Influence of Ischemic Preconditioning on Intracellular Sodium, pH, and Cellular Energy Status in Isolated Perfused Heart

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The possible relationships between intracellular Na^+ (Na_i^+), bioenergetic status and intracellular pH (pH_i) in the mechanism for ischemic preconditioning were studied using ^{23}Na and ^{31}P magnetic resonance spectroscopy in isolated Langendorff perfused rat heart. The ischemic preconditioning (three 5-min ischemic episodes followed by two 5-min and one 10-min period of reperfusion) prior to prolonged ischemia (20 min stop-flow) resulted in a decrease in ischemic acidosis and faster and complete recovery of cardiac function (ventricular developed pressure and heart rate) after 30 min of reperfusion. The response of Na_i during ischemia in the preconditioned hearts was characterized by an increase in Na_i^+ at the end of preconditioning and an accelerated decrease during the first few minutes of reperfusion.

During post-ischemic reperfusion, bioenergetic parameters (PCr/P_i and $\beta ATP/P_i$ ratios) were partly recovered without any significant difference between control and preconditioned hearts. The reduced acidosis during prolonged ischemia and the accelerated decrease in Na_i^+ during reperfusion in the preconditioned hearts suggest activation of Na^+/H^+ exchanger and other ion transport systems during preconditioning, which may protect the heart from intracellular acidosis during prolonged ischemia, and result in better recovery of mechanical function (LVDP and heart rate) during post-ischemic reperfusion.

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Several theories have been proposed to explain the effect of brief periods of ischemia (ischemic preconditioning) to reduce necrosis in the heart during subsequent prolonged ischemia. The roles of adenosine, bradykinin, opioids, catecholamines, prostaglandins, free radicals, stress proteins, protein kinase C, sarcolemmal and mitochondrial K_{ATP} channels, glycolytic rate, etc. have been discussed as potential mechanisms by which preconditioning protects ischemic heart (see reviews 1–3). The possible role of ionic alterations (Na^+, Ca^{2+}, H^+) for ischemic preconditioning was first described by Steenbergen *et al.* (4). Others have demonstrated that Ca^{2+} overload and ischemic acidosis are important intracellular alterations, which could lead to damage in ischemic cardiomyocytes (5, 6). Na^+ is involved in regulating both Ca^{2+} and H^+ concentrations in the cell through Na^+/H^+, Na^+/Ca^{2+}, Na^+-K^+-$2Cl^-$ and Cl^-/HCO_3^- ion transport mechanisms. Furthermore, Na^+ is an important regulator of bioenergetic processes in healthy and diseased cardiomyocytes (7).

There is still controversy concerning the time and magnitude of changes in intracellular sodium (Na_i^+), which occur during preconditioning, post-preconditioning ischemia, and post-ischemia recovery (8–10). Steenbergen *et al.* (4) have demonstrated that preconditioning attenuated the increase Na_i^+ during ischemia, and that there was no difference in the rate of Na_i^+ recovery during reperfusion. Ramasamy *et al.* (11) have demonstrated that preconditioning did not reduce Na_i^+ accumulation during ischemia, but the decline in Na_i^+ during reperfusion was significantly greater in the preconditioned hearts compared to the control. Na^+ transport systems (Na^+/K^+-ATPase, Na^+/H^+ and Na^+/Ca^{2+} exchangers and Na^+-K^+-$2Cl^-$ co-transporter) could play a key role in preconditioning, most likely by limiting intracellular acidosis and protecting intracellular Ca^{2+} overload.

The goal of our investigation was to examine the relationships between the changes in Na_i, cellular energy status, and pH during ischemic preconditioning, prolonged ische-

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mia, and post-ischemia recovery during reperfusion in the Langendorff perfused rat heart. We employed ^{23}Na nuclear magnetic resonance (NMR) spectroscopy and the paramagnetic shift reagent (SR) thulium(III) 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylene phosphonate) ($TmDOTP^{5-}$) for measurement of Na_i^+ and ^{31}P MRS for measurement of phosphocreatine (PCr), adenosine triphosphate (ATP), inorganic phosphate (P_i), and intracellular pH_i.

Methods

Isolated Perfused Heart. Male Sprague-Dawley rats (220–270 g, $n = 10$) were obtained from the Charles River Co. The study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania and conforms with the PHS *Guide for the Care and Use of Laboratory Animals* and the *U.S. Interagency Research Animal Committee Principles for the Utilization and Care of Research Animals.* The rats were anesthetized by an intraperitoneal injection of sodium Nembutal (100 mg/kg). Hearts (1.26 ± 0.07 g) were quickly removed and perfused via the aorta using the Langendorff method (100 mmHg at 37°C) with modified Krebs-Henseleit buffer: NaCl 118 m*M*, KCl 4.7 m*M*, $MgSO_4$ 1.2 m*M*, $CaCl_2$ 2.0 m*M*, Na_2·EDTA 0.5 m*M*, $NaHCO_3$ 25 m*M*, glucose 11 m*M*. The buffer was equilibrated with 95% O_2/5% CO_2.

A latex balloon (size 4, Kent Scientific Corp., Litchfield, CT) filled with the perfusate buffer containing 10 m*M* $TmDOTP^{5-}$ and 108 m*M* methylphosphonic acid (MPA) was inserted through the mitral valve and secured in the left ventricle. The balloon was connected to a pressure transducer (Ohmeda Medical Devices, Madison, WI) and a physiological monitor (Columbia Instruments, Columbus, OH) for monitoring the heart rate and left ventricular developed pressure (LVDP, systolic minus diastolic pressure). Heart rate and LVDP were used as indices of mechanical function. The coronary flow rate was measured and quantitated by collecting the coronary artery effluent in a calibrated graduated cylinder.

