Importance of Metabolic Inhibition and Cellular pH in Mediating Preconditioning Contractile and Metabolic Effects in Rat Hearts

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The pathophysiological mechanisms by which brief periods of flow interruption before a prolonged ischemic period, ischemic preconditioning (IPC), increase myocardial tolerance to ischemia and improve myocardial function during reperfusion are not completely understood. To test whether short periods of metabolic inhibition in the absence of a flow reduction induce similar protective effects, we studied cardiac function and metabolism using $^{31}$P nuclear magnetic resonance spectroscopy in isolated isovolumic rat hearts. Fifteen hearts underwent IPC, consisting of two 5-minute ischemia-reperfusion cycles (IPC group); 18 hearts underwent brief metabolic inhibition by exposure to two 5-minute infusions of 10 mmol/L sodium cyanide (CN group); and 15 hearts served as controls. Subsequently, all hearts were subjected to 30 minutes of total global ischemia at 37°C followed by reperfusion. At the end of the ischemic period, creatine phosphate and ATP levels did not differ among the groups. Cellular pH, however, plateaued at a higher level in the CN group (6.51±0.03) and IPC group (6.12±0.06) than in the control group (5.84±0.01, P<.001). IPC and CN hearts had better functional and metabolic recovery than the control hearts. Improved contractile recovery correlated with coronary flow rates at reperfusion (r=.7, P<.001) and with pH, values at 30 minutes of ischemia (r=.8, P<.001) but not with increased ATP levels during ischemia. Additional control hearts were reperfused at 15 mL/min so as to match the flow rates of IPC and CN groups, but this did not result in improved performance. To test the hypothesis that the preconditioning effect was related to pH, during ischemia, additional IPC and CN hearts underwent the same preconditioning protocol, except that the cellular pH at the end of the ischemic period was lowered by the use of hypercarbic superfusion during ischemia or by the use of bicarbonate-free perfusate just before sustained ischemia. Both of these interventions resulted in significantly lower contractile and metabolic recoveries than those observed in other IPC and CN hearts. Therefore, the preconditioning effect does not require reduced coronary flow but can be effectively elicited by metabolic inhibition per se in this model. The protective effect is not dependent on preservation of global myocardial energy stores but, rather, on reduced acidosis during the prolonged ischemic period. (Circ Res. 1994;74:139-150)

Key Words • pH • high-energy phosphates • sodium cyanide • myocardial ischemia • preconditioning

Some periods of ischemia induce increased myocardial tolerance to subsequent prolonged ischemic insults. This phenomenon, known as "ischemic preconditioning," was first described by Murry et al. in 1986, who demonstrated that four 5-minute coronary occlusions separated by equal periods of reperfusion before a subsequent sustained occlusion resulted in a 75% reduction of infarct size in dogs. This preconditioning effect seems to be an important mechanism of myocardial adaptation to ischemia and is present among different species of mammals, including dogs, rabbits, pigs, and rats. The beneficial effects of ischemic preconditioning have been subsequently expanded to additional aspects reflecting increased myocardial protection during and after ischemia, including preservation of left ventricular (LV) systolic function, decreased myocardial enzyme release, increased high-energy phosphate stores, and improved electrical stability. There is also evidence for the existence of preconditioning in humans.

Although the beneficial effects of ischemic preconditioning have been well described, the responsible mechanism(s) is not known. Proposed mediators include preservation of myocardial high-energy stores during ischemia, lessened tissue acidosis, adenosine receptor stimulation, activation of ATP-sensitive K$^+$ channels, and altered release of oxygen-derived free radicals. A common finding in all of these experiments is that the protective effects have been elicited by prior transient reductions in coronary flow. Flow-dependent mechanical effects on the myocardium such as the "garden hose" effect have been well described, and endothelial tissue is also known to respond to various stimuli, including vascular flow-dependent mechanical alterations. Therefore, it is conceivable that the mediators responsible for the beneficial effects of preconditioning could be released in response to, or only in the presence of, some of these flow-dependent changes. Although lowered coronary flow can result in mechanical changes in vascular and myocardial tissues, the prime effect of transient ischemia is altered myocardial metabolism. Myocardial metabolism can also be altered by inhibitors of oxidative metabolism, such as cyanide, which reduce contractility and high-energy phosphate levels to an extent similar to that observed during ischemia but without the vascular changes or the same degree of anaerobic metabolic product accumulation. The latter difference is probably the result of the continuous washout of catabolites provided by coronary flow.
flow in hearts exposed to metabolic inhibition as opposed to very little washout associated with ischemic interruption of flow. In this regard, metabolic inhibition can act as a paradigm for some of the metabolic effects of cardiac ischemia and can therefore be used to assess the relative significance of such effects in the absence of alterations in flow.

