Post-conditioning Preserves Glycolytic ATP During Early Reperfusion: A survival Mechanism for the Reperfused Heart

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Key Words

Post-conditioning • Iodoacetate • Glycolysis • Reperfused heart • ATP

Abstract

Background: Glycolytic activity during the transition period from anaerobic to aerobic metabolism has been demonstrated to be critical for heart recovery in isolated reperfused hearts. The purpose of this work was to investigate the relevance of the glycolytic pathway in preserving the cardiac function of post-conditioned hearts. **Methods:** The activation of the glycolytic pathway in post-conditioned hearts was evaluated by measuring GLUT-4 insertion, glucose consumption and lactate production. Iodoacetic acid and 2-deoxy-D-glucose were administered to the working hearts to evaluate the effect of glycolytic inhibition in the post-conditioning protective effect. **Results:** Post-conditioning maneuvers applied to isolated rat hearts, after prolonged ischemia and before reperfusion, promoted recovery of cardiac mechanical function with sustained increase of GLUT-4 translocation and activation of the glycolytic pathway during ischemia and early reperfusion. Iodoacetate

inhibited the protective effect of post-conditioning, without affecting the mitochondrial oxidative capacity. Glycolysis contribution to maintain mechanical function at early reperfusion was observed in post-conditioned hearts perfused with 2-deoxy-D-glucose and in hearts in which iodoacetate was administered only during reperfusion. **Conclusion:** It is concluded that in the post-conditioned heart, a functional compartmentation of anaerobic energy metabolism, at early reperfusion, plays a significant role in cardiac protection against reperfusion damage.

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Introduction

Paradoxically, although rapid initiation of reperfusion is the most effective treatment to reduce infarct size resulting from myocardial ischemia, it has the potential to induce additional lethal injury that is not evident at the end of the ischemia. This process is known as reperfusion injury [1-3].

Reperfusion injury is the consequence of numerous mechanisms activated in the intracellular and extracellular

environment. Owing to its diverse and numerous causes, reperfusion injury is expressed physiologically in equally diverse ways, including endothelial and vascular dysfunction, sequels of impaired blood flow, metabolic dysfunction, contractile dysfunction, dysrhythmia, cellular necrosis, and apoptosis. Several strategies have been developed to contend with reperfusion injury. Zhao et al. 2003 [4] reported that brief periods of ischemia-reperfusion, applied at the onset of the coronary reflow, significantly reduce the infarct size. This mechanical maneuver is known as “post-conditioning” [4]. Elucidation of the molecular mechanisms activated by “post-conditioning” is the subject of intensive research in several laboratories. Some studies indicate that post-conditioning cardioprotection is dependent on protein kinase C (PKC) [5], $\text{mitoK}_{\text{ATP}}$ opening [6] and myocardial acidosis [7].

It has been reported that glycolytic activity during the transition period from anaerobic to aerobic metabolism is critical for myocardial recovery in isolated reperfused hearts after 20 min of global ischemia [8]. There are also evidences indicating that heart recovery after ischemia and short reperfusion has improved in conditions of higher availability of glycolytic substrates [9, 10]. Further, it has been suggested that glycolytic ATP could be relevant in maintaining calcium homeostasis during reperfusion [11, 12]. Thus, the purpose of this work was to investigate the role of glycolysis in the cardioprotection exerted by post-conditioning in isolated reperfused rat hearts.

Materials and Methods

This investigation was performed in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (US-NIH).

Male Wistar rats (400-450 g) were anesthetized with sodium pentobarbital (60 mg/kg) and sodium heparine (1000 U/kg). Five minutes after heparin injection, a midsternal thoracotomy was performed and the heart was rapidly excised and placed in ice-cold Krebs-Henseleit buffer solution of pH 7.4, consisting of 118 mM NaCl, 4.75 mM KCl, 1.18 mM KH_2PO_4 , 1.18 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 mM CaCl_2 , 25 mM NaHCO_3 , supplemented with 5 mM glucose, and 100 μM sodium octanoate unless otherwise indicated. The heart was mounted onto a Langendorff heart perfusion system and perfused retrogradely, via the aorta at a constant flux of 12 ml/min with Krebs-Henseleit solution, which was continuously bubbled with 95% O_2 and 5% CO_2 at 37°C. Mechanical work was measured at left ventricular end-diastolic pressure of 10 mm Hg using a latex balloon inserted into the left ventricle and connected to a pressure transducer. All variables were recorded using a computer acquisition data system designed by the Instrumentation and Technical Development Department of the

National Institute of Cardiology (México, D.F., México).

