: summary data for 7 experiments, showing the abundance of the cleaved relative to the non-cleaved forms, normalized to 7 h normoxic control (means + SEM). Student's t-test was performed. * $p < 0.05$ and ** $p < 0.01$, indicates significant difference from that in parallel normoxic controls.

shown). In accordance with these protective effects, both PostC and acidic reperfusion were associated with an about 50% reduction of the infarct size (Fig. 3, Table 1). We next asked whether these protective effects of PostC and acidic reperfusion could be mimicked by inhibition of NHE1. As seen in Fig. 2c-d, in contrast to these treatments, the NHE1 inhibitor EIPA significantly decreased LVDP and also a modest increase of LVEDP was detected (Fig. 2a), whereas the flow rate was unaffected (data not shown). Infarct area was increased by 20 % to $48.5 \pm 4.3\%$ compared to $40.9 \pm 1.9\%$ in controls (Fig. 3, Table 1).

Thus, in isolated, perfused rat hearts, mechanical PostC and 15 min of acidic (pH 6.5) reperfusion were protective against I/R damage, protection with acidic reperfusion consistently tending to be greater than with PostC for infarct size, LVDP, and LVEDP. In contrast, inhibition of NHE1 was detrimental to heart functional parameters.

Brief acidic reperfusion is not protective against cellular reperfusion damage in cultured HL-1 cardiomyocytes in the presence of HCO_3^-

To assess the usefulness of the cultured HL-1 cardiomyocytes as a cellular model for acidic postconditioning we exposed these cells to a simulated I/R protocol (hereafter: I/R) mimicking as closely as possible the conditions in the perfused intact hearts. The

cardiomyocytes were exposed to ischemia (0.5% O₂, 5% CO₂, simulated ischemia Ringer) for 5 h, followed by 2 h of reperfusion at pH 7.4. The first 15 min of reperfusion the HR pH was pH 7.4 (ctrl.), or was reduced to pH 6.0 (acidic reperfusion), or contained the NHE1 inhibitor.

Fig. 5. Simulated ischemia and reperfusion induces pH- and NHE1-independent activation of Akt and JNK1/2 but not of p38 MAPK in HL-1 cardiomyocytes. Cells were exposed to 5 h simulated ischemia followed by 15 min of acidic (pH_o 6.0) or pH-neutral (pH_o 7.4) reperfusion before another 1 h 45 min of pH-neutral reperfusion. During reperfusion cells were treated with or without 5 μ M EIPA. Cells were lysed and kinase activation assessed by Western blotting using phospho- and total antibodies against a: Akt, b: JNK1, c: JNK2, and d: p38 MAPK. *Top* a-d: representative Western blots. *Bottom* a-d: summary data for 3-4 experiments per condition, showing phospho- relative to total kinase abundance, normalized to normoxic control (means $\pm$ SEM). Student's t-test was performed. * p < 0.05 and ** p < 0.01, indicates a significant difference from that in parallel normoxic controls.

Normoxic controls were carried out in parallel, in HR Ringer pH 7.4.

Necrotic HL-1 cell death, assessed as G6PD release to the surrounding medium, was significantly increased by I/R (Fig. 4a) but 15 min acidic reperfusion was not protective against the I/R-induced necrotic cell death. Induction of apoptotic cell death pathways was assessed by monitoring cleavage of caspase-3 from pro-caspase to active form, and PARP1 from its 116 kDa full length form to the 89 kDa fragment. As seen, both caspase-3- and PARP1 cleavage were increased by I/R (Fig. 4b). Similar to what was seen for necrotic cell death, 15 min acidic reperfusion could not protect HL-1 against caspase-3- and PARP1 cleavage. The lack of protective effect of brief acidic reperfusion was verified using chromatin condensation assays ($n=4$, data not shown). The presence of EIPA in the first 15 min of reperfusion to inhibit NHE1 had no significant effect on I/R-induced caspase-3- and PARP1 cleavage, suggesting that NHE1 activity does

not modulate apoptotic death of HL-1 cardiomyocytes under these conditions.