In their early descriptions of preconditioning, Murry et al.\textsuperscript{15} recognized that preconditioning critically reduced myocardial energy demand during the subsequent ischemic period, but they could not distinguish whether preserved ATP levels or reduced cellular load of catabolates was primarily responsible for delaying ischemic cell death.\textsuperscript{15} In more recent studies using \textsuperscript{31}P nuclear magnetic resonance (NMR) spectroscopy in the intact pig model, Kida et al.\textsuperscript{16} observed both preserved high-energy phosphate levels and cellular pH in previously preconditioned hearts during ischemia and suggested that the pH effect may be more important because it was more prolonged. To date, however, no studies have independently altered ATP levels or pH during ischemia in order to unequivocally assess their relative contribution to the preconditioning effect. Therefore, the present experiments were performed in the isolated rat heart model, which permits the rapid and simultaneous measurement of systolic and diastolic pressures, oxidative metabolism, and coronary flow, which are chief determinants of infarct size and functional recovery after ischemia. Another advantage of this model is that it also permits independent primary manipulations of ATP concentration and cellular pH.

The goal of the present investigation was twofold: (1) to determine whether short periods of metabolic inhibition induced by cyanide exposure in the absence of flow reduction would result in protective metabolic and contractile effects similar to those caused by ischemic preconditioning and (2) to investigate the relative contribution of preconditioning-induced changes in cellular pH versus ATP levels during ischemia to the improved functional and metabolic recovery observed in this model.

Materials and Methods

Isolated Heart Preparation

Nonfasting, exbreeder, male Wistar rats were anesthetized with 100 to 150 mg intraperitoneal pentobarbital. The hearts were excised, rapidly mounted on a Langendorff apparatus, and retrogradely perfused with oxygenated solution at 37°C at a constant perfusion pressure of 70 mm Hg. The perfusate contained (mmol/L) Na\textsuperscript{+}, 144; Ca\textsuperscript{2+}, 1.5; K\textsuperscript{+}, 5; bicarbonate, 17.5; Mg\textsuperscript{2+}, 1.2; chloride, 134; and glucose, 5; along with 0.05 U/mL insulin and 5 μg/mL lidocaine. The perfusate was not recirculated, and pH was adjusted to 7.4 by bubbling with a gas mixture containing 95% O\textsubscript{2}-5% CO\textsubscript{2}. The hearts were paced at 4 Hz using a Grass SD-9 stimulator via a KCl-wick electrode placed into the right ventricle. To minimize temperature changes during ischemia, the hearts were superfused throughout the experiment with a solution identical to the perfusate except for the absence of glucose and insulin and in equilibrium with a 5% CO\textsubscript{2}-95% N\textsubscript{2} gas mixture. The temperature of the air flowing around the tube that contained the heart was also controlled by a thermocouple accessory present within the NMR probe. A latex balloon, connected to PE-190 tubing, was inserted into the LV through the mitral valve. It contained a solution of 100 mmol/L phenylphosphonic acid at pH 7.4, which served as a \textsuperscript{31}P NMR standard, and was filled in increments so as to obtain the maximum developed pressure, which usually occurred at an end-diastolic pressure of 8 to 14 mm Hg. The balloon line was connected to a Gould P23Db transducer for measurements of LV pressure. Mean coronary flow was calculated by timed collections of the returning fluid.

\textsuperscript{31}P NMR Spectroscopy

The hearts were positioned in a 20-mm probe of a Bruker AM 360-WB spectrometer in a field strength of 8.5 T. Magnetic field homogeneity was optimized during observation of the water proton signal using the decoupler coil. Proton-decoupled minimally saturated \textsuperscript{31}P NMR spectra were obtained with a 2.1-second delay between pulses of 22-microsecond duration at a flip angle of 60°. Cycles of 64 pulses were collected during =2.5 minutes. Relative metabolite quantification was obtained by the use of an automated iterative time-domain nonlinear least-squares fitting routine in the Sunview environment of a Sun Sparcstation.\textsuperscript{24} Known frequencies of the metabolites of interest at baseline were used as starting values in a model of decaying oscillations.\textsuperscript{25} An illustrative example of the results of the fitting routine is shown in Fig 1. Results are expressed as percentage of the baseline values. pH\textsubscript{w} was measured by the chemical shift of the inorganic phosphate (P\textsubscript{i}) peak relative to the creatine phosphate (PCr) peak. The chemical shift values in parts per million were converted to pH units as previously described.\textsuperscript{26,27}

Experimental Protocols

The hearts were initially allowed to stabilize in the spectrometer for at least 20 minutes during calibration. Baseline spectra and hemodynamic measurements were recorded, and the hearts were then allocated to either one of the preconditioning protocols (see below) or to an equivalent period of flow with normal perfusate at a constant perfusion pressure (control group). All hearts were then subjected to a period of 30 minutes of continuous normothermic ischemia caused by clamping the perfusion lines, followed by reperfusion at the baseline perfusion pressure. The hearts were paced throughout the experiment except for the first 30 minutes of reflow, when the stimulator was turned off to reduce the incidence of arrhythmias.