Krebs-Henseleit buffer was perfused for 20 min to stabilize the heart. The ischemic and reperfused hearts were subjected to global ischemia for 30 min by turning off the pumping system. The post-conditioning maneuver consisted of five cycles of ischemia-reperfusion, 30 seconds of ischemia and 30 seconds reperfusion per cycle (PC), followed by 60 min of reperfusion ($\text{I/R}_{60} + \text{PC}$). Hearts without post-conditioning were reperfused for additional 60 min after ischemia (I/R_{60}).

Hearts that developed arrhythmia before ischemia were discarded and replaced. Thus, all analyses represent hearts that did not show electrical dysfunction before ischemia.

GLUT-4 immunoblot analysis

At the end of different treatments (Figure 2A), the rat hearts were quickly frozen and stored in liquid nitrogen. Cardiac tissue was powdered with a pre-chilled pestle in a frozen mortar and dissolved in an ice-cold buffer of pH 7.4 containing 0.2 M Tris-HCl, 10 mM EDTA, 0.25 M sucrose, 0.2 mg/ml benzamidine, 10 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, and 1 mM sodium orthovanadate. The homogenates were centrifuged at 8700 x g for 20 min. The pellet containing sarcolemma membranes was resuspended in the buffer, homogenized again, and centrifuged at 750 x g for 10 min and then at 270 x g for 10 min each. The resulting supernatants from the two centrifugations were combined and centrifuged for 30 min at 10 000 x g, and the resulting supernatant was centrifuged again at 48 000 x g for 60 min. GLUT-4 insertion was measured by western blot technique using a primary polyclonal anti-GLUT-4 antibody (1:2,000) (Sigma-Aldrich, MO) and a detection system of enhanced chemiluminescence. Control loading was achieved by incubating the membranes against anti- $\text{Na}^+/\text{K}^+ \text{ATP}_{\text{ase}}$ (Santa Cruz, CA) polyclonal antibodies.

Glucose consumption and lactate production

Glucose utilization was measured as the formation of [^3H] water during perfusion using [$5\text{-}^3\text{H}$] glucose as substrate as described by Liang et al [13]. Briefly, the hearts were stabilized for 10 minutes in Krebs-Henseleit buffer of pH 7.4, before 5 mM glucose (1 $\mu\text{Ci}/\text{ml}$) was perfused for additional 20 minutes. Then the hearts were subjected to global ischemia for 30 min and to the post-conditioning maneuver, followed by 60 min of reperfusion with the [^3H] Krebs-Henseleit Buffer. At 10-min time intervals, 500 μl of perfusate were collected in Eppendorf tubes and mixed with 25 μl of 0.6 N HCl, then they were placed inside 20-ml scintillation capped-vials containing 2 ml of distilled H_2O . The formed [^3H] water was allowed to diffuse for 72 hours at 37°C, then the inside tube was removed and 10 ml of scintillation liquid cocktail was added for counting. Diffusion efficiency was calculated using a known amount of tritiated water.

Lactate content was measured as an indicator of glycolysis activation. Acid extracts obtained from heart eluates were neutralized with KOH, and 0.2 ml of each extract was incubated in a medium of pH 9.0 containing 0.4 M hydrazine, 0.5 M glycine, 10 mM NAD^+ . Lactate dehydrogenase activity was spectrophotometrically evaluated as NADH production at 340

Fig. 1. Post-conditioning protects myocardial performance against reperfusion injury. After a 20-min equilibration time, the hearts were subjected to global ischemia for 30 min. Then the perfusion pump system was restarted and the hearts were reperfused for 60 min. Post-conditioned hearts were subjected to five cycles of ischemia-reperfusion, each of 30 s of ischemia and 30 s of reperfusion, before prolonged ischemia and reperfusion. (■) Control; (▲) I/R₆₀ and (●) I/R₆₀ + PC. Data represent the mean of 14 experiments ± S.D. * $P \leq 0.05$ vs Control.

nm. NADH content was calculated according to a reference curve. Lactate content is expressed as $\mu\text{mol/ml}$.