Magnetic Resonance Spectroscopy. A Bruker 9.4-Tesla magnet interfaced to a state-of-the-art Bruker AVANCE 400 DMX console was used. Isolated perfused beating hearts were placed in a 20-mm NMR tube for acquisition of ^{23}Na and ^{31}P spectra. ^{23}Na NMR spectra were obtained using a 34-μsec excitation pulse (90° flip angle) followed by acquisition of 3,072 data points over a spectral width of 10 kHz. One hundred free induction decays (FIDs) were averaged over 25 sec using 0.2-sec repetition time. The Na_i^+ and extracellular sodium (Na_e^+) resonances were discriminated with the paramagnetic SR $TmDOTP^{5-}$ (3.6 m*M*) added to the perfusate buffer. ^{31}P NMR spectra were acquired using a 40-μsec excitation pulse (60° flip angle) followed by acquisition of 16k data points over a spectral width of 16 kHz. One hundred twenty-eight FIDs were averaged over 3 min using 1.5-sec repetition time.

Experimental Protocol. As shown in Figure 1, two groups of hearts, one with preconditioning (PC group) and the other without preconditioning (CON group) were examined. After 15 min of baseline perfusion, preconditioning was produced over 35 min. For preconditioning, two 5-min ischemic episodes were each followed by 5 min of reperfusion. Another 5-min ischemic episode was then followed by 10 min of reperfusion. The control hearts were perfused normally during the 35 min preconditioning period. Both the CON ($n = 6$) and PC ($n = 4$) hearts were subjected to 20-min stop-flow ischemia followed by 30 min of reperfusion.

In the beginning of perfusion two ^{31}P spectra were obtained to determine the cellular energy status of the isolated hearts (Fig. 1, *step I*). After addition of dimethyl methylphosphonate (DMMP) and phenylphosphonic acid (PPA), which were used for measurement of intracellular volume (12), usually two ^{31}P and two ^{23}Na spectra were collected

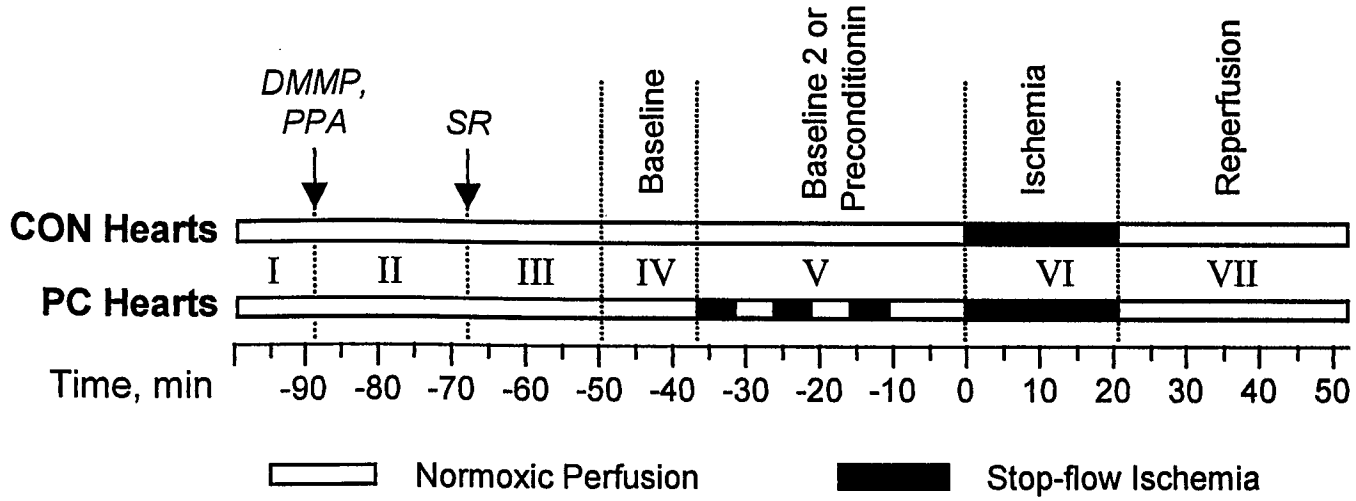

Figure 1. Schematic representation of the experimental protocols. DMMP (dimethyl methylphosphonate), PPA (phenylphosphonic acid), and SR (shift reagent) were added in perfusion buffer. I–VII are the steps of the experimental protocol described in the Methods.

(*step II*). The SR, $TmDOTP^{5-}$ (3.6 m*M*) was added to the perfusate to separate Na_i^+ and Na_e^+ signals. Additional $CaCl_2$ (1.3–1.6 m*M*) was added to the perfusate to bring the free $[Ca^{2+}]$ in physiological range. ^{23}Na spectra were continually acquired during the SR equilibration period, which took 20–25 min (*step III*). After a steady-state separation between the Na_i^+ and Na_e^+ resonances was achieved, one ^{31}P spectrum was collected to determine the effects of SR on cellular bioenergetics and pH, and two ^{23}Na spectra were obtained to determine the initial Na_i^+ level (baseline period, 15 min, *step IV*). During preconditioning, six ^{23}Na spectra were collected in PC hearts: one for each 5-min ischemic episode and one for each reperfusion episode (3 ischemia/reperfusion episodes) (preconditioning period, 35 min, *step V*). During the third reperfusion episode in the preconditioning period, one ^{31}P spectrum was obtained to determine the effect of preconditioning on cellular energetics. In the CON group, the same number of ^{23}Na and ^{31}P spectra were collected during the 35-min regular perfusion (*step V*). During 20-min ischemia (*step VI*), four ^{23}Na spectra followed by one ^{31}P spectrum were obtained. Similarly, during the 30-min reperfusion (*step VII*), six ^{23}Na spectra followed by one ^{31}P spectrum were collected. The coronary flow rate was measured at the end of the initial stabilization period, SR equilibration period, preconditioning, and reperfusion.

