Conservation of phosphorylation state of cardiac phosphofructokinase during in vitro hypothermic hypoxia

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Phosphofructokinase (PFK) throughout prolonged hypothermic hypoxia in porcine hearts. Hearts from 35 to 40 kg pigs were flushed with one of the following five solutions: St. Thomas’ Hospital solution (STHS); modified University of Wisconsin (UW) solution; and three solutions containing modified UW plus 90 mM of histidine, bicine, or BES. The hearts were then stored at 4°C for 10 h. After 10 h of hypoxic hypoxia, lactate values were 6.7–12.9 μmol/g higher than control; this reflected an increase in anaerobic end product of 35–67%. The consequences of enhanced anaerobic metabolism were higher ATP, total adenylate, Energy Charge, and ATP/ADP ratios in most of the buffered groups after 4–10 h cold storage; effectiveness of the buffers employed correlated with buffering capacity (BES proved to be the most effective). PFK remained activated throughout most of the 10-h period in hearts stored with buffers and did not undergo the rapid inactivation experienced by hearts stored in STHS. Conservation of PFK integrity with buffering agents was not related to a pH-mediated event; changes in kinetic parameters suggested that this protection was due to an irreversible posttranslational modification, specifically a dephosphorylation event.

Tissue energy levels during the preservation period are a net result of the delicate balance between energy production by the organ and energy utilization. Strategies to improve energetics can take the form of increasing ATP synthesis or decreasing its consumption. In clinical practice, both of these approaches are feasible and can be used together in a complementary manner. Because all enzymatic reactions within the cell are temperature dependent, cooling the organ slows down cellular metabolism resulting in less ATP consumption by these reactions. Typically, biological reactions decrease twofold for every 10°C temperature decline (12); hence the metabolic rate of a flushed heart will drop to approximately one-tenth of in vivo levels during cold storage. However, decreasing the rate of metabolism via hypothermic exposure is not sufficient to maintain myocardial viability during storage, tissue energy levels must be conserved. Stringham and colleagues (16) showed that ischemic myocardial contracture correlated with a decrease in cellular ATP to less than 80% of preischemic values. In addition, high levels of ATP are needed by the heart in the early reperfusion period in order for excitation-contraction coupling to function effectively. Therefore, for the heart to sustain the patient’s circulation upon reimplantation, the myocardium must either have an immediate supply of ATP or the means to quickly regenerate ATP.

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storage must be a primary target when attempting to develop novel preservation solutions.

Present methods of cardiac transplantation require a period of cold ischemia lasting from the time the heart is removed from the donor, until perfusion is reestablished in the recipient. During this time, the only available source of energy needed by the cardiomyocyte to maintain viability is through anaerobic glycolysis. However, energy production through glycolytic flux is limited; various potential mechanisms include lactate transport out of the cell, substrate availability, cold lability of enzymes, the accumulation of metabolic inhibitors (ammonium and citrate ions), and the accumulation of protons under anaerobic conditions. It has been shown that enzymes controlling flux through the glycolytic pathway, particularly phosphofructokinase (PFK), are reversibly inhibited by the pH decline that occurs during ischemia (7, 10). However, Churchill and Kneteman (3) found in other tissues that buffering agents can influence the apparent phosphorylation status of such regulatory enzymes during ex vivo storage. In the present study, we hypothesized that increasing the buffering capacity of preservation solutions would enhance anaerobic energy production by relieving the pH inhibition of key enzymes required for continued metabolism through the glycolytic pathway and by maintaining the phosphorylation state of PFK.

MATERIALS AND METHODS

Biochemicals and animals. All chemicals were Analytic Reagent grade and were purchased from Sigma or BDH. The cardiac donors were 35–40 kg adult Landrace-Yorkshire pigs from the University of Alberta swine farm. All pigs were treated humanely in accordance with the Animal Protection Act regulations set forth by the Canadian Council on Animal Care. The experiments were approved by the University Health Sciences Animal Welfare Committee.