Hexokinase activity

A coupled hexokinase/glucose-6-phosphate dehydrogenase (G6PDH) reaction was used to evaluate hexokinase activity in myocardial extracts. NADPH production was detected spectrophotometrically ($\epsilon = 6.22 \text{ mM}^{-1}$), in 1 mL KME buffer (100mM KCl, 50 mM MOPS, 0.5 mM EGTA, pH 7.0) plus 2U G6PDH, 1 mM NADP⁺, 15 mM MgCl₂, glucose and 0.02-0.1 mg of myocardial cytoplasm. The reaction was started with 3mM glucose and initial velocity was calculated as $\text{mU/mg} = \text{nmol/min} \cdot \text{mg}$. Also, tissue samples were mixed with RIPA Buffer containing the anionic detergent IGEPAL (0.5%) and a mixture of protease inhibitors. Samples containing 20 μg of protein and SDS-PAGE loading buffer with 5% β -mercaptoethanol were boiled during 5 minutes and loaded onto 12.5% polyacrilamide gels. Electrophoresis to PVDF membranes (Millipore, MA) was followed by immunoblotting with anti-HXK (1:1 000) and anti- α Tubulin (1:500) (Santa Cruz, CA) polyclonal antibodies. Respective secondary antibody (1:2 000) conjugated with peroxidase (Santa Cruz, CA) was added and the signal was detected by chemiluminescence using the ECL-Plus Detection System (Amersham Bioscience, NJ).

Glycolytic inhibition

To evaluate the effect of glycolytic inhibition in the post-conditioning protective effect, 100 μM of iodoacetic acid (IAA) was perfused to the hearts for 20 min prior to the ischemic period. Another group of hearts was perfused with pyruvate and 5 mM 2-deoxy-D-glucose for 20 min before the ischemia. The hearts were then subjected to post-conditioning and to prolonged reperfusion without the compound. As inhibition of glycolysis before induction of ischemia may compromise recovery by enhancement of myocardial injury occurring during

ischemia, rather than during reperfusion, a group of hearts was perfused after ischemia and after the post-conditioning cycles with 100 μM IAA or with 5 mM 2-deoxy-D-glucose.

Measurement of ATP and phosphocreatine content

Cardiac tissue was extracted with perchloric acid and was neutralized with 3M TRIS-KOH to pH 7.3. ATP content was measured fluorometrically as NAD⁺ reduction in a medium of pH 7.4, containing 50 mM TRIS, 10 mM MgCl₂, 5 mM EDTA, 50 U/ml hexokinase, 50 U/ml glucose 6-phosphatase, and 0.5 ml of neutralized extracts ($\lambda_{\text{ex}} = 340 \text{ nm}$ and $\lambda_{\text{em}} = 460 \text{ nm}$). NADH content was calculated according to a reference curve as described by Wanders et al.[14]. Phosphocreatine content was also determined in neutralized acid extracts from cardiac tissue by using 19 U/ml creatine kinase, 10 μM ADP, and 2.4 μM N-acetyl-cysteine, according to Bergmeyer [15].

Data analysis

Data are presented as means $\pm$ SD for each experimental protocol. Significance ($P \leq 0.05$) was determined for discrete variables by analysis of variance (ANOVA), using the data analysis and technical program Microcal™ Origin™ from Microcal Software, Inc. (1999).