Data Analysis and Statistics. NMR data were transferred to a personal computer and processed using NMR Utility Transform Software (Acorn NMR, Fremont, CA) program for Windows 95/NT. Line broadenings of 25 Hz for ^{31}P and 10 Hz for ^{23}Na were applied, and the resulting FID was Fourier transformed. The resonance areas in ^{31}P and ^{23}Na spectra were determined by integration. Intracellular pH (pH_i) was calculated using the following relation (13):

$$pH_i = 6.75 + \log[(\delta_{Pi-\alpha ATP} - 10.85)/13.25 - \delta_{Pi-\alpha ATP})].$$

Volumes in the beating, perfused hearts were determined, using ratios of DMMP, MPA, and PPA resonance areas in ^{31}P MRS spectra (12). The intracellular volume was calculated from the difference between the total water volume and extracellular volume.

All data are presented as the mean ± SE. Statistical analyses of the data were performed by two-way analysis of variance (ANOVA) (Statistica/w 5.1 program). *Post hoc* comparisons among the experimental groups were then performed using least-significant differences (LSD) test. A P value ≤ 0.05 was used to denote a statistical significance.

Results

Cardiac Function. Table I shows the heart rate, LVDP, and coronary flow rate before and after the addition of SR and during preconditioning, ischemia, and reperfusion for the CON and PC hearts. During each 5-min preconditioning ischemic episode, the LVDP decreased to approximately 3–4 mmHg. However, heart rate and LVDP returned back to baseline values during each preconditioning reperfusion episode. At the end of ischemic preconditioning, heart rate, LVDP and coronary flow were not significantly different from the values before preconditioning. Significant differences were found between CON and PC hearts during reperfusion after the 20-min ischemia. After 5 min of reperfusion, LVDP was recovered to 62 ± 16% compared to the baseline value in the PC hearts. In contrast, it recovered to only 31 ± 16% in the CON hearts ($P \leq 0.05$ CON vs. PC). After 30 min of reperfusion the difference in LVDP was even larger and more significant (PC, 82 ± 13% recovery vs. CON, 38 ± 16% recovery, $P \leq 0.01$) (Fig. 2). Two-way ANOVA showed a significant effect of preconditioning on LVDP between CON and PC hearts ($F(1, 24) = 11.69$, $P < 0.002$) during post-ischemic reperfusion. Repeated-measure ANOVA showed changes in LVDP values

Table I. Myocardial Function in Isolated Perfused Heart*

Function	Heart rate (beats/min)		Left ventricle developed pressure (LVDP, mmHg)		Coronary flow (ml/min)	
	CON	PC	CON	PC	CON	PC
Baseline	253 ± 9	249 ± 7	31 ± 6	32 ± 6	9.4 ± 0.2	9.8 ± 1.3
Baseline (after shift reagent)	277 ± 14	249 ± 7	18 ± 3	22 ± 4	8.8 ± 0.8	8.9 ± 1.1
Preconditioning						
1–5-min ischemia				3.8 ± 1.5		0
1–5-min reperfusion		242 ± 11		17 ± 2		♣
2–5-min ischemia				3.5 ± 0.4		0
2–5-min reperfusion		245 ± 11		16 ± 2		♣
3–5-min ischemia				3.7 ± 0.6		0
3–10-min reperfusion	271 ± 13♠	229 ± 16	19 ± 2♠	18 ± 3	9.8 ± 1.5♠	9.3 ± 1.5
Ischemia, 20 min	0	0	2.5 ± 1.3	2.7 ± 1	0	0
Reperfusion, 5 min	♣	♣	5.5 ± 1.8	14 ± 3*	8.0 ± 0.4	8.2 ± 2.2
Reperfusion, 30 min	189 ± 31#	229 ± 12	6.8 ± 1.8	18 ± 3**	8.3 ± 0.3	♣

* Note: $n = 6$ for CON and $n = 4$ for PC hearts. Values are reported as mean ± SE. Significance: CON, # $P \leq 0.05$ (reperfusion, 30 min vs. baseline); * $P \leq 0.05$, ** $P \leq 0.01$ (CON vs. PC hearts).
♣ Parameters were not stable or were not measured.
♠ The value in the CON group which are corresponded to the time of last preconditioning episode in the PC group.

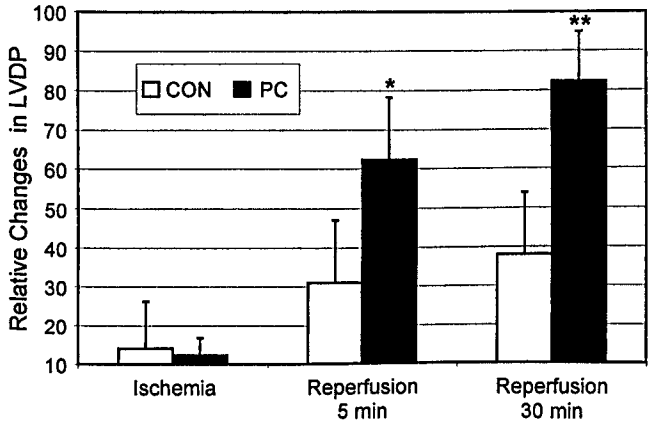

Figure 2. Relative changes in left ventricular developed pressure (LVDP) in control (CON) and preconditioned (PC) hearts during ischemia (20 min of stop-flow) and reperfusion (30 min). Baseline LVDP is normalized to 100%. The PC hearts showed 82 ± 16% recovery of LVDP after 30 min of reperfusion compared to only 39 ± 13% in CON hearts; $n = 6$ for CON and $n = 5$ for PC hearts. Values are reported as Mean ± SE. Significance: $^{*}P < 0.05$, $^{**}P < 0.01$ (CON vs. PC hearts).

($F(2, 24) = 9.50$, $P < 0.0004$) with time in both the groups and effect of preconditioning with time ($F(2, 24) = 3.00$, $P < 0.069$). In addition, the recovery of heart rate was also better in PC hearts. After 30 min of reperfusion, the heart rate was recovered to 229 ± 12 beats/min in PC hearts and only to 189 ± 31 beats/min in CON hearts (Table I).