I/R in cultured HL-1 cardiomyocytes is associated with pH-independent activation of Akt and JNK, but not of p38 MAPK

The divergent effects of acidic postconditioning on death between whole hearts and cultured cardiomyocytes could reflect the absence of hypercontracture-related damage in HL-1 cells, but also differences in upstream signaling can play a role. We evaluated the total and active, phosphorylated levels of Jnk1/2, p38 MAPK, and Akt after I/R with or without 15 min at pH 6.0 at onset of reperfusion, and in parallel normoxic controls in HL-1 cardiomyocytes. As noted above, these kinases have been assigned major roles in control of cell death/survival balance after I/R [6, 24, 36], and have been reported to be regulated by pH_i (JNK, p38 MAPK) and by NHE1 (Akt). Furthermore, altered activity of these kinases during

postconditioning procedures has been reported [37, 38]. The activity of Akt was increased by I/R, and this was unaffected by brief acidic reperfusion, in the absence or presence of EIPA (Fig. 5a). Both JNK1 and JNK2 activity was significantly increased by I/R, in a manner unaffected by reperfusion pH or EIPA (Fig. 5b-c). The activity of p38 MAPK was not affected by I/R under any of the conditions tested (Fig. 5d). Thus, Akt and JNK1/2 activity is increased in HL-1 cardiomyocytes after I/R, and this activation is insensitive to brief acidic reperfusion and to NHE1 inhibition. In contrast, under these conditions, there was no net effect of I/R on p38 MAPK activity.

Discussion

Although PostC has been shown to have, under some conditions, strong protective effects against reperfusion injury after cardiac ischemia, the mechanisms involved are incompletely understood. Recent studies have suggested a role for delayed recovery of pH_i in the protective effect of PostC [8, 11, 12, 39]. Inhibition of NHE1, which at least in some cases will also lead to delayed pH_i recovery after ischemia, is similarly strongly protective against reperfusion injury in some models of cardiac ischemia [7]. A role for NHE1 inhibition by the cGMP/PKG pathway in PostC was recently suggested, albeit not experimentally verified in the intact heart [12].

Here, we therefore asked the central question of whether PostC and acidic pH_o (which will also lead to a reduction of pH_i) exerts similar protective effects against I/R damage in Langendorff perfused hearts, and whether these effects can be mimicked by inhibiting NHE1. For statistical analysis of the Langendorff data we employed both conventional one-way ANOVA with Tukey-Kramer post-test at the 120 min time point, and the bootstrapping technique to evaluate for possible differences over the entire 120 min reperfusion range. Notably, 15 min exposure to pH 6.5 followed by normal reperfusion at neutral pH exerted a protective effect in this model that consistently tended to be more pronounced than that of ischemic PostC. This was particularly marked for LVEDP, where we observed a near-significant difference ($p < 0.077$, bootstrap analysis) between the two treatments. Also maximal systolic pressure (LVDP+LVEDP; data not shown) was significantly lower after ischemic postconditioning compared to acidic reperfusion (t -test, $p < 0.05$). We furthermore observed a numerical 15% decrease in maximal systolic pressure after ischemic postconditioning compared to control hearts. This

demonstrates that the mechanisms accounting for the cardioprotective effects of acidic reperfusion differ from those of ischemic postconditioning and also that there might be detrimental side effects of the procedure of mechanical ischemic postconditioning. Notably, already during the first few minutes of the 15 min treatment phase, LVEDP in the pH 6.5-treated hearts was significantly lower than that of control hearts. This demonstrates an almost immediate protective effect of the acidic exposure that was not seen for the PostC protocol. Future studies should address the marked protective effect of brief acidic reperfusion, and the mechanisms by which it might partially differ from that of PostC. Importantly, in contrast to the protective effect of acidic reperfusion and PostC, 15 min exposure to EIPA augmented reperfusion damage. A pertinent conclusion of this study therefore is that, in the perfused heart model employed, the protective effects of PostC and of acidic reperfusion do not reflect inhibition of NHE1 and cannot be mimicked by inhibiting this transporter. Moreover acidic reperfusion is more protective than PostC by increasing the improvements of the functional recovery of the hearts.