Results

Cardiac function

Cardiac mechanical work of hearts, subjected to 30 min of ischemia and 60 min of reperfusion (I/R₆₀), decreased dramatically from the first few minutes of reperfusion, compared to the hearts subjected to post-conditioning maneuvers (I/R₆₀ + PC) (Figure 1). After reperfusion from minute 20 to minute 60, there was no

Fig. 2. Post-conditioning effect on GLUT-4 insertion into the plasmatic membranes of reperfused hearts. The content of GLUT-4 was evaluated by western blot. Approximately 100 μ g of protein was loaded into each lane of the SDS-PAGE gels and was transferred to PVDF membranes. The blots were incubated with polyclonal anti-GLUT-4 antibodies (1:2000) in TRIS-buffered saline (TBS) of pH 7.0, supplemented with 0.1% Tween 20. Then, the membranes were incubated with a biotin-conjugated secondary antibody and with streptavidin-peroxidase before detecting enhanced chemiluminescence. The gray bar represents the mean of the intensity of the signal obtained in post-conditioned hearts before reperfusion. Data represent the mean $\pm$ S.D of four different experiments. * $P \leq 0.05$ vs Control.

significant difference between the mechanical works of I/R₆₀ + PC hearts and control hearts.

GLUT-4 translocation to the sarcolemma membranes is maintained during post-conditioning

To determine whether increased flux of glucose could be relevant to the recovery of ischemic myocardium in post-conditioned hearts, GLUT-4 insertion levels in the sarcolemmal fraction of those hearts were measured. As expected, ischemia increased the amount of GLUT-4 in the sarcolemmal fraction. The activation of the glycolytic pathway, in terms of increased glucose uptake, represents the metabolic response of a jeopardized myocardium to meet the energy demand. Interestingly, the hearts that were subjected to post-conditioning before prolonged reperfusion (PC) showed similar GLUT-4 content than those that experienced ischemia and it show a tendency to return to basal levels only after prolonged reperfusion (I/R₆₀ + PC). Conversely, the hearts that were reperfused for 60 min (I/R₆₀) without post-conditioning treatment showed a dramatic decrease of GLUT-4 compared to that observed in control hearts (Figure 2B).

Glucose metabolism is involved in heart myocardial recovery after post-conditioning

To determine the contribution of the activation of the glycolytic pathway in post-conditioning myocardial protection, we perfused hearts with IAA, which at low

doses produces a selective inhibition of GADPH [16]. 5 mM of pyruvate was used as substrate for oxidative metabolism, instead of glucose in glycolysis inhibition experiments, because it was reported that the buildup of glycolytic intermediates (sugar phosphates), following maneuvers that inhibit glycolysis, can negatively affect high-energy phosphate levels and thereby cardiac function [17, 18]. In these experiments, IAA addition would block usage of potential glycogen stores available during ischemia. In this respect it has also been described that pyruvate inhibits glucose utilization [19]. Blockage of glycolysis severely impaired the functional recovery of post-conditioned hearts at early reperfusion - e.g., 100 mm Hg x beat/min (I/R₆₀ + PC + IAA) vs 20 000 mm Hg x beat/min (I/R₆₀ + PC) - and remained so at those values after 1 h. Post-conditioned hearts, using pyruvate as substrate, recovered their post-ischemic function to control levels, after the first 10 min of reperfusion. Control hearts perfused with IAA showed similar function as that shown by control hearts without any treatment, confirming that at the doses used, IAA does not inhibit mitochondrial oxidative capacity (Figure 3). To evaluate if glycolysis remained activated during early reperfusion in the post-conditioned hearts and if it contributed to maintain mechanical function, 100 μ M IAA was added to the reperfusion solution along with pyruvate as oxidative substrate after ischemia and after the post-conditioning cycles. IAA perfusion was maintained throughout the reperfusion period. Under such conditions, post-

Fig. 3. Effect of glycolysis inhibition on the mechanical performance of post-conditioned hearts. The hearts were subjected to the protocols described in Figure 1. Additionally, some hearts were perfused, prior to ischemia, with 100 μ M of iodoacetic acid for 20 min, and then subjected to post-conditioning and to prolonged reperfusion without the compound. Control hearts $\pm$ IAA ($\blacksquare$); I/R₆₀ + PC ($\bullet$); I/R₆₀ ($\blacktriangle$); I/R₆₀ + PC + IAA ($\blacktriangledown$). Data represent the mean $\pm$ SD of three different experiments. *P $\leq$ 0.05 vs Control.