Experimental groups. The pigs were assigned to five experimental groups with n = 4–6 for each group. The groups differed only in the solution used to flush and store the hearts with other factors being held constant. STHS II served as the control group because of its limited buffering capacity. The remaining three groups used solutions composed of UW (BES), UW-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES), and were also snap frozen in liquid nitrogen by using Wollenberger clamps. All samples were stored at −65°C until it was time to process them.

Sample preparation and metabolite assays. Tissue samples were homogenized in 6% perchloric acid (containing 1 mM EDTA); a 50-μl volume of the well-mixed homogenate was then removed and added to 100 mM Na-acetate for subsequent determination of glycogen contents. Precipitated protein in the remaining homogenate was removed by centrifugation (15 min at 20,000 g). The protein pellet remaining was used for determination of wet-to-dry weight ratios. The acid extracts were neutralized with 3 M KOH-0.4 M Tris-0.3 M KCl and recentrifuged (15 min at 20,000 g). The neutralized extracts were used immediately for assays of phosphocreatine (PCr), ATP, ADP, and AMP. Extraction procedure and metabolite assays were conducted as described previously (8, 11). Metabolites were assayed enzymatically based on the absorbance of NADH at 340 nm.

Enzyme assays. Tissue samples (50–100 mg) were homogenized in buffer (in mM: 50 imidazole-HCl, pH 7.2, 30 mercafortoeholn, 5 EDTA, 5 EGTA, and 100 NaF) in the presence of a few crystals of phenylmethylsulfonyl fluoride (~1 mM). Phosphorylase assays were performed as described previously (11) based on a spectrophotometric assay with/without the addition of physiological adenylate (ATP, ADP, AMP) levels at each respective time point. PFK was assayed as previously described (11) with the addition of physiological adenylate levels and by maintaining fructose 6-phosphate levels at a constant 10 mM and varying ATP levels. The kinetic parameter determination for Michaelis-Menten constant (K_vmax)/ATP and maximal velocity (V_max) was based on the Michaelis-Menten equation for reaction kinetics.

Statistics. Data were reported as means ± SE; n = 4–6. Metabolite levels were reported in terms of micromole per gram wet weight. Changes in levels were assessed at each time point compared with the control group using an ANOVA followed by Dunnett’s post hoc comparison test; P < 0.05 was reported.

RESULTS

Tissue water contents revealed no significant changes over the 10-h experimental time-course within or between groups; water contents of all samples pooled together averaged 64.7 ± 0.07%. Glycogen levels in hearts at t = 0 ranged from 36–44 μmol/g wet wt (measured as glucosyl units). After 10 h, levels had dropped significantly by 14–22
μmol/g in all groups; although levels in all groups significantly dropped throughout the 10-h time course, no statistical differences in net glycogen decline were detected.

Buffering capacities of experimental solutions. Buffering capacities of the preservation solutions used in this study were examined between pH 7.4 and 6.2 and ranged from 4.5 mmol H⁺/l (STHS) to 66.0 mmol H⁺/l (BES). In relative terms, buffering capacity for the STHS was 30% of modified UW and 19, 9, 7% of the bicine-, histidine-, and BES-supplemented groups, respectively.

Lactate accumulation. Anaerobic metabolism in the myocardium was measured through the accumulation of tissue lactate over the 10-h experimental time course (see Fig. 1). All groups showed significant increases in lactate within the first 1 h of storage (P < 0.05). Increases continued for all groups from 2–10 h indicating continuous flux (albeit at varying rates) through the glycolytic pathway. After 2 h, only histidine- and BES-treated hearts exhibited a significantly greater accumulation of lactate than in STHS-treated hearts. By 4 h, the presumed effect of buffering on glycolytic activity was apparent; levels of lactate were 6.3 (UW), 10.1 (bicine), 9.6 (histidine), and 13.2 (BES) μmol/g greater than the levels of the STHS-treated hearts. Final levels after 10 h of storage were significantly greater than the STHS group in only the bicine, histidine, and BES groups; lactate accumulated 35, 39, and 67% more than in the STHS group (P < 0.05).

