Influence of heat stress on myocardial metabolism and functional recovery after cardioplegic arrest: a $^{31}$P N.M.R study

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Abstract

Objective: Heat stress and induction of heat shock proteins confer protection against myocardial ischemia-reperfusion injury; however the precise mechanisms of this effect remain unknown. We investigated the influence of heat stress on metabolic and functional recovery after cardioplegic arrest, in a protocol mimicking clinical donor heart preservation. Methods: Langendorff perfused rat hearts in control group (C, n = 6) and heat stressed (24 h prior to experiment) group (HS, n = 6) were subjected to 4 h of ischemia at 4°C following cardioplegic arrest (St. Thomas' No. 1). $^{31}$P nuclear magnetic resonance spectroscopy was used to follow changes in ATP, phosphocreatine and inorganic phosphate concentrations during the pre-ischemic, ischemic and reperfusion periods. Myocardial adenine nucleotide levels in hearts at the end of experiments and purine catabolite release in coronary effluent during reperfusion, were evaluated using high performance liquid chromatography. Mechanical function in the pre-ischemic and reperfusion periods was evaluated using an intraventricular balloon. Western immunoblotting was used to quantitate HSP70 expression. Results: Although baseline concentrations of ATP and phosphocreatine were similar in C and HS groups, the rate of high-energy phosphate depletion was attenuated during the early phase of ischemia in HS groups. On reperfusion, recovery of ATP was 10–20% greater in HS versus C groups; phosphocreatine levels also recovered better in the HS group, transiently reaching levels 40% higher in HS versus C groups. The concentrations of adenine nucleotides in hearts were significantly higher in the HS versus C groups. These changes were associated with an attenuation of total purine catabolite release in the coronary effluent in HS versus C groups. A significant improvement in relative recovery of developed pressure was shown in HS versus C groups in the post-ischemic periods. Conclusions: Heat stress causes beneficial changes in high-energy phosphate metabolism in the rat heart subjected to cardioplegic arrest and ischemia. Improved mechanical recovery in HS versus C groups was associated with a decreased rate of high-energy phosphate depletion and increased recovery of ATP and phosphocreatine levels during reperfusion. Changes in energy metabolism may play a role in the mechanism of cardioprotection by heat stress during prolonged hypothermic cardiac arrest. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Heat shock proteins; Myocardial ischemia; Myocardial reperfusion injury; Cardioplegia; Energy metabolism; Nuclear magnetic resonance

1. Introduction

Heat stress enhances endogenous protective mechanisms against ischemia-reperfusion injury in the heart and the induction of heat shock proteins (HSPs) is believed to play a crucial role in this phenomenon [1,2]. HSPs are a family of proteins which are constitutively
expressed in cells and have an essential role in intracellular protein folding and translocation and are thus termed molecular chaperones [3]. HSP levels are increased under various conditions of stress, including heat stress, ischemia and toxins [4–6] and HSP70 is particularly important for cardioprotection.

We have previously shown that HSP70 is associated with improved recovery of both ventricular and coronary endothelial function following prolonged cardioplegic arrest and reperfusion [7,8]. This recovery was greatest when the interval between heat stress and ischemia was 24 h, correlating with maximal levels of HSP70 induction [9].

The mechanisms by which heat stress confers cardioprotection after ischemia-reperfusion remains unclear [10]. In addition to increased expression of HSP70, proposed mechanisms include attenuation of free-radical damage due to induction of antioxidant enzymes [11] and enhancement of nitric oxide production [12]. Heat stress has also been shown to attenuate the calcium paradox following ischemia-reperfusion, indicating a possible role for HSP70 in intracellular calcium handling [13].

Some studies have indicated that changes in myocardial energy metabolism after heat stress is related to improved mechanical function following normothermic ischemia [14,15]. However, detailed analysis of high-energy phosphate metabolism and its relationship to mechanical recovery has not been performed in hearts subjected to heat stress prior to cardioplegic arrest and prolonged hypothermic ischemia.