Fig. 4. Effect of glycolytic inhibition on the mechanical function in post-conditioned hearts. Iodoacetic acid (IAA) or 2-deoxy-D-glucose (2-DG) was perfused into post-conditioned hearts only during reperfusion. Control hearts with and without 2-DG ($\blacksquare$); I/R₆₀ + PC ($\bullet$); I/R₆₀ + PC + IAA ($\blacktriangledown$) and I/R₆₀ + PC + 2-DG ($\blacklozenge$). Data represent the mean $\pm$ SD of three different experiments. *P $\leq$ 0.05 vs Control.

conditioning partially protected heart function (Figure 4). After 10 min of reperfusion, the recovery in the mechanical function of IAA-treated hearts was only 20% of that observed in post-conditioned hearts without the glycolytic inhibitor. An increase in heart function was observed after longer reperfusion, although maximum recovery was only 60% of the functional recovery in post-conditioned hearts without IAA. Another group of hearts received pyruvate and 5 mM of 2-deoxy-D-glucose instead of glucose, and then were subjected to ischemia and to the post-conditioning protocol before reperfusion. Post-ischemic function in these hearts was severely compromised as in the hearts perfused with IAA. Conversely, mechanical work in control hearts perfused with these substrates remained unchanged along all the

protocol (Figures 3 and 4).

Glucose utilization and lactate production

Glucose utilization was determined as the formation of tritiated water from [5-³H] glucose in cardiac effluents. Glucose utilization was greatly enhanced during the first three minutes of reperfusion and it remained significantly greater (P $\leq$ 0.05) during the reperfusion phase in the post-conditioned hearts (Figure 5). We also measured lactate in the eluates of the isolated hearts at the indicated times. Lactate production was almost the same before ischemia in post-conditioned hearts and in hearts without post-conditioning (Figure 6). During the first minutes of reperfusion, an important increase in lactate production was observed in I/R₆₀ hearts that diminished after 10

Fig. 5. Glycolysis measurement in post-conditioned hearts. Production of tritiated water from [5-³H] glucose. Control hearts (■); I/R₆₀ (▲); I/R₆₀ + PC (●). Each value is the mean ± SD of three different experiments in duplicate. *P ≤ 0.05 vs control.

Fig. 6. Lactate content in post-conditioned hearts. Lactate content in eluates from post-conditioned hearts was determined as described under “Materials and Methods”. In the insert is shown lactate efflux during the post-conditioning cycles. I/R₆₀ (▲); I/R₆₀ + PC (●). Data represent the mean ± S.E. of three different experiments. * P ≤ 0.05 vs Control.

minutes of reperfusion, and remained significantly lower than lactate content in post-conditioned hearts ($P \leq 0.05$). Conversely, in the post-conditioned hearts, lactate was slowly washed out from the myocardium during the post-conditioning cycles. As a consequence, at the first minute of long reperfusion, lactate efflux in I/R hearts was 4.3 higher than that in post-conditioned hearts ($3.9 \pm 0.5 \mu\text{mol/ml}$ vs $0.9 \pm 0.2 \mu\text{mol/ml}$).

Hexokinase activity in post-conditioned hearts

As hexokinase has been pointed out as a control point in glycolytic flux in the working heart, its activity was measured in hearts subjected to post-conditioning. A remarkable increment in hexokinase activity was determined only during ischemic conditions, but it dropped during reperfusion in both post-conditioned and in non post-conditioned hearts. Accordingly, the enzyme content

Fig. 7. Hexokinase activity in post-conditioned hearts. A) Activity was determined in cytoplasmic fractions by using a coupled hexokinase/glucose-6-phosphate dehydrogenase reaction. Data represent the mean of two different preparations in duplicate. B) Hexokinase content in the same fractions was evaluated by western blot and compared with tubulin content.

in the hearts subjected to the different protocols showed no differences, except in the ischemic hearts (Figure 7).

ATP and phosphocreatine content in post-conditioned hearts

Table 1 shows that post-conditioned hearts, as compared to control hearts, preserved ATP content and phosphocreatine levels up, to after 60 min of reperfusion.