^{23}Na MRS of Perfused Heart. Figure 3 shows ^{23}Na spectra from an isolated, perfused heart before and after addition of 3.6 m*M* TmDOTP^{5-} to the perfusate. The chemical shift of tissue Na^+ before addition of the SR was set to 0 ppm, while the signal from Na^+ in the reference bulb containing 10 m*M* TmDOTP^{5-} was shifted to 10.5 ppm. Ten minutes after addition of SR, one shifted peak became visible at ~2.7 ppm (spectrum 2). It is likely that this shifted peak was from Na^+ in the perfusate and the unshifted peak contained contributions from both intracellular and interstitial sodium in the heart. A few minutes later, a second shifted peak appeared at ~1.5 ppm (spectrum 3). This shifted peak may largely be from interstitial Na^+ in the heart. The two shifted ^{23}Na peaks from the perfusate and interstitial Na^+ converged after 16 min (spectrum 4). Only a single shifted Na_e^+ peak was visible at about 20 min, when a steady state was reached (spectra 5 and 6). This is in contrast with a recent report in which multiple Na_e^+ peaks were observed even after 75 min of perfusion with the same SR but Ca^{2+} free perfusate (14). In our experiments 3.5 m*M* TmDOTP^{5-} shifted the Na_e^+ signal sufficiently to obtain adequate resolution for reliable measurement of changes in Na_i^+ signal intensity.

Figure 4 shows relative changes in Na_i^+ signal intensity for the PC and CON hearts during preconditioning, ischemia, and reperfusion. The three ischemia/reperfusion episodes during preconditioning progressively increased Na_i signal intensity. During the third ischemic episode of preconditioning, the Na_i signal intensity in PC hearts (132 ± 8.4%) was 33.0% higher ($P < 0.01$) compared to CON hearts (98.8 ± 4.0%). Na_i^+ signal intensity increased from 126 ± 12.9% to 174 ± 8.6% during the 20 min of ischemia in PC hearts and from 108 ± 4.0 to 151 ± 22.2% in CON hearts. The increase in Na_i^+ signal intensity relative to the pre-ischemic value was not significantly different in the two groups. During the later periods of reperfusion Na_i signal intensity decreased to similar levels in both the experimental groups. However, as shown in Figure 5, during the first few minutes of reperfusion, the decrease in Na_i^+ was faster in the PC hearts compared to CON hearts. Na_i^+ decreased by 31.3 ± 8.3% (from 174 ± 8.6% to 143 ± 12.2%) during the initial 3-min of reperfusion in the PC hearts, but it

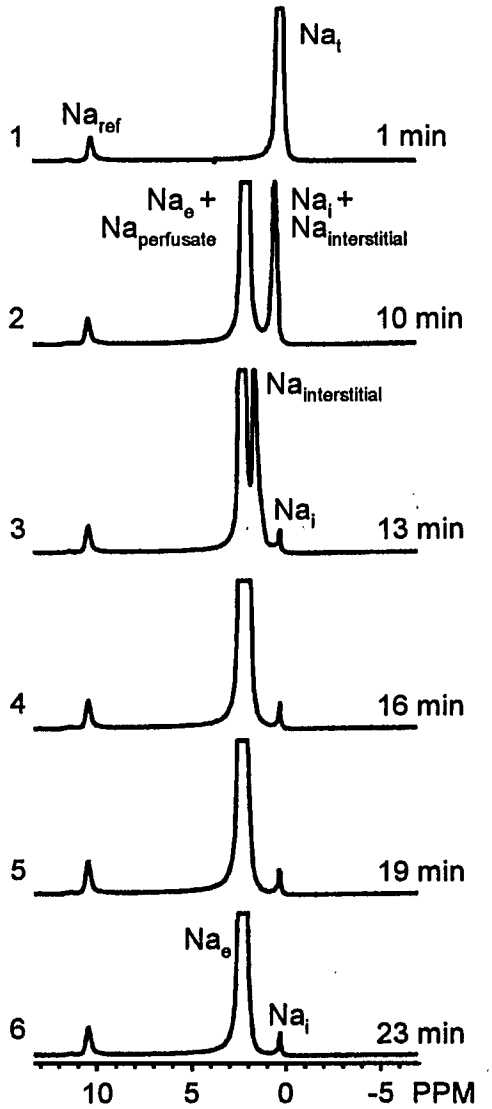

Figure 3. Stacked-plot of typical ^{23}Na spectra from an isolated perfused rat heart after addition of the sodium shift reagent (SR), TmDOTP^{5-} (3.6 m*M*). Na_i, intracellular sodium; Na_t, total sodium; Na_e, extracellular sodium; Na_{ref}, signal from reference capillary containing 10 m*M* TmDOTP^{5-}. Time after TmDOTP^{5-} addition is indicated on the right. Addition of the SR initially produced two shifted ^{23}Na resonance from perfusate and interstitial sodium (spectrum 3). However, the two shifted Na_i^+ signals coalesced into a single sharp resonance after 20–25 min (spectra 5 and 6).

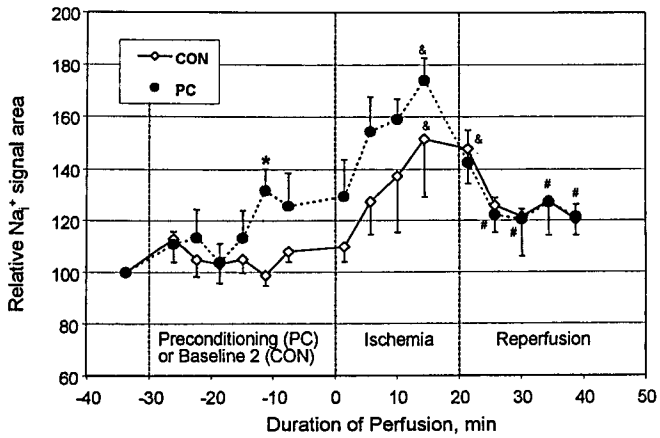

Figure 4. Relative changes in intracellular sodium (Na_i^+) resonance area as a function of time in control (CON, $n = 6$) and preconditioned (PC, $n = 4$) hearts. Na_i baseline is normalized to 100. The Na_i^+ level in the PC hearts was increased by approximately 33% at the end of preconditioning. On reperfusion after prolonged ischemia, the decrease in Na_i^+ was faster compared to the CON hearts. Significance: $^*P < 0.01$ (PC vs. CON), $^\#P < 0.05$ (PC group vs. end of ISC), $^\&P < 0.01$ (vs. pre-ischemic level for each group).