Phosphocreatine. The PCr levels in all groups immediately after the flush (t = 0) were 6.1–6.7 μmol/g (see Fig. 2). Over the first 1 h of storage, levels dropped by 50–61% of initial values (P < 0.05). Although there was a transient relative increase of PCr in bicine-treated hearts at 2 h (P < 0.05), a prolonged positive effect of BES treatment on this high-energy phosphagen was seen after 4–10 h of storage (P < 0.05). Despite this significant difference compared with STHS-stored hearts, absolute PCr quantities in BES-stored hearts never exceeded 0.6 μmol/g greater than STHS.

ATP. Initial ATP levels did not differ between STHS control (4.85 μmol/g) and experimental groups; values ranged from 4.5–5.0 μmol/g (see Fig. 3). Levels in STHS group dropped by 34% over the first 2 h and continued to decline an additional 7% throughout the 10-h period. ATP levels in the UW group remained at initial values for the first 2 h and subsequently fell to values not significantly different from the control group (STHS). Levels in the buffered groups remained not significantly different from initial t = 0 values (P < 0.05) even after 2 h. The bicine- and histidine-treated groups exhibited their largest decline between 2 and
levels, TA in the BES-treated group were consistently greater than STHS-treated hearts ($P < 0.05$) and at no time declined from initial values.

**Energy charge and ATP/ADP ratios.** Energy charge (EC) values are important in predicting and influencing glycolytic regulatory control, whereas ATP/ADP ratios are more relevant to the regulation and status of oxidative phosphorylation. EC values remained at initial levels until at least 1 h; this correlated well with ATP levels in most experimental groups. In the control group (STHS), EC started to exhibit a decline by 2 h. Values dropped by 0.11 ($P < 0.05$) from the initial value of 0.89. By the final 10-h time point, EC had fallen to 0.72 ($P < 0.05$). At 2 h all experimental groups were significantly greater than STHS; values ranged from 0.80 to 0.87. Although there were no differences at 4 h, values in the buffered groups after 10 h were significantly greater than control. The greatest difference occurred with the most highly buffered treatment group, BES, followed by histidine and bicine; UW treatment showed no differences compared with control. ATP/ADP ratios exhibited declines in all groups at earlier time points than with EC values, clearly reflecting the negative effects of anoxia on the ability of the heart to maintain ATP levels. In the STHS group ATP/ADP values began to decline immediately with 1 h of storage from an initial value of 5.90; values continued to drop over the first 2 h and appeared to level off within a range of 2.03–2.68 ($P < 0.05$) after 2 h. By 2 h, all experimental groups (including UW-treated hearts) were significantly greater than the control group, but as time progressed only the three buffered groups (bicine, histidine, BES) were statistically greater than STHS. The final 10-h values for ATP/ADP increased with the degree of buffering capacity of all solutions, presumably reflecting less of a metabolic stress on oxidative phosphorylation than with the gold standard cardiac preservation solution STHS. The correlation between buffering capacity (assessed between pH = 7.4 and 6.2) and ATP/ADP ratio was $r = 0.933$ based on linear correlation analysis (see Table 1).

### Table 1. Energy charge and ATP/ADP ratios throughout 10 h of cold storage

<table>
<thead>
<tr>
<th>Time, h</th>
<th>STHS</th>
<th>UW</th>
<th>Bicine</th>
<th>Histidine</th>
<th>BES</th>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
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<td>0.89±0.01</td>
<td>0.88±0.01</td>
<td>0.90±0.01</td>
<td>0.87±0.01</td>
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<td>0.86±0.01</td>
<td>0.87±0.01</td>
<td>0.87±0.01</td>
</tr>
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<td>2</td>
<td>0.78±0.01*</td>
<td>0.86±0.01†</td>
<td>0.83±0.01**</td>
<td>0.80±0.03†</td>
<td>0.87±0.01†</td>
</tr>
<tr>
<td>4</td>
<td>0.79±0.01*</td>
<td>0.82±0.01*</td>
<td>0.79±0.01*</td>
<td>0.82±0.02*</td>
<td>0.53±0.02*</td>
</tr>
<tr>
<td>10</td>
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<td>0.73±0.02*</td>
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<td>0.82±0.02**</td>
<td>0.84±0.01†</td>
</tr>
<tr>
<td>ATP/ADP ratio</td>
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<td></td>
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</tr>
<tr>
<td>0</td>
<td>5.90±0.39</td>
<td>4.85±0.32</td>
<td>5.64±0.31</td>
<td>4.47±0.38†</td>
<td>5.06±0.44</td>
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<td>4.94±0.69</td>
<td>3.99±0.18*</td>
<td>4.23±0.14</td>
<td>4.46±0.43</td>
</tr>
<tr>
<td>2</td>
<td>2.15±0.09*</td>
<td>4.31±0.58†</td>
<td>3.67±0.29†</td>
<td>3.43±0.45†</td>
<td>4.21±0.29†</td>
</tr>
<tr>
<td>4</td>
<td>2.68±0.13*</td>
<td>3.14±0.21*</td>
<td>2.52±0.18*</td>
<td>3.18±0.30*</td>
<td>3.67±0.43†</td>
</tr>
<tr>
<td>10</td>
<td>2.03±0.18*</td>
<td>2.23±0.24*</td>
<td>2.85±0.15†</td>
<td>3.17±0.30**</td>
<td>3.26±0.21†</td>
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</tbody>
</table>