Three groups of hearts were studied initially:

Group 1: Control hearts. After the baseline measurements were obtained, 15 hearts were maintained for 20 minutes at 70 mm Hg of perfusion pressure and then underwent total global

Fig 1. A Fourier-transformed spectrum of a typical 5-minute acquisition is displayed in A. The spectrum resulting from utilization of the automated least-squares fitting algorithm on the data from the same acquisition is shown in B. C shows each of the fitted peaks individually and the baseline correction, whereas D shows the results of the subtraction of the actual spectrum from the fitted spectrum. Peaks are identified from left to right as phenylphosphonic acid (PPA), inorganic phosphate (Pi), creatine phosphate (PCr), and [β-ATP] of ATP (β-ATP, used for quantification purposes).
interruption of the coronary flow for 30 minutes. This was followed by 45 minutes of reperfusion at the baseline perfusion pressure.

**Group 2: Ischemia preconditioned hearts.** After the baseline measures, 15 hearts underwent an ischemic preconditioning protocol composed of two 5-minute periods of total global ischemia, each followed by 5 minutes of reperfusion. Subsequently, each heart underwent 30 minutes of total ischemia followed by reperfusion at the baseline perfusion pressure.

**Group 3: Cyanide preconditioned hearts.** To determine whether metabolic inhibition alone could induce increased ischemic tolerance, 18 hearts underwent transient metabolic inhibition by cyanide exposure with intact flow in lieu of the brief periods of total ischemia. This consisted of two 5-minute infusions of 10 mmol/L NaCN and 10 minutes of washout with no interruption of coronary flow before the sustained 30 minutes of total ischemia and subsequent reperfusion.

**Statistics**

Statistical analyses were performed using commercially available computer software (sas, Statistical Analysis Institute, Cary, NC). Results of continuous variables are reported as mean±SEM. Each parameter was evaluated initially by one-way or repeated-measures ANOVA for differences among the groups. When the resulting F values indicated that significant differences were present among the groups, the analysis proceeded by the use of the Tukey's multiple-comparisons procedure at an appropriate level of significance. Least-squares linear regression was used to correlate two continuous variables. Two-tailed values of P<.05 were considered significant.

**Results**

**Control and Ischemic and Cyanide Preconditioning Experiments**

**LV function.** Mean LV developed pressure at baseline did not differ among the groups and was 161±2 mm Hg. Ventricular pressures did not change significantly in the control group (n=15) during the first 20 minutes of constant perfusion pressure. Shortly after the onset of each 5-minute interruption of coronary flow during the ischemic preconditioning protocol (n=15), the LV developed pressure rapidly fell to 0 mm Hg and recovered to some extent during each subsequent reperfusion period (Fig 2A). Contractile recovery was less after the first preconditioning interruption of coronary flow than after the second (69±3% and 80±2% of the initial developed pressures) in hearts that underwent ischemic preconditioning. In the hearts submitted to metabolic inhibition (n=18, group 3), developed pressure fell during each cyanide infusion, but it was not abolished. In both groups, LV contractile function also recovered partially at the end of the preconditioning periods (80±2% of the initial developed pressure in group 2 and 75±2% in group 3, P<.05 from baseline by paired analysis, P=NS between groups), thus reflecting the presence of a modest and similar degree of myocardial contractile dysfunction before sustained ischemia in both preconditioning protocols. Because of the decline in LV developed pressure caused by the preconditioning protocols, ventricular pressure was significantly higher in the control group immediately before the long ischemic insult (P<.05 versus cyanide- and ischemia-preconditioned groups, Fig 2A). During sustained ischemia, all groups demonstrated a homogeneous and rapid decrease in developed pressure, which fell to <5 mm Hg after 2.5 minutes of ischemia in all hearts.

After 30 minutes of sustained ischemia, LV contractile recovery was significantly better in ischemia- and cyanide-preconditioned hearts than in control hearts (Fig 2A). LV contractile function recovery was poor in the hearts of the control group during the reperfusion period (8±2% recovery of the initial LV developed pressure after 30 minutes of reflow). Developed pressure in hearts submitted to the ischemic preconditioning protocol recovered to 54±6% of the initial value at 30 minutes of reperfusion (P<.001 versus control hearts), whereas hearts that underwent metabolic inhibition preconditioning without any interruption of coronary
flow preceding the prolonged ischemic period recovered to 68±3% of the initial LV developed pressure (P<.001 versus control group, P<.05 versus ischemia preconditioned group).