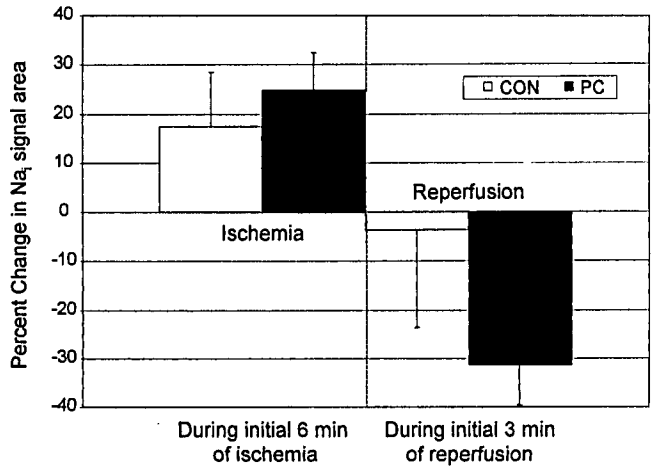

Figure 5. Relative changes in normalized intracellular sodium (Na_i^+) resonance area at the beginning of ischemia and reperfusion in control (CON) and preconditioned (PC) hearts. Areas are derived from data shown in Figure 4. The Na_i^+ increase during initial 6 min of ischemia was similar in CON and PC hearts. However, PC hearts showed a faster initial recovery of Na_i^+ during reperfusion compared to the CON hearts.

changed by only 3.75 ± 19.7% (from 151 ± 22.2% to 148 ± 13.3%) in the CON hearts.

Two-way ANOVA showed significant differences in Na_i^+ between CON and PC hearts during preconditioning ($F(1, 48) = 6.15$, $P < 0.0167$) and ischemic periods ($F(1, 32) = 8.46$, $P < 0.0065$) but not during post-ischemic reperfusion ($F(1, 43) = 0.025$, $P < 0.875$).

^{31}P MRS of Perfused Heart. *Cellular energetics.* Figure 6 shows PCr/P_i and βATP/P_i for the PC and CON hearts during initial perfusion, and at the end of preconditioning, ischemia, and post-ischemic reperfusion. At the beginning of perfusion (baseline) the cellular energy status of the PC and CON hearts was similar. The PCr/P_i was 3.39 ± 0.11 in PC hearts and 3.23 ± 0.17 in CON hearts, and βATP/P_i was 2.95 ± 0.2 in PC hearts and 2.96 ± 0.24 in CON hearts. Addition of PPA, DMMP, and $TmDOTP^{5-}$ to the perfusate did not produce significant changes in the cellular energy status in either group (data are not presented). After preconditioning, in the PC hearts, PCr/P_i and βATP/P_i were 1.79 ± 0.75 and 1.33 ± 0.44, respectively, and, in the CON hearts, 2.68 ± 0.54 and 2.07 ± 0.83, respectively. Preconditioning produced a significant decrease in ATP/P_i in the PC hearts compared to the baseline value. However, there was no significant difference in the cellular energy status between the PC and CON hearts before prolonged ischemia. At the end of ischemia PCr/P_i and βATP/P_i levels were reduced to almost zero in both the groups. At the end of reperfusion PCr/P_i and βATP/P_i ratios were partly recovered without any significant difference between the PC and CON hearts. PCr/P_i and ATP/P_i were 1.2 ± 0.3 and 0.4 ± 0.1, respectively, in PC hearts and 1.0 ± 0.4 and 0.31 ± 0.05, respectively, in CON hearts. Two-way ANOVA did not show significant differences between CON and PC hearts with respect to PCr/P_i ($F(1, 28) = 0.316$, $P < 0.579$) and βATP/P_i ($F(1, 28) = 0.656$, $P < 0.425$).

Intracellular pH. Figure 7 shows pH_i (calculated from ^{31}P spectra) for the PC and CON hearts during initial perfusion, and at the end of preconditioning, ischemia, and post-ischemic reperfusion. At the beginning of perfusion, pH_i of the PC (7.06 ± 0.05) and CON (7.09 ± 0.06) hearts was similar. At the end of preconditioning, pH_i fell to 6.75 ± 0.15 in the PC hearts ($P < 0.05$). At the same time point, pH_i did not change significantly in the CON hearts (6.97 ± 0.10). The end-ischemic pH_i was decreased compared to the baseline values in both the groups ($P < 0.001$). However, compared to the pre-ischemic levels, ischemia did not change pH_i significantly in the PC group and it decreased pH_i by 0.59 ± 0.08 unit ($P < 0.001$) in CON group. At the end of 20 min of ischemia, pH_i was significantly acidic in the CON hearts (6.38 ± 0.27) compared to the PC hearts (6.65 ± 0.12) ($P < 0.05$). Reperfusion returned pH_i to approximately the same levels in PC (6.98 ± 0.19) and CON (6.90 ± 0.15) hearts. Two-way ANOVA showed significant differences between CON and PC hearts with respect to pH_i in different experimental periods of perfusion ($F(3, 33) = 4.19$, $P < 0.013$).

Cellular volume. The intra- and extracellular volumes in the beating, perfused hearts were 0.36 ± 0.07 and 6.46 ± 1.3 ml, respectively, and did not change significantly during preconditioning, ischemia, and reperfusion.