Values are means ± SE (μmol/g wet wt). STHS, St. Thomas’ Hospital solution; UW, University of Wisconsin solution; BES, N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid. *Significantly different from $t = 0$, $P < 0.05$; †significantly different from corresponding time point in STHS group, $P < 0.05$. **Significantly different from $t = 0$, $P < 0.05$. ‡Significantly different from corresponding time point in STHS group, $P < 0.05$.
EFFECTS OF BUFFERING AGENTS ON PFK

Table 2. Activities of key regulatory enzymes of glycolysis: glycogen phosphorylase and PFK

<table>
<thead>
<tr>
<th>Time</th>
<th>Glycogen Phosphorylase</th>
<th>Phosphofructokinase</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Activity – PA</td>
<td>Physiological activity</td>
</tr>
<tr>
<td>0 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STHS</td>
<td>3.5 ± 0.4</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>UW</td>
<td>3.2 ± 0.4</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>BIC</td>
<td>3.4 ± 0.1</td>
<td>1.8 ± 0.2†</td>
</tr>
<tr>
<td>HIS</td>
<td>4.1 ± 0.3</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>BES</td>
<td>3.5 ± 0.5</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>1 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STHS</td>
<td>2.4 ± 0.2*</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>UW</td>
<td>1.6 ± 0.1†</td>
<td>1.1 ± 0.1*</td>
</tr>
<tr>
<td>BIC</td>
<td>2.6 ± 0.1*</td>
<td>1.8 ± 0.1†</td>
</tr>
<tr>
<td>HIS</td>
<td>2.3 ± 0.1*</td>
<td>1.5 ± 0.1*</td>
</tr>
<tr>
<td>BES</td>
<td>2.6 ± 0.1</td>
<td>1.5 ± 0.1*</td>
</tr>
<tr>
<td>4 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STHS</td>
<td>3.1 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>UW</td>
<td>3.3 ± 0.3</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>BIC</td>
<td>4.1 ± 0.1†</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>HIS</td>
<td>4.0 ± 0.1†</td>
<td>1.7 ± 0.1†</td>
</tr>
<tr>
<td>BES</td>
<td>4.4 ± 0.2†</td>
<td>1.6 ± 0.1†</td>
</tr>
<tr>
<td>10 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STHS</td>
<td>2.3 ± 0.2*</td>
<td>1.7 ± 0.1*</td>
</tr>
<tr>
<td>UW</td>
<td>2.8 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>BIC</td>
<td>3.8 ± 0.2†</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>HIS</td>
<td>3.6 ± 0.2†</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>BES</td>
<td>3.8 ± 0.3‡</td>
<td>1.7 ± 0.1*</td>
</tr>
</tbody>
</table>

Values are means ± SE of U/g wet wt (enzyme activities) or μM (K_m). Physiological activity denotes enzyme activities measured in the presence of physiological adenylate levels as assessed in tissue at the respective time points. – PA denotes the absence of physiological adenylate levels. *Significantly different from t = 0 within each group; P < 0.05; †significantly different from corresponding value (same time point) in the STHS group; P < 0.05.