Diastolic pressures were initially similar in all groups before ischemia and were unchanged after the preconditioning interventions (Fig 2B). Diastolic pressure increased during the prolonged ischemic period in all hearts. It increased faster, however, in hearts preconditioned with cyanide. The time of onset of ischemic contracture, determined by an increase of >2 mm Hg in the ventricular pressure, was similar in the ischemia preconditioned and control hearts (12±2 and 14±1 minutes, respectively) but was significantly shorter in hearts previously exposed to cyanide (6±1 minutes, P<.05). During reperfusion, the LV diastolic pressure recovered significantly better in preconditioned hearts (Fig 2B). The hearts submitted to metabolic inhibition had the lowest diastolic pressures during reperfusion (18±2 mm Hg), followed by ischemia-preconditioned hearts (32±4 mm Hg) and control hearts (74±3 mm Hg at 30 minutes of reflow, P<.001 among the groups).

**Coronary flow rates.** The mean coronary flow rate at baseline was 18±1 mL/min and was similar in the three groups (17±1 mL/min for the control group and 20±1 and 18±1 mL/min for ischemia- and cyanide-preconditioned groups, respectively; P=NS). During reperfusion, the mean coronary flow rate of the control hearts was significantly lower than that of the ischemia- and cyanide-preconditioned groups (11±1 mL/min for control hearts versus 15±1 and 16±1 mL/min for the ischemia- and cyanide-preconditioned hearts, respectively; P<.05; Fig 2C).

**High-energy phosphate levels.** PCr levels declined significantly during each of the preconditioning interventions, but the levels declined more in the hearts of the cyanide-preconditioned than the interrupted-flow (ischemia-preconditioned) group (21±2% of the initial values for the ischemia-preconditioned group versus 16±1% for the cyanide-preconditioned group during the first preconditioning intervention and 27±2% versus 19±2%, respectively, during the second preconditioning intervention) and recovered promptly after each brief episode (Fig 3A). Immediately before ischemia, there was some PCr overshoot in the cyanide-preconditioned group (121±2% of the initial values, P<.05 versus baseline) but not in the interrupted flow group. Since there was a moderate decrease in ATP levels during both preconditioning protocols, these were higher immediately before ischemia in the control group (99±2% of the initial levels) than in the ischemia preconditioned group (87±3%) and in the cyanide-preconditioned group (80±2%, P<.01 among groups).

PCr levels fell rapidly after the final interruption of coronary flow and were undetectable in all hearts after 5 minutes of total global ischemia. ATP levels decreased progressively after the interruption of coronary flow and were undetectable in all groups at the end of the 30-minute period of total ischemia. However, the hearts that had been treated with cyanide had significantly lower ATP levels during the first 20 minutes of sustained ischemia (Fig 3B).

Similar to the improved recovery of ventricular contractile performance, preconditioning was also associated with improved recovery of myocardial high-energy phosphate levels during reperfusion (as shown in the example presented in Fig 3 and in the group data displayed in Fig 4A and 4B). PCr levels at 30 minutes of reflow were highest in the cyanide-preconditioned group (105±3% of the initial values), followed by the ischemia-preconditioned group (90±4%) and the control group (52±4%, P<.001 among groups). Analogous
Fig 4. Relative creatine phosphate (PCr) levels are presented in A, ATP levels in B, and pH in C. On reperfusion, ischemia-preconditioned (○) and cyanide-preconditioned (△) hearts demonstrated better metabolic recovery than did control hearts (□), as evidenced by higher PCr and ATP levels and a less acidic cellular pH. Note that both preconditioning interventions resulted in significantly less intracellular acidosis during ischemia than that observed in control hearts and that cyanide-preconditioned hearts had lower ATP levels during the first 20 minutes of ischemia when compared with the other groups. Time 0 indicates the onset of ischemia; B, baseline measurements; and P, preconditioning episodes.

Fig 5. Expanded portions of 31P nuclear magnetic resonance spectra depicting the inorganic phosphate region at 15 minutes of ischemia are presented for the same control (A), ischemia-preconditioned (B), and cyanide-preconditioned (C) hearts displayed in Fig 3. These are presented because pH, can be calculated from the chemical shift position of the inorganic phosphate peak. The cellular pH was 6.05 for the control heart, 6.19 for the ischemia-preconditioned heart, and 6.41 for the heart exposed to cyanide.

715±77% in the ischemia-preconditioned group, and 811±88% in the cyanide-preconditioned group (P<.01 for cyanide-preconditioned versus control groups, P=NS for ischemia-preconditioned group versus cyanide-preconditioned or control groups). At 30 minutes of reperfusion, however, P, levels were highest in the control hearts (286±26% of the initial levels), were lower in the ischemia-preconditioned group (183±21%, P<.01 versus control group), and had returned to near baseline levels in hearts preconditioned with cyanide infusion (146±14%, P<.01 versus control group; P=NS for the ischemia-preconditioned group versus the cyanide group). Baseline PCr to P, ratios uncorrected for saturation and nuclear Overhauser effects (NOE) were similar in the three groups (3.1±0.2 for control hearts, 3.4±0.3 for ischemia-preconditioned hearts, and 2.7±0.3 for the cyanide-preconditioned group). Thirty minutes after reperfusion, this ratio was significantly lower in control hearts (0.6±0.1) than in the ischemia-preconditioned hearts (2.0±0.3) and cyanide-preconditioned hearts (2.8±0.4, P<.01).