Discussion

Cardiac Function and Intracellular pH. The results reported here show that the hearts that were subjected to ischemic preconditioning have a smaller decrease in pH_i during prolonged ischemia and better recovery of mechanical function (LVDP and heart rate) during reperfusion after prolonged ischemia compared to hearts without preconditioning. These data support the well-known effect that short

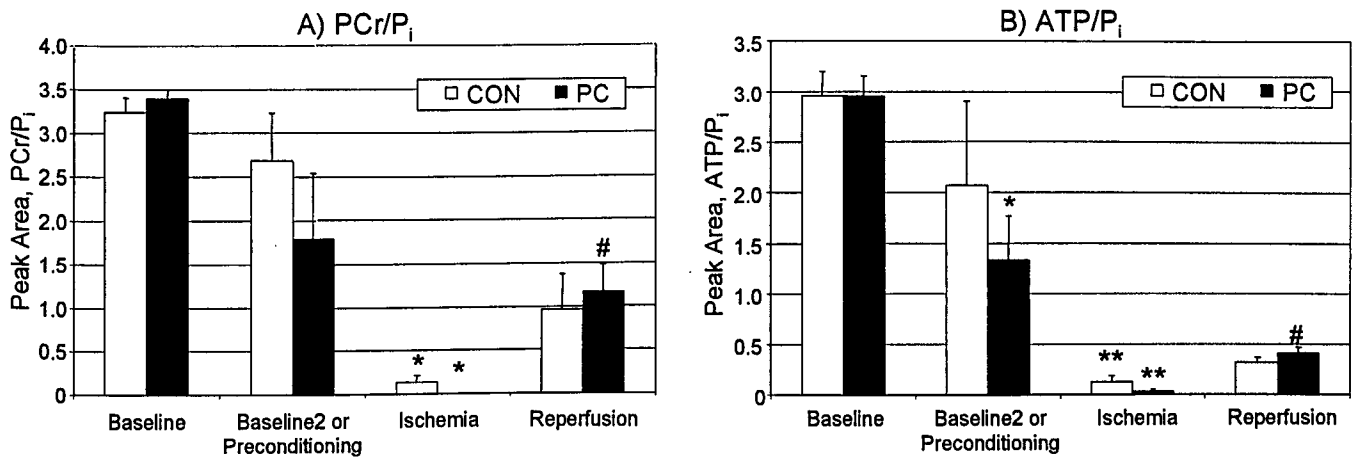

Figure 6. Changes in PCr/P_i (A) and ATP/P_i (B) ratios in control (CON) and preconditioned (PC) hearts (mean ± SE, $n = 6$ (CON), $n = 4$(PC)). After preconditioning, ATP/P_i were reduced compared to the baseline value in the PC heart. At the end of ischemia, PCr/P_i and ATP/P_i were reduced to almost zero in both CON and PC groups. At the end of post-ischemic reperfusion, PCr/P_i and ATP/P_i were partly recovered without any significant difference between the two groups. Significance: (i) PCr/P_i data, $^*P < 0.01$ (ischemia vs. baseline), $^\#P < 0.05$ (reperfusion-PC vs. ischemia-PC); (ii) ATP/P_i data, $^*P < 0.05$ (preconditioning vs. baseline), $^{**}P < 0.01$ (ischemia vs. baseline), $^\#P < 0.01$ (reperfusion-PC vs. ischemia-PC).

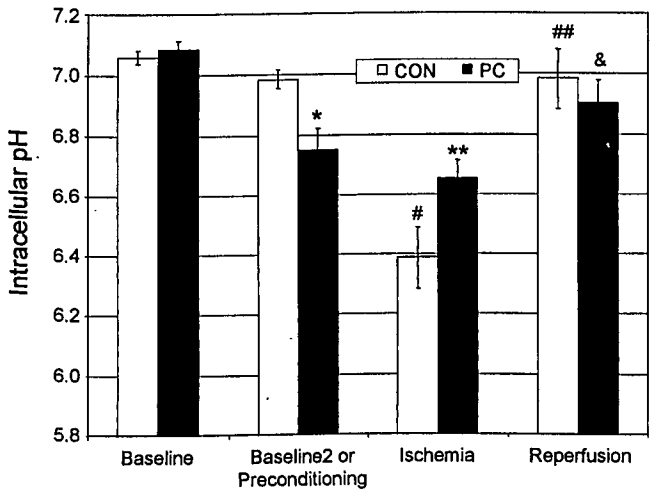

Figure 7. Changes in intracellular pH in control (CON) and preconditioned (PC) rat hearts. At the end of preconditioning, pH_i was significantly reduced compared to baseline. Compared to pre-ischemic levels, ischemia decreased pH_i by 0.59 ± 0.08 unit in CON hearts but it did not change pH_i significantly in the PC hearts. Reperfusion returned pH_i to approximately the same levels in PC and CON hearts. Significance: $^*P < 0.05$ (PC vs. CON (baseline 2)), $^{**}P < 0.05$ (CON (ischemia) vs. PC (ischemia); $^\#P < 0.001$ (CON: ischemia vs. baseline2), $^{\#\#}P < 0.01$ (CON: reperfusion vs. ischemia); $^\&P < 0.05$ (PC: reperfusion vs. ischemia).

ischemic episodes protect the heart from ischemic/reperfusion injury and reduce infarct size (15–17).

LVDP recovery during reperfusion after ischemia is determined by many factors, including pH (18). In our experiments there was an initial rapid recovery of LVDP during post-ischemic reperfusion in PC hearts (reaching 64% of the pre-ischemic level within 5 min and 82% within 30 min of reperfusion). A better recovery of post-ischemic LVDP could be explained by improved pH homeostasis most likely via Na^+-coupled net H^+ efflux (Na^+-HCO^{3-} co-influx and Na^+/H^+ exchange) (19) along with contribution from lactate and CO_2 efflux (20). In non-preconditioned hearts LVDP did not return back to the pre-ischemic level even after 30 min of reperfusion following prolonged ischemia (only 39% compare to 100% pre-ischemic value), despite the recovery of intracellular pH.