Enzyme activities and kinetics. Enzymes were assayed according to the established procedures for V_{max} and also in the presence of physiological adenylate levels to examine the net effect of adenylates as allosteric modifiers. Although pH is clearly a major controlling factor for glycolytic activity, any effects due to posttranslational modification would not have been observed if enzyme activities were assessed under conditions of varying pH; hence pH of the assays were held constant (see Table 2).

Glycogen phosphorylase. Phosphorylase levels (without physiological adenylates) dropped after 1 h of storage in two of three buffered groups, but resumed values that were significantly greater than STHS after 4 h, but not greater than initial values in each group. The effect of physiological adenylate levels muted many of the changes in phosphorylase activities in all groups; values ranged from 1.0–1.8 U/g with minor fluctuations throughout the 10-h period.

Phosphofructokinase. Alterations in PFK activities revealed kinetic changes that have implications on the regulatory control of the glycolytic pathway during cold storage of cardiac tissue. V_{max} levels for PFK in the STHS group revealed a progressive decline in enzyme activity throughout 10 h, from 68 to 39 U/g (P < 0.05). In the buffered groups after 4 h, V_{max} values had not dropped and were equal to or greater than initial t = 0 values. The effect of physiological adenylate levels was quite apparent in the STHS group; physiological activities dropped rapidly from 24 to 3.6 U/g over the first hour and remained significantly reduced throughout 10 h. The UW group exhibited markedly lower physiological activities than the three buffered groups after 1 h. Values in the buffered treatment groups remained elevated throughout most of the first 4 h. This maintenance of PFK activity was greatest in BES and bicine groups followed by histidine (the effect of histidine was equal to the other two buffers with the exception of a transient decline at 1 h). By 10 h levels had dropped significantly to a range of 3.4–9.5 U/g; BES showed significantly greater PFK activity than STHS even after 10 h storage. Michaelis-Menten constant, K_m (ATP), doubled within the first 1 h in the STHS group and significantly elevated for the remainder of the 10-h period. K_m values for the buffered groups showed no change until 4 h at which point the K_m for bicine and histidine almost doubled; K_m for BES did not increase until the final 10-h time point. Increases in K_m values are typically representative of a posttranslational modification, probably a protein phosphatase-mediated dephosphorylation event. Assuming this, PFK remained phosphorylated (active) for the longest period in the BES group (10 h), followed by bicine and histidine (4 h); the absence of alterations in K_m for the UW group is unclear, especially in light of considerably reduced activity when assayed in the presence of physiological adenylate levels.

DISCUSSION

Current techniques utilized for cardiac transplantation involve a period of ischemic cold storage during which time the nutrient and oxygen supply to the
organ is interrupted. Under these conditions, damage to the myocardium inevitably occurs and is cumulative with time. The injury sustained by the myocardium is at least in part related to the depletion of high-energy molecules (ATP, PCr) as well as damage to the associated enzyme machinery. Depletion of the tissue energy status leads to intracellular ionic dyshomeostasis and is the result of a lack of fuel for the cellular ion pumps that regulate intracellular ion concentrations (particularly Na$^+$ and Ca$^{2+}$) (13). Experimental evidence suggests that ischemic contracture of the graft with resultant nonfunction can occur with ATP drops as little as 20% from preischemic levels (16). Levels of high-energy molecules in the cold-stored myocardium reflect a balance between the production and utilization of these compounds. The current study addresses the production aspect of this delicate balance. In the anaerobic state, glycolysis becomes the primary source of energy production. In terms of energy yield, glucose metabolism through this pathway is inefficient, producing only 5–8% (depending on the source of glucose) of the ATP produced by oxidative metabolism (9). To compensate for this relative inefficiency, flux through glycolysis must increase to meet the organ’s energy demands. Hence, during anaerobic conditions of organ storage, protons quickly accumulate, which is an event of direct consequence to anaerobic energy production. By providing intracellular buffers in the preservation solution, as proposed by Bretschneider et al. (1), we have the practical means available to “soak up” some of these excess protons and therefore lessen the pH decline that results in an inhibition of anaerobic energy production.