pH. Initial pH, was similar in all groups (mean pH, 7.17±0.01) and comparable to other previously reported values for normal myocardial pH.37 During each preconditioning 5-minute interruption of coronary flow, cellular pH fell an average of 0.4 pH units to 6.8±0.01 and returned to normal values after reflow (Fig 4C). Cyanide metabolic inhibition caused less intracellular acidosis (pH 7.1±0.01, P<.05 versus baseline and P<.01 versus ischemic preconditioning). Importantly, the fall in cellular pH during the 30-minute global ischemic period was less in hearts from both preconditioning protocols (see example in Fig 5 and group data in Fig 4C). The mean rate of decrease in cellular pH during
the first 20 minutes of ischemia was highest in the control group (3.4±0.01 pH units per hour) followed by the ischemia- and cyanide-preconditioned groups (2.2±0.2 and 1.1±0.1 pH units per hour, respectively; \( P < .001 \) among groups). In addition, \( pH \) eventually plateaued at a higher value (6.12±0.06) during the 30 minutes of total ischemia in the ischemia-preconditioned hearts than in the control hearts (5.84±0.01, \( P < .01 \)). Hearts that had been subjected to prior metabolic inhibition alone developed even less intracellular acidosis during ischemia, with a final \( pH \) of 6.51±0.03 (\( P < .01 \) versus control and ischemia-preconditioned hearts). During reperfusion, \( pH \) recovered to a greater extent in the ischemia- and cyanide-preconditioned hearts (7.15±0.02 and 7.18±0.01, respectively; 7.08±0.01 in the control hearts at 30 minutes of reflow; \( P < .01 \)).

**Correlations to the return of contractile function.** To gain insight into the mechanism of improved mechanical recovery in preconditioned hearts, metabolic and perfusion parameters were correlated with contractile recovery. As shown in Fig 6A, improved LV contractile performance was associated with increased coronary flow rates during reperfusion \( (r=.7, P<.001) \) and linearly related to the cellular \( pH \) value achieved during ischemia (LV developed pressure=122·\( pH - 6.84 \), \( r = .8 \), \( P < .001 \), Fig 6C). There was no relation between higher ATP levels at 10 minutes of ischemia (or at 5 and 15 minutes, data not shown) and better contractile recovery at reperfusion (Fig 6B). The best preservation of LV contractile function was present in the hearts that had been exposed to cyanide, which, paradoxically, had the fastest decline in ATP levels during ischemia.

Therefore, the studies described thus far demonstrate that ischemic preconditioning as well as transient cyanide exposure induce increased myocardial tolerance during subsequent prolonged ischemia in this model. Improved mechanical recovery after ischemia is associated with both higher cellular \( pH \) during ischemia and increased coronary flow rates during reperfusion in preconditioned hearts. Therefore, two additional sets of experiments were performed.

First, to assess whether the diminished contractile recovery of the control hearts was due to their lower coronary flow at reperfusion, four hearts underwent exactly the same protocol as in the control group until the end of the 30-minute ischemic period, when they were reperfused at the constant coronary flow of 15 mL/min so as to match the higher flow rate of the preconditioned hearts measured during reperfusion.

Second, to evaluate the importance of improved preservation of \( pH \) during sustained ischemia to the increased performance of the preconditioned hearts, two different interventions designed to lower the \( pH \) of preconditioned hearts during ischemia were performed. Prior experimental data as well as theoretical modeling work have demonstrated that ischemic myocardial \( pH \) can be affected by an increase in the superficial \( CO_2 \) tension.28-29 Therefore, to increase the accumulation of \( CO_2 \) and primarily affect ischemic tissue \( pH \), five ischemia-preconditioned hearts and 11 cyanide-preconditioned hearts were exposed to a hypercarbic superfusate solution (mmol/L: \( Na^+ \), 144; \( Ca^{2+} \), 1.5; \( K^+ \), 5; bicarbonate, 0; \( Mg^{2+} \), 1.2; chloride, 152; and lidocaine, 5 \( \mu g/mL \); saturated with a 30% \( CO_2 \)–70% \( N_2 \) gas mixture) during the 30 minutes of sustained ischemia. At reperfusion, the superfusate was switched back to a solution in equilibrium with 95% \( N_2 \)–5% \( CO_2 \) at \( pH \) 7.4, and these hearts were reperfused at 70 mm Hg for 45 minutes. Ten additional ischemia- and six cyanide-preconditioned hearts underwent a different intervention to lower the extracellular buffering capacity and hence affect \( pH \) during ischemia. These hearts were perfused with buffer-free perfusate for 30 seconds before the sustained ischemic period. After reperfusion, the hearts were returned again to bicarbonate buffer perfusion.
Matched Coronary Flow Experiments