The LVDP in our perfused heart preparations was significantly less than the normal values for the isolated perfused rat heart (Table I). This was perhaps due to the combined effects of the anesthesia and the sodium SR. All SRs, including $TmDOTP^{5-}$, are known to complex with extracellular Ca^{2+} and decrease the free Ca^{2+} concentration. To avoid this problem we added extra 1.3–1.6 m*M* $CaCl_2$ to the perfusion medium, but that did not circumvent the problem. Despite this problem, the other mechanical functions of the heart (heart rate, 249–277 beats/min, and coronary flow, 8.8–8.9 ml/min) during baseline perfusion were within physiological range in our experiments. Furthermore, these mechanical functions (in preconditioned hearts) and the intracellular pH (in both the experimental groups) were recovered after post-ischemic reperfusion.

Na^+ Accumulation During Preconditioning and Ischemia/Reperfusion. In our experiments, preconditioning resulted in an increase in Na_i^+ during the last (third) ischemic episode. Intracellular sodium during preconditioning has also been measured in other studies (4, 9, 11). A study by Tosaki *et al.* (9) using destructive chemical analysis and another study by Steenbergen *et al.* (4) using NMR did not show significant changes of Na_i^+ during preconditioning. This may be due to (i) large errors associated with the measurement of Na_i^+ by atomic absorption method (9), (ii) use of calcium chelator 5F-BAPTA in the perfusion media (for measurement of free intracellular Ca^{2+}), which can lead to decreased developed pressure throughout the experiment, and/or (iii) presence of high concentration of NH_4^+ (20 m*M*) as a counter-cation with $TmDOTP^{5-}$ (4). Our results are similar to the data reported by Ramasamy *et*

al. (11), who used $DyTTHA^{3-}$ as a Na^+ SR and showed a significant increase in Na_i^+ at the last (fourth) ischemic episode during preconditioning compared to CON hearts. The preconditioning-induced increase in Na_i^+ may be due to the activation of Na^+/H^+ exchanger.

To establish that preconditioning activates Na^+/H^+ exchanger, the ratios of changes in Na_i^+ to changes in pH_i ($\Delta Na_i/\Delta pH_i$) during preconditioning, prolonged ischemia, and post-ischemic reperfusion periods were calculated. In CON hearts, $\Delta Na/\Delta pH$ values were −88, −75, and −52 during preconditioning, ischemia, and reperfusion periods, respectively, and were largely unchanged. In PC hearts, $\Delta Na/\Delta pH$ was −75 during the preconditioning period, which is similar to the value for CON group. However, during prolonged ischemia the value increased to −482 in preconditioned hearts. During reperfusion it reduced to −209 but was still higher than the control hearts. The preconditioning induced increase in $\Delta Na/\Delta pH$ was due to an increase in Na^+ influx (Fig. 3) and a relatively small change in pH_i (Fig. 6). These data support our hypothesis that preconditioning activated the Na^+/H^+ exchanger in the isolated perfused heart.

In our experiments, both PC and CON hearts showed a rapid increase in Na_i during prolonged ischemia. Compared to the baseline period (i.e., before preconditioning) the relative increase in Na_i^+ at the end of ischemia was 50% for the CON hearts versus 75% for the PC hearts (Fig. 4). However, the relative increase in Na_i^+ compared to the values before prolonged ischemia was similar in the two groups (+43.3% for the CON group and +47.9% for PC group). Ramasamy *et al.* (11) have shown similar results using a different preconditioning protocol (four 5-min ischemia and 5-min reperfusion episodes) and $Dy(TTHA)^{3-}$ as SR. Preconditioning-induced increase in Na^+/H^+ exchanger activity did not lead to a significant difference in the change in Na_i^+ level between the two experimental groups during prolonged ischemia because of a dramatic decrease in cellular energy metabolites (Fig. 6) resulting in a decrease in Na^+/K^+-ATPase activity, which is most important in maintaining the transmembrane Na^+ gradient (21). In contrast to our data and the data reported by Ramasamy *et al.* (11), Steenbergen *et al.* (4) and Imahashi *et al.* (10) observed an attenuated increase in Na_i^+ during ischemia in PC hearts compared to CON hearts. Tani *et al.* (8) have suggested that the increase in Na_i^+ during ischemia may be age related. They reported an attenuated increase in Na_i^+ during ischemia in preconditioned hearts from 12-week-old rats but not from 50- and 100-week-old animals. We used 9- to 10-week-old rats and observed identical relative increase in Na_i^+ during prolonged ischemia in PC and CON hearts and at the same time observed cardioprotective effect of preconditioning on mechanical function.

Very interestingly, our data show that in the beginning of post-ischemic reperfusion, the rate of Na_i recovery was faster in the PC hearts compared to the CON hearts (Fig. 5). This is in contrast with Steenbergen *et al.* (4) who did not find any difference in the rate of post-ischemic Na_i^+ recovery. On the other hand, Tosaki *et al.* (9) demonstrated unchanged Na_i^+ levels during post-ischemic reperfusion in both control and preconditioned hearts. Our data are consistent with Ramasamy *et al.* (11), who reported an accelerated recovery of Na_i^+ during reperfusion in preconditioned hearts. We have shown that most of the decrease in Na_i^+ occurred during the initial 10 min of post-ischemia reperfusion and that the changes in Na_i^+ during the later 20 min were similar in PC and CON groups. Na_i^+ remained approximately 20% higher compared to baseline after 30 min of post-ischemic reperfusion in both CON and PC groups. Thus, the most important and significant response of Na_i^+ in the PC hearts was an increase in Na_i^+ at the end of preconditioning and a rapid decrease Na_i^+ during the first few minutes of post-ischemic reperfusion compared to the CON hearts. These data suggest that changes in ion transport processes involving Na^+ are altered in the PC hearts and may protect the heart from ischemic damage.