When IAA was administrated during reperfusion a significantly diminution in ATP was observed, sustained the idea that glycolysis remained activated during reperfusion. Similarly, it was observed that ATP and phosphocreatine contents were depleted after reperfusion at lower values than those detected after 30 min of ischemia. That ATP derived from glycolysis is paramount for heart recovery is demonstrated in Figure 3. In such conditions, pyruvate-oxidizing hearts, perfused with the glycolytic inhibitor IAA and subjected to post-conditioning after ischemia-reperfusion, did not recover heart function and showed ATP values close to ischemic-reperfused

nmol/mg protein	Control	Ischemia	I/R ₆₀	I/R ₆₀ +PC	I/R ₆₀ +PC+IAA
ATP	19.6±2.2	10.6±1.4*	5.4±1.6*	17.6±2.4	9.2±1.6
PCr	27.3±2.5	8.3±2.8*	2.3±0.3*	22.1±4.7	n.e

Table 1. Cytosolic ATP and phosphocreatine content in post-conditioned hearts. High energy phosphates were measured as described under “Materials and Methods”. Data represent the mean ± S.D of three different experiments. * $P \leq 0.05$ vs Control. n.e = no evaluated.

hearts (9.2 ± 1.6 nmol ATP/mg protein vs 5.4 ± 1.6 nmol ATP/mg protein). As mentioned before, control hearts perfused with IAA or 2-deoxy-D-glucose along with pyruvate, showed similar function as that shown by control hearts without any treatment, discarding and inhibitory effect of these compounds on mitochondrial oxidative capacity.

Discussion

In the normal heart, the large amount of ATP necessary to sustain contractile function and basal metabolism is generated primarily by mitochondrial oxidative metabolism. The ability of mitochondria to sustain ATP synthesis is highly compromised during ischemia and in such a situation, anaerobic glycolysis becomes an important source for ATP production. It is known that under low oxygen availability, mammalian cells activate adaptation responses, including transcriptional activation of several hypoxia-inducible genes: erythropoietin, glycolytic enzymes, and glucose transporters (GLUT). As a result, glycolysis becomes the major source of ATP production instead of oxidative phosphorylation. Therefore, the aim of this study was to investigate the relevance of the glycolytic pathway for preserving cardiac function in the post-conditioned heart. The results obtained here showed an increase in the insertion of GLUT-4 during ischemia, which remained so up to early reperfusion and showed a tendency to return to control values in post-conditioned hearts only after 60 min of reperfusion (Fig. 2). This is indicative of an enhancement of glycolytic flux by post-conditioning during ischemia and early reperfusion. Previous reports indicate that enhanced glucose uptake and augmented insertion of GLUT-4 transporter, mediated by p38 MAP-kinase, occur due to brief episodes of ischemia before prolonged

ischemia-reperfusion (pre-conditioning) [20]. Also, pre-conditioning increases lactate production and glucose uptake during low flow ischemia [21] and facilitates glycolysis in an early period of reperfusion in the rat heart [22].

A higher rate of glucose oxidation in the post-conditioned hearts may reflect an increase of viable myocytes as compared to I/R hearts. In this respect, our experiments showed that glucose consumption was significantly increased during the early reperfusion phase in the post-conditioned hearts (Figure 5). This result is comparable to that obtained in isolated hearts of transgenic mice over-expressing hexokinase B, in which a sustained increase in glycolysis and lactate production was maintained during the reperfusion phase [13]; however, we did not observe and increase in hexokinase activity in the post-conditioned hearts.