This study differs from other whole perfused heart studies that have shown a protective effect of EIPA primarily in the timing and duration of the exposure to EIPA, with most (albeit not all, e.g. [40]) other studies having EIPA present in the ischemic phase [see 7]. It is interesting, however that the present findings agree well with the results of clinical trials, which have failed to show a protective effect of the NHE1 inhibitor cariporide [20, 21]. In contrast, PostC has generally been found to be protective in clinical trials, although only a few such trials exist at this point [22]. It has been suggested that NHE1 activity is reduced or absent during ischemia *per se*, reflecting ATP-depletion and reduced pH_o [see 7]. However, both parameters recover rapidly during early reperfusion, restoring NHE1 activity [7, 41]. Hence, it is highly unlikely that the lack of protective effect of NHE1 inhibition during reperfusion reflects that NHE1 is not active in this phase. It is also important in this regard that inhibition of NHE1 during I/R in fact does not necessarily lead to a delay in pH_i recovery [7, 41]. This likely reflects both the increased requirement for Na^+/K^+ ATPase activity and hence (H^+ producing) ATP consumption resulting from NHE1 activity, and contributions from other H^+ extruding transporters, such as Na^+ , HCO_3^- cotransporters, which play a major role in pH_i regulation in the heart [see 7, 15].

Our study has some limitations. Firstly, we cannot exclude that in other perfused heart models, such as a

working heart model, or in other species, the balance between protective and detrimental effects of NHE1 inhibition would be different, resulting in a net protective role of NHE1 inhibition. Second, although we used low concentrations of EIPA (3–5 μM), we cannot rule out that non-NHE1 specific effects of EIPA might contribute to our findings, as proposed in studies of NHE1 inhibitors added during the ischemia phase *per se* [42]. Reported non-NHE1 effects of NHE1 inhibitors include inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange (NCX) and cardiac Na^+ channels, as well as mitochondrial effects. The former, however, would be expected to exert protective rather than detrimental effects [see [7] and hence are unlikely to explain the effects observed in the present study. The mitochondrial effects have been reported for both EIPA and the more NHE1-specific cariporide [43, 44], indicating that cariporide would not offer much advantage in terms of possible side effects, and also, in our view, hinting that such suggested non-NHE1 effects may in fact be NHE1-related.

To map the mechanisms leading to the protection by PostC and potentially exploit these in a clinically relevant form, it is necessary to understand how PostC treatments affect the individual cardiomyocyte. HL-1 atrial cardiomyocytes in many ways resemble adult cardiomyocytes [30], and are a convenient and widely used tool for cellular level analysis of cardiac function. However, both contracting and non-contracting versions of these cells exhibit a number of differences from adult cardiomyocytes that could compromise their usefulness for studies of PostC. These include major differences in mitochondrial dynamics and a greatly increased fractional dependence on glycolysis rather than oxidative phosphorylation, compared to adult cardiomyocytes [31–33]. To assess the usefulness of HL-1 as a model for cardioprotection by acidic reperfusion, we mimicked as closely as possible the protocol used in the Langendorff heart model while taking into account that compared to hearts exposed to no-flow ischemia, cultured HL-1 cardiomyocytes seem to require a substantially longer period of simulated ischemia to induce I/R damage [e.g. 18], presumably because of their reduced dependence on oxidative phosphorylation [33]. The HL-1 cells were exposed to 5 h of simulated ischemia (0.5% O_2 , 5% CO_2 , HCO_3^- buffering to pH 6.0, increased K^+ , and no glucose), followed by 120 min reperfusion, of which the first 15 min was either at pH 7.4 or pH 6.0, and in the absence or presence of EIPA. HL-1 cells undergoing ischemia followed by 120 min neutral reperfusion exhibited a marked increase in both necrotic and apoptotic cell death