Our data show that ischemia decreased pH_i by approximately 0.6 unit compared to the pre-ischemic value in the control hearts but did not produce a significant change in the PC hearts. Ischemic preconditioning may stimulate Na^+/H^+ exchanger, which could potentially reduce intracellular acidosis during prolonged ischemia. Activation of Na^+/H^+ exchanger in this case could increase acid extrusion during ischemia and support faster recovery of mechanical function in PC hearts during post-ischemic reperfusion. In PC hearts the $\Delta Na_i/\Delta pH_i$ value, which represents Na^+/H^+ exchange activity, was decreased during post-ischemic reperfusion compared to ischemia, but it was still higher than the value during the baseline period. It has been shown by others that short repetitive intracellular acidification (a likely preconditioning stimulus) activates proton efflux via Na^+/H^+ exchanger (23). Activation of Na^+/H^+ exchanger may be related to activation of protein kinase C during preconditioning. Liu *et al.* (24) have shown that protein kinase C-dependent phosphorylation is necessary for the protective effects of preconditioning. In addition, Wallert *et al.* (25) have shown that the cardiac Na^+/H^+ exchanger is stimulated by activation of protein kinase C and that stimulation of the Na^+/H^+ exchanger normalized pH_i after an acid load. Thus, preconditioning may activate protein kinase C, which in turn activates the Na^+/H^+ exchanger. Another possibility is that preconditioning or repeated intracellular acidification increases the number of sites or copies of the exchanger in the sarcolemma (23).

Contrary to our hypothesis that activation of Na^+/H^+ exchange during preconditioning could be beneficial for reducing intracellular acidosis, recently, the use of Na^+/H^+ exchanger antagonists has been suggested as a mechanism to prevent ischemic damage to the myocardium (16, 26, 27). Use of Na^+/H^+ exchange inhibitors in human clinical trials, however, have shown disappointing results; the reasons for this are not clear (28, 29). Our results support the viewpoint that use of Na^+/H^+ exchange inhibitor would likely be detrimental in patients with ischemic heart disease and possibly

explain the poor efficacy or no efficacy of such inhibitors in preventing ischemic damage.

The pH_i at the end of ischemia was higher in our experiments compared to what has been reported in some previous publications (30–32). However, other publications have reported similar end-ischemic intracellular pH values as we observed. For example, Steenbergen *et al.* reported a pH_i of 6.3 in control hearts and 6.5 in preconditioned hearts after 30 min of ischemia (4). The exact reason for the difference in end-ischemic pH_i in various studies is not clear. It does not appear to be related to the different buffer systems ($NaHCO_3/HCO_3^-$ vs. $NaH_2PO_4/H_2PO_4^-$) or substrate(s) (glucose only vs. glucose plus pyruvate) in the perfusion medium used in the previous studies. Another possibility is that the different studies used different equations for calculating the pH from ^{31}P NMR data. We recalculated pH_i using different equations that have been used previously for the heart but that did not change our pH_i values significantly.

In addition to Na^+/H^+ exchanger, functional coupling between Na^+-K^+-$2Cl^-$ co-transporter and Cl^-/HCO_3^- exchanger may be involved in controlling the pH during ischemia in PC hearts. Our data show that Na_i^+ is increased during preconditioning. This increase may result from an increase in the activity of Na^+-K^+-$2Cl^-$ co-transporter in addition to other ion transport mechanisms. If this is true, then intracellular Cl^- should also increase during preconditioning. The increased Cl^- during preconditioning may facilitate transport of HCO_3^- into the cells via Cl^-/HCO_3^- exchanger, thus buffering the cell (11, 20) during prolonged ischemia.

We have shown in the present study that Na_i^+ is increased at the end of preconditioning and further increased during prolonged ischemia in PC hearts compared to CON. One traditional view is that the increase in Na_i^+ in cardiomyocytes can stimulate the Na^+/Ca^{2+} exchanger, leading to Ca^{2+} overloading in the myocardium, and increase susceptibility to ischemia/reperfusion injury (33). However, we observed better recovery of mechanical function (LVDP and heart rate) and less ischemic acidification in PC hearts despite the higher Na_i^+ level. Consistent with our data, a previous study by Tosaki *et al.* (9) has shown that ischemic preconditioning causes less accumulation of Ca^{2+} by cells during post-ischemic reperfusion and better recovery of cardiac function during reperfusion. One explanation for this phenomenon is that during ischemia, the Na^+/Ca^{2+} exchanger may work in reverse direction, i.e., transport Ca^{2+} out of the cells and Na^+ into the cells (34).

Steenbergen *et al.* (4) have shown that there is a good correlation between tissue ATP levels and Ca^{2+} concentration during ischemia. Lundmark *et al.* (23) have shown that preconditioning or repetitive acidosis attenuates the decrease of ATP during prolonged ischemia in isolated perfused rat hearts. Thus, the higher ATP level during ischemia in preconditioned hearts may help in maintaining the cellular Ca^{2+} level. A higher ATP level during ischemia may also be responsible for the faster recovery of Na_i^+ during the first few minutes of reperfusion in the preconditioned hearts. In our experiments the cellular ATP and PCr levels in the PC and CON hearts were similar during the preconditioning, ischemia, and reperfusion periods. Perhaps we did not observe better bioenergetics in the PC hearts compared to the CON hearts because we collected ^{31}P NMR spectra only at the end of each experimental period, as our main goal was to monitor the changes in Na_i^+ continuously.

Conclusion

Ischemic preconditioning resulted in decreased cellular acidosis during prolonged ischemia and faster and more complete recovery of cardiac function upon reperfusion. The response of Na_i^+ in the PC hearts was an increase in Na_i^+ at the end of preconditioning and an accelerated decrease during the first few minutes of post-ischemic reperfusion. These data suggest that PC stimulated preliminary activation of ion transport processes involving Na^+ may protect the heart from intracellular acidosis during prolonged ischemia, promote rapid recovery of pH_i during reperfusion, and thus result in better recovery of mechanical function (LVDP and heart rate) during post-ischemic reperfusion.

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