In this study, we investigated the effects of buffering capacity of preservation solutions on the maintenance of high-energy molecules (ATP, PCr) and key regulatory enzyme activity during a prolonged period of ischemic cold storage. A maximum storage time of 10 h was chosen because this duration represents an approximate doubling of the 4–6 h, which is currently considered safe for cardiac allografts in the clinical transplant setting. The data presented in this study are consistent with our hypothesis that tissue energetics of cold-stored myocardium can be enhanced through increased buffering of the preservation solution. STHS was our control solution and is widely used in clinical cardiac transplantation centers in Canada; the STHS utilizes NaHCO$_3$ as its primary buffer (15). We measured the buffering capacity of our experimental solutions over a pH range of 6.0–7.4; the capacities of our experimental solutions were considerably higher, ranging between 333% (UW) and 1,430% (BES) compared with the STHS control group. Over the first 4 h of storage, there was a clear and distinct difference with respect to ATP levels between STHS and BES (with the highest buffering capacity tested); ATP remained at the initial value, whereas all other groups exhibited considerable declines. After 10 h, ATP levels in the other two buffers tested (bicine and histidine) remained constant compared with 4-h levels, whereas ATP in STHS continued to fall. Hence, after prolonged storage (10 h) there was a definite pattern: ATP levels in the buffered groups appeared to closely follow the buffering capacity of the three most highly buffered solutions. In increasing order of buffering capacity, ATP levels were 47, 56, and 82% greater than hearts stored in STHS for bicine, histidine, and BES treatments, respectively. There was a strong correlation between the accumulation of lactate and ATP levels after 10 h of storage (Fig. 5); the correlation coefficient between these two parameters was $r = 0.997$. Notably, the UW solution did not fit the correlation pattern. Though energy-utilizing processes are still in operation (albeit at a markedly reduced rate during hypothermic exposure), glycolytic ATP production must be increased to a point at which the ATP demand is met, before ATP levels will begin to increase (denoting an ATP excess). Hence, there is likely a minimum requirement for increased anaerobic glycolytic activity, and more specifically in this case, buffering capacity that must be met to positively affect cellular ATP levels after prolonged (10 h) hypothermic hypoxia. In addition to superior ATP levels after 10 h of storage, total adenylate, energy charge, and ATP/ADP levels clearly reflected the superior maintenance of energetics achieved with the supplemental buffering agents; in all cases, BES was the most efficacious agent even after 10 h of cold storage. Interestingly, the ATP/ADP ratios after 10 h of storage exhibited a linear correlation ($r = 0.933$) with buffering capacity of the preservation solutions; this suggests that these supplemental buffers may indeed benefit the regeneration of energy homeostasis upon reperfusion due to less metabolic stress on oxidative pathways, in addition to benefiting anaerobic ATP production during storage. The energy charge in the nonbuffered hearts was maintained at a relatively higher level by decreasing the total adenylate pool, presumably through AMP deaminase activity. The concomitant accumulation of NH$_4^+$ might be implicated in reduced viability after prolonged periods of storage; the possibility of incorporating an inhibitor of the enzyme AMP deaminase should be explored in future studies to assess this potentially detrimental metabolic consequence of low-buffering capacity in STHS and UW solutions.

Fig. 5. The relationship between ATP and lactate levels after 10 h of hypothermic hypoxia. Correlation coefficient ($r$) is denoted.
An important consideration with respect to lactate accumulation is the inhibitory effects that it has on anaerobic metabolism. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key NAD-requiring enzyme of glycolysis that catalyses the conversion of glyceraldehyde-3-phosphate to 3-phosphoglyceroyl phosphate. Activity of this enzyme has been shown experimentally to be inhibited both by pH decline and by lactate (10, 14). Mochizuki and Neely (10) showed a 50% inhibition of GAPDH activity in hearts perfused with 20 mM lactate. However, the linear relationship between lactate and buffering capacity (r = 0.94) in this study demonstrates the practical importance of maintaining tissue pH during static organ storage to maximize the contribution of anaerobic ATP production to energy homeostasis. Despite the dominant role of lactate in GAPDH inhibition, our data show that maintaining pH with high-capacity buffering agents can alleviate the inhibition of glycolysis not only at PFK but also at GAPDH due to increasing lactate levels.