As described above, four additional control hearts were studied under conditions identical to the original control conditions except that these hearts were reperfused at a constant flow rate of 15 mL/min, which matched the higher reperfusion flow of the preconditioned hearts. As expected, no differences in high-energy phosphate levels or cellular pH were observed during baseline or the 30 minutes of total ischemia. Despite the higher coronary flow rates after reperfusion, there was no improvement in either LV systolic and diastolic pressures or in high-energy phosphate metabolites as compared with the prior control group (shown in Fig 7). LV developed pressure recovered to only 5±3 mm Hg at 30 minutes of reflow (P=NS versus control group and P<.01 versus ischemia-preconditioned group), and the diastolic pressure at reperfusion was high (86±1 mm Hg at 30 minutes, P=NS versus control group and P<.01 versus ischemia- and cyanide-preconditioned groups). High-energy phosphates at reperfusion were not different from those of the initial control group (44±6% versus 51±4% [PCr] and 27±5% versus 25±3% [ATP] of the initial values 30 minutes after reflow, P=NS versus control group, and P<.01 versus preconditioned groups). At 30 minutes of reperfusion, P, levels were 183±14%, and the PCR to P, ratio was 0.5±0.1. Thus, matching the coronary flow rate of the control group to that of the preconditioned hearts did not improve contractile or metabolic performance after ischemia.

Preconditioning Plus Increased Acidosis During Ischemia

The hearts of this group were treated the same as the other ischemia-preconditioned hearts until just before the onset of the sustained ischemic insult. Five hearts were exposed to a hypercarbic superfusion during ischemia, which resulted in an ischemic pH of 5.86±.02 at the end of 30 minutes of ischemia, which was significantly lower than that achieved by the first ischemia-preconditioned group (P=NS versus control group and P=.03 versus ischemia-preconditioned group). Ten other ischemia-preconditioned hearts were perfused with a bicarbonate-free solution just before sustained ischemia and had an ischemic pH of 5.98±.03 (P=.06 versus original ischemia-preconditioned group; see Fig 8). Contractile and metabolic recoveries during reperfusion were significantly depressed in both of these groups of hearts (as shown in Fig 8). LV developed pressures at 30 minutes of reperfusion were only 24±5 and 52±8 mm Hg, respectively, for the hypercarbic superfusion and buffer-free groups (versus 89±10 mm Hg of the first ischemia-preconditioned group, P<.05). Hearts exposed to hypercarbia also had higher LV diastolic pressures than those of the first ischemia-preconditioned group (56±3 versus 32±4 mm Hg, 30 minutes after reflow, P<.05). Both of these pH-lowering interventions resulted in lower PCR levels after reflow (68±7% and 74±4% for hypercarbic and bicarbonate-free groups versus 90±4% for the first ischemia-preconditioned group at 30 minutes, P<.05). There was also a
trend for lower ATP levels during reperfusion in this group (26±6% and 30±2% versus 35±3% at 30 minutes of reflow in the first ischemia-preconditioned group).

Experiments similar to those described above were performed in cyanide-preconditioned hearts. These also resulted in lower myocardial pH during ischemia and poorer functional and metabolic recoveries on reperfusion (Fig 9). pH, at the end of the ischemic period was significantly lowered to 6.37±0.04 (P<.01 versus conventional cyanide-preconditioned group) by the hypercarbic solution and to 6.22±0.04 by buffer-free perfusion (P<.01 versus conventional cyanide-preconditioned group). LV developed pressures 30 minutes into reperfusion were 93±5 and 80±13 mm Hg for the hypercarbic and bicarbonate-free cyanide groups, respectively (versus 114±5 mm Hg for the original cyanide-preconditioned group, P<.05). Diastolic pressures after 30 minutes of reflow were 22±3 and 34±3 mm Hg for the hypercarbic and buffer-free cyanide-preconditioned hearts, respectively (P=.13 and P<.01 versus 18±2 mm Hg for the original cyanide-preconditioned hearts). PCr at 30 minutes of reflow was also lower than in the first cyanide-preconditioned hearts (91±4% and 88±4% for hypercarbic and low-bicarbonate cyanide-preconditioned hearts, respectively, versus 105±3% for the original cyanide-preconditioned group; P<.05). At the same time, ATP levels were 32±4% and 42±5% for the hypercarbic and low-buffer cyanide-preconditioned hearts, respectively (P=NS versus first cyanide experiments).

Therefore, ischemic preconditioning and transient cyanide exposure both limit the fall in cellular pH during ischemia and are associated with improved contractile and metabolic recoveries during reperfusion in this model. The independent lowering of pH during ischemia by two unrelated maneuvers offsets both the beneficial contractile and metabolic effects of these preconditioning interventions.