compared to normoxic controls, consistent with previous reports by us and others [3, 18, 45]. However, in marked contrast to the findings in whole perfused hearts, neither apoptotic nor necrotic cell death of HL-1 cells under these conditions was attenuated by 15 min acidic reperfusion. This is in apparent contradiction to one earlier study in HL-1 cells, in which a brief acidic treatment was found to exert significant protection against I/R damage [45]. Notably, however, where we employed true hypoxia in combination with physiologically relevant $\text{CO}_2/\text{HCO}_3^-$ buffering, this study [45] employed a “simulated ischemia” protocol in which glucose- and mitochondrial metabolism is poisoned by cyanide and 2-deoxyglucose (2-DOG) to completely block both. Furthermore, pH was buffered artificially with HEPES rather than by $\text{CO}_2/\text{HCO}_3^-$, which means that all HCO_3^- dependent pH regulation was prevented [45]. It may be noted that if we conduct our HL-1 cell experiments in the absence of HCO_3^- , we also observe a protective effect of acidic pH against I/R-induced chromatin condensation ($n=3$, data not shown). It thus seems likely that the difference stems from this marked difference in experimental conditions. The lack of protective effect of acidic pH in HL-1 cardiomyocytes in the present study – which with respect to buffering and hypoxia resemble more closely those of the native cardiomyocyte – may reflect that hypercontracture-induced damage does not contribute to death induced by I/R in the non-contracting HL-1, or it can be due to differences in signaling via upstream effectors in the native heart and in the HL-1 cells. It has been reported that PostC attenuates cardiomyocyte apoptosis through inhibition of JNK and p38 MAPK [36]. In contrast, Liu and coworkers [38] demonstrated that p38 MAPK was activated as a response to hypoxic postconditioning in rat primary cardiomyocytes. Both JNK and p38 MAPK have been shown to be strongly pH sensitive, being more active at alkaline than at acidic pH [28, 46]. Furthermore, Akt, another kinase implicated in PostC, is regulated by NHE1 [17]. In perfused rat hearts Akt activation was increased both after ischemia/reperfusion and, to a greater extent, after ischemic postconditioning [37]. We therefore tested the effects of brief acidic reperfusion on these kinases. I/R of HL-1 cardiomyocytes was, in line with earlier reports [37, 38], associated with a marked increase in the activity of JNK1/2 and Akt (but not of p38 MAPK), which were unaffected by post ischemic acidic or EIPA treatment. Thus, in HL-1 cardiomyocytes exposed to I/R, these kinases are not pH sensitive in the range tested.

Considered the marked protective effect of acidic reperfusion/ischemic postconditioning in intact hearts and

the lack of such effects in HL-1 cardiomyocytes, caution must clearly be exerted in using HL-1 cells for studies of simulated PostC, at least in the form of acidic reperfusion. Although other factors, such as the role of reduced hypercontracture in PostC protection in the intact heart [8] are likely to contribute, we speculate that the major reason for this discrepancy is the markedly different metabolism and mitochondrial function between HL-1 cells and adult cardiomyocytes [31-33]. Also a 15 min treatment with EIPA failed to protect the HL-1 cells from reperfusion damage. This is, to our knowledge, the first study of brief, post-ischemic NHE1 inhibition. The lack of protection by NHE1 inhibition is in good agreement with recent studies by us and others pointing to a modest protective, rather than a detrimental role of NHE1 after cardiac ischemia [18, 19]. Addressing this question in the intact heart will necessitate tight spatial and temporal control of cardiac pH_i and pH_o , a methodologically challenging feat – but should be carried out in future studies.

In conclusion, a 15 min acidic treatment at the onset of reperfusion is at least as protective as PostC against

I/R damage in intact rat hearts, whereas inhibition of NHE1 is detrimental. In contrast, in HL-1 cardiomyocytes, necrotic and apoptotic death induced by I/R in the presence of HCO_3^- are not attenuated by a 15 min acidic exposure at the onset of reperfusion, arguing against the use of this model for PostC studies. Finally, I/R in HL-1 cardiomyocytes is associated with a pH- and NHE1-independent increase in the activity of JNK1/2 and Akt, but not of p38 MAPK.

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