Our results showed that ATP content after the post-conditioning maneuver and before long reperfusion, significantly increased as compared to reperfused hearts without post-conditioning. The relevance of glycolytic ATP in heart recovery after reperfusion has been demonstrated by several groups [8]. For example, Wang et al. 2005 showed that perfusion with high glucose/high insulin improves cardiac efficiency in the isolated reperfused heart after low-flow ischemia [23]. Cross et al. 1995 demonstrated that the utilization of extra glycolytic substrate, either during ischemia or during early reperfusion, enhances functional heart recovery [24]. Accordingly, we showed here that perfusion of IAA or 2-deoxy-D-glucose inhibited the protective effect of post-conditioning (Fig. 3). These data are comparable to Jeremy's findings that the inhibition of glycolysis during reperfusion impairs heart recovery and induces a marked rise in left ventricular end-diastolic pressure, indicating that glycolysis plays a crucial role during functional and metabolic recovery of the post-ischemic myocardium [8]. However, addition of IAA prior to ischemia, would likely affect heart function during ischemia as well as during reperfusion, if less ATP was produced from anaerobic glycolysis. Indeed, our results showed that the ATP produced during prolonged ischemia contributed to sustain 60 % of control heart function at the end of reperfusion (Figure 4). Certainly, IAA is not a specific glycolytic inhibitor, but several reports indicate that a low concentration of IAA, as the one used here, neither inhibits other enzymes, such hexokinase, phosphofructokinase, pyruvate kinase [25], succinate dehydrogenase [26], nor does it inhibit mitochondrial oxidative capacity from reperfused hearts even at higher doses (e.g., 0.3 mM

IAA) [27]. In this sense, we suggested that glycolysis also contributed to maintain the mechanical function at early reperfusion in the post-conditioned hearts, as the protective effect of the maneuver was significantly inhibited in hearts in which iodoacetate was administered only during reperfusion.

However, as previous works have associated iodoacetate-inhibition of anaerobic glycolysis with marked contracture-rigor, it was possible that IAA-poisoned hearts would result in cell death, hindering the inhibition of post-conditioning protection. It is worth noting that, in canine hearts treated with IAA, depletion of ATP was observed very early at ischemia without lactate accumulation or obvious ultra structural evidence of lethal injury. This suggests that totally ischemic myocytes tolerate a very low level of high energy phosphates if lactate is not accumulated [28]. Consistent with this concept is the reduction in contracture associated with increased lactate efflux in reperfused hearts, in which the use of myocardial carbohydrate was stimulated [29]. The results obtained here show that lactate clears up within the short cycles of post-conditioning, avoiding the deleterious effect of concomitant acidosis, but glycolysis remained activated, providing a complementary source of ATP. In this respect, a speculative explanation could be that hexokinase-mitochondrial interaction during post-conditioning could have a non-enzymatic role in cardioprotection. Binding to hexokinase to the mitochondrial permeability transition pore has been proposed to provide a link between glucose phosphorylation and mitochondrial oxidative phosphorylation, thereby preventing lactate accumulation [30]. The continuous glycolytic ATP production could be used to maintain Na^+/K^+ ATPase activity, preventing the injury caused by Na^+ accumulation via Na^+/H^+ exchange. This has been suggested to occur in models of lactate-induced damage, in which reduction of glycolytic flux during low-flow ischemia leads to inhibition of Na^+/K^+ ATPase activity, increased intracellular Na^+ , and decreased post-ischemic functional recovery [31]. Also, preferential fueling of Na^+-K^+ -ATPase from glycolytically derived ATP has been suggested to sustain normal Na^+ homeostasis in the perfused rat heart [16]. Besides, the prevailing notion is that glucose-derived ATP is directed toward ion transport processes [8]. Calcium transport in sarcoplasmic reticulum (SR) membranes was demonstrated to depend on glycolytic ATP [22] and recently Zima et al. reported an important functional role for glycolysis in intracellular calcium regulation, in terms of the close proximity of the glycolytic enzymes and the Ca^{2+} transport systems of the

sarcoplasmic reticulum [32]. They showed that sugar phosphates, metabolized during glycolysis, modulate local sarcoplasmic reticulum (SR) Ca^{2+} release in cat ventricular myocytes. The effect of sugar phosphates on SR Ca^{2+} release was mediated by altering the activity of two major Ca^{2+} transport systems of the SR, the RyR, and the SR Ca^{2+} -ATPase. Other workers reported that accumulation of sugar phosphates due to inhibition of glycolysis caused intracellular Ca^{2+} overload [17]. The authors' data, considered in the light of the foregoing observations, support the notion that in the post-conditioned heart, a functional compartmentation of energy

metabolism could play a significant role in cardiac protection against reperfusion damage.

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