Discussion

The increased ischemic tolerance observed in preconditioned hearts has been previously associated with a slower metabolic rate in these hearts during ischemia, which leads to preserved ATP stores and reduced catabolite accumulation after coronary occlusion. Both the extent and duration of depressed high-energy phosphate levels and intracellular acidosis are known determinants of myocardial ischemic injury, and the improvement of either could theoretically result in the protection seen in preconditioned hearts. By independently manipulating pH, and high-energy phosphate stores during ischemia, we have been able to separate one effect from the other. The use of brief cyanide infusions instead of short episodes of ischemia to condition myocardium was associated with less intracellular...
Acidosis and lower ATP levels during ischemia and resulted in improved recovery of the contractile function and energetic metabolism as compared with that seen in standard ischemia-preconditioned or control hearts. The substitution of the standard superfusion solution by one containing increased H⁺ concentration and increased CO₂ tension as well as the utilization of a buffer-free perfusate solution just before sustained ischemia significantly lowered myocardial pH at the end of 30 minutes of ischemia in hearts that had undergone both ischemic and cyanide preconditioning interventions, without affecting myocardial high-energy levels during ischemia. These resulted in significantly poorer metabolic and functional recoveries during reperfusion as compared with those present in the standard preconditioned hearts.

Intracellular acidosis is an important cause of ischemic contractile dysfunction and myocardial injury. Increased intracellular proton concentration can cause direct structural damage to cells. Additionally, intracellular acidosis is believed to be one of the main mechanisms for Ca²⁺ accumulation in myocardial tissue during ischemia and reperfusion. Acidosis enhances the release of Ca²⁺ from the sarcoplasmatic reticulum and can lead to accumulation of Na⁺ (via H⁺-Na⁺ exchange) and, therefore, Ca²⁺ (via Na⁺-Ca²⁺ exchange). Elevated Ca²⁺ levels are believed to have a role in the activation of several destructive mechanisms responsible for cell damage in these settings. Steenbergen et al reported that the decreased intracellular proton concentration observed with preconditioning is associated with less Na⁺ and Ca²⁺ accumulation during ischemia in 5F-BAPTA–loaded rat hearts. Recently, decreased intracellular acidosis was observed in preconditioned in vivo hearts and was related to diminished ultrastructural damage.

The exact mechanism by which preconditioning leads to decreased H⁺ accumulation during ischemia is still unclear. Theoretically, either increased buffering capacity of the myocardial cells or diminished production of protons could result in better preservation of cellular pH during ischemia. Little is known of how the buffering capacity is regulated in ischemic myocytes. One mechanism proposed to result in consumption of protons during ischemia is the hydrolysis of PCr. However, the importance of this as a “proton sink” is limited, since it is rapidly depleted during the first few minutes of ischemia. In the present study, PCr levels quickly fell during total global ischemia and were undetectable after 5 minutes of interrupted coronary flow. It is probable that preconditioning results in decreased proton production in ischemic tissues. The pathways of proton generation during ischemia have been reviewed recently. One proton-producing mechanism that could be affected by preconditioning is the turnover of glycolytically produced ATP. Less lactate accumulation and less glycogen breakdown during regional ischemia in preconditioned dogs suggest that preconditioning results in diminished glycolytic rates during ischemia.

Some investigators correlated the diminished infarct size caused by preconditioning with the amount of
glycogen depletion before the sustained ischemic insult and observed that the loss of these benefits was temporally associated with glycogen repletion in hearts allowed to recover after preconditioning.\textsuperscript{39} Indeed, some interventions, such as anoxic preperfusion, that, like preconditioning, result in glycogen depletion have been shown to be protective during later ischemia and reperfusion.\textsuperscript{40} However, Schneider and Taegtmeyer\textsuperscript{41} reported that the increase in glycogen stores caused by fasting results in improved rather than decreased recovery after ischemia in rat hearts. Furthermore, depletion of glycogen alone resulted in decreased acidosis during ischemia but did not improve recovery in isolated hearts.\textsuperscript{42} Therefore, the hypothesis that diminished glycogen stores per se limit glycolysis during later ischemia and/or afford protection does not explain all the experimental data available. Depletion of glycogen may be necessary but does not seem to be sufficient for the activation of the protection seen in preconditioned hearts during later ischemia and reperfusion. It is conceivable that the previous episodes of ischemia/anoxia/metabolic inhibition trigger some other mechanism important for the protective effect that is not present in glycogen depletion alone.