The two key regulatory enzymes that control flux through the glycolytic pathway in an active manner (i.e., responsive to hormonal cues) when endogenous substrate is utilized under ischemic conditions are glycogen phosphorylase and PFK. These enzymes respond directly to hormonal and/or neural cues via kinase and/or phosphatase-mediated phosphorylation and/or dephosphorylation events, thereby effecting a dominant level of regulatory control in addition to the secondary level of fine enzyme control involving pH and allosteric modification (9). The pH sensitivity of PFK in particular has been well documented in the context of cardiac storage (2, 7); as cellular pH drops, PFK undergoes a reversible hysteretic loss of activity as a direct effect of a pH-induced depolymerization event. However, the preservation and maintenance of PFK integrity demonstrated with the selected buffering agents in this study were not related to pH-induced hysteresis (depolymerization) because all assays were conducted in vitro at a constant pH. The changes in kinetic parameters $V_{\text{max}}$, $K_m$ (ATP), and physiological activity strongly indicate the effect of the buffers was due to an irreversible posttranslational modification, most likely a dephosphorylation event. This was not entirely unexpected because stringent hormonal control on cellular phosphorylation status is clearly absent in an isolated organ. This type of progressive dephosphorylation has been observed with glycogen phosphorylase in the liver, and the presence of buffering agents (histidine and bicine) has been protective in maintaining the phosphorylation status of this enzyme throughout 10 h of cold storage (3, 4). However, by which mechanism the buffering agents confer protection of PFK during cold storage in the present study is unclear. Although the protective effects of BES and bicine were superior to that of histidine, the conservation of PFK activity was apparent with all three buffers (and even UW to a minor degree) compared with STSIS, which has the lowest buffering capability. This suggests that the protection may not necessarily be compound-specific, but rather pH dependent; hence the mechanism likely involves pH effects on PFK-related kinases (phosphorylating) or more likely phosphatases (dephosphorylating). The elevated intracellular pH may not be favorable for PFK phosphatase activity thereby maintaining PFK activity for a longer duration; this idea is reasonable because it would be metabolically futile (and perhaps lethal to the tissue) to reduce the rate of energy producing pathways during such a period of energy stress. During such an insult, cellular pH is likely to be the dominant regulatory stimulus of PFK in the absence of exogenous hormonal cues, because changes in intracellular pH are typically transient events.

Similar to procurement methods used clinically, our study focused on a static storage system whereby the heart is simply flushed with the preservation solution and then stored hypothermically at (0–4°C). With no perfusion during storage, lactate accumulates as we and others have already shown. Although buffering addresses the problem of pH inhibition of glycolytic flux, metabolism will eventually be limited by other end products such as lactate. Hence lactate cannot accumulate indefinitely. One possible solution to this problem (with respect to clinical application) would be to intermittently flush the stored heart during prolonged periods of storage with additional preservation solution supplemented with a selected buffering agent (such as BES). This would allow removal of inhibitory end products such as lactate and NH$_4^+$ thereby maximizing the potential benefits of solution buffering on energy levels and preservation of the phosphorylation state of the key regulatory enzyme of glycolysis, PFK.

In summary, the conservation of enzyme machinery involved in regulating carbohydrate catabolism and anaerobic energy metabolism is clearly an important aspect of cardiac preservation in the absence of oxygen. Previous studies documenting a reversible loss of PFK activity (due to depolymerization) have suggested that there is a >85% return of enzyme activity (2, 7). However, our study documents the effect of probable alterations in PFK phosphorylation status which is irreversible and which has direct implications on energy metabolism and metabolic regulation of such processes during short- and long-term cardiac storage. The benefits of buffering not only include a reduction in the pH-mediated inhibition of glycolysis but also the maintenance of the phosphorylation status of PFK. This previously undocumented effect of buffering agents in the realm of ex vivo organ storage needs to be examined further in other important cellular processes controlled via posttranslational phosphorylation and/or dephosphorylation mechanisms.

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REFERENCES