Although emphasis has been given to the preservation of high-energy phosphate levels of the cell in the genesis of the protection afforded by preconditioning, increased high-energy phosphate levels during ischemia are not consistently observed in preconditioned hearts,\textsuperscript{12,37,39} and when observed, they occur in a very restricted time window.\textsuperscript{15,16} Despite improved contractile and metabolic recovery, we did not observe increased high-energy phosphate levels during global ischemia in rat hearts preconditioned by two brief ischemic episodes. Moreover, the best contractile recovery was seen in the hearts preconditioned by metabolic inhibition alone, and they, paradoxically, demonstrated the lowest ATP levels during the first 20 minutes of ischemia (as shown in Fig 4B). \textsuperscript{31}P NMR spectroscopy techniques can provide reliable and accurate repetitive measurements of global myocardial high-energy phosphate levels. Despite the finding of unchanged or even diminished total high-energy phosphate levels in preconditioned hearts, we cannot exclude the possibility that the preconditioning interventions resulted in improved preservation of a hypothetical compartimentalized portion of the ATP pool responsible for the maintenance of some specific vital energy consuming reaction within the cell. Nevertheless, recovery of function at reperfusion correlated with diminished intracellular proton concentration but not with total ATP levels during ischemia. Therefore, we conclude that the protection afforded by preconditioning in this model is not mediated by improved preservation of global myocardial high-energy phosphate levels.

There is conflicting evidence regarding the effect of ischemic preconditioning on myocardial stunning, the prolonged postischemic contractile dysfunction observed in viable myocardium. Some investigators reported improved systolic function in stunned viable myocardium after ischemia in preconditioned dogs,\textsuperscript{7} whereas others did not.\textsuperscript{43} The reason for the disparity of these findings is still unclear. We report increased contractile recovery on reperfusion in preconditioned hearts submitted to 30 minutes of normothermic ischemia. Since the protocol we used required 30 minutes of total ischemia, which is enough to cause cell death and considerable necrosis of rat myocardial tissue,\textsuperscript{44} we cannot distinguish whether the improved contractile function at reperfusion observed in this model is the result of decreased myocardial necrosis alone, improved contractile recovery of stunned viable tissue, or a combination of both effects.

Myocardial ischemia is known to impair not only the systolic but also diastolic cardiac properties.\textsuperscript{45} Diastolic dysfunction can lead to significant pathophysiological consequences during myocardial ischemia and reperfusion and is of recognized clinical importance in patients with ischemic heart disease.\textsuperscript{46} Preconditioned hearts demonstrated remarkably better recovery of LV diastolic function, as reflected by significantly lower diastolic pressures during reflow. Postischemic diastolic dysfunction has been associated with increased Ca\textsuperscript{2+} levels during reperfusion\textsuperscript{32}; therefore, it is probable that lower diastolic pressures after reperfusion are related to diminished cellular Ca\textsuperscript{2+} accumulation in preconditioned hearts, as previously reported.\textsuperscript{37}

Others have also reported that preconditioning is associated with improved contractile recovery despite the earlier development of ischemic contracture.\textsuperscript{37} We observed that ischemic contracture occurred significantly sooner in hearts previously subjected to cyanide exposure, which also exhibited the most rapid decline in ATP levels during ischemia. Therefore, to the extent that Ca\textsuperscript{2+} gain during the ischemic period is mediated by the level of intracellular acidosis at that time, our findings agree with those of Koretsune and Marban,\textsuperscript{48} which suggested that ischemic contracture is temporally associated with diminished ATP levels rather than with Ca\textsuperscript{2+} accumulation during ischemia, as previously suggested.\textsuperscript{49} Ischemic contracture, however, was not associated with decreased postischemic contractile or metabolic recovery in this model, and that casts some doubt on the notion that contracture is a pathognomonic sign of the formation of permanent myofilament crossbridges and loss of myocardial viability.

The preconditioning protective effect, as reported so far, has been triggered by brief coronary occlusion-reperfusion cycles and, therefore, in the presence of ischemia and reoxygenation phenomena. We report in the present study that rat myocardium can be effectively protected from ischemic injury by transient metabolic inhibition alone, caused by cyanide infusion, and therefore that this phenomenon can occur in the absence of interrupted coronary flow. The application of cyanide preconditioning to cellular and subcellular experimental models that do not support true ischemia and reperfusion conditions may permit more basic studies of preconditioning phenomena in the future.

There is now clear evidence that the diminished cellular accumulation of protons during ischemia is a prime mechanism responsible for the cardiac protection afforded by preconditioning. Not only have we observed that the preservation of pH\textsubscript{i} during ischemia in preconditioned hearts is associated with improved recovery of contractile function and energetic metabolism but also that the manipulation of the intracellular concentration of protons during ischemia can alter the protection...
induced by preconditioning. We believe that the preservation of global myocardial energy stores during ischemia is not responsible for the protection observed in preconditioned hearts and that these findings provide a strong argument in favor of a causal relation between the decreased intracellular accumulation of protons during ischemia and the improved performance seen in preconditioned hearts during reperfusion.

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Importance of metabolic inhibition and cellular pH in mediating preconditioning contractile and metabolic effects in rat hearts.
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