In this study we used nuclear magnetic resonance spectroscopy (N.M.R.) to investigate the sequential changes in ATP, phosphocreatine (PCr) and intracellular inorganic phosphate (Pi) levels in heat stressed (HS) and control (C) rat hearts in a model mimicking cardiac preservation for clinical transplantation together with evaluation of mechanical recovery.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats (280–320 g) were used in all studies. Animals received humane care in compliance with the ‘Principles of Laboratory Animal Care’ formulated by the National Society for Medical Research; the ‘Guide for the Care and Use of Laboratory Animals’ prepared by the Institute of Laboratory Animal Resources (N.I.H. publication No. 86–23, revised 1985); and the ‘European Convention on Animal Care’ guide. The study was approved by the institutional ethics committee on animal research.

2.2. Experimental protocol

Control (n = 6) and heat stressed (n = 6) hearts were studied to determine metabolic and functional changes before, during and after 4 h cardioplegic arrest at 4°C, as shown in Fig. 1. After an initial 40 min normoxic perfusion required for optimization of the N.M.R. and collection of initial spectra, pre-ischemic mechanical function was evaluated using a balloon catheter. Subsequently, hearts were arrested by infusion of 4°C cardioplegia at a constant pressure of 70 mmHg for 6 min. Hearts were immersed in cardioplegia and maintained at 4°C with the aid of the temperature control unit of the N.M.R. probe.

After 4 h of cardioplegic arrest, hearts were reperfused with Krebs–Henseleit buffer at 37°C and coronary effluents were collected during the first 25 min of reperfusion. After 40 min of reperfusion, post-ischemic mechanical function was evaluated. At the end of the experiments, hearts were freeze-clamped in liquid nitrogen for high performance liquid chromatographic (H.P.L.C.) analysis.

Hearts were not paced during the entire protocol; pre- and post-ischemic heart rates were recorded after
60 min of perfusion and 60 min reperfusion, respectively.

2.3. Induction of heat stress

Rats were subjected to heat stress after induction of anesthesia with sodium pentobarbitone (intraperitoneal, 50 mg/kg) using a temperature-controlled heating pad (I.M.S., Cheshire, UK). Body temperature (monitored with a rectal probe) was maintained between 42 and 42.5°C for 25 min as described previously [7]. Control animals underwent anesthesia but not heat stress. On recovery, animals were re-hydrated with normal saline (intraperitoneal, 10 ml/kg) and used for experiments 24 h later.

2.4. Heart perfusion

Rats were anesthetized with diethyl ether and sodium heparin (1000 IU/kg) injected via the femoral vein. Hearts were rapidly excised, placed in ice-cold Krebs–Henseleit buffer, immediately attached to a Langendorff apparatus and perfused with filtered Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 24 mM NaHCO₃, 11 mM glucose, 1.2 mM CaCl₂ pH 7.4) at a constant pressure of 100 cmH₂O and continuously gassed with a 95%O₂/5%CO₂ mixture at 37°C, as described previously [16]. Perfusion was carried out inside the bore of the magnet to determine changes in high-energy phosphate levels, as described below.

2.5. Cardioplegic solution

St. Thomas' Hospital cardioplegic solution No. 1, supplied as a concentrate (Martindale, Essex, UK), was diluted (1:50) in Ringer's solution (Travenol Labs, Norwalk, UK) and filtered before use.

2.6. ³¹P Nuclear Magnetic Resonance Spectroscopy

Changes in myocardial ATP, PCr and Pi were followed using ³¹P N.M.R. (Bruker AMX-400 wide bore vertical system, ³¹P frequency 161.9 MHz). Fully relaxed spectra were acquired at 20 min of normoxic perfusion (36 scans, 90° angle and 15 s interpulse delay). Subsequently, saturated spectra (24 or 280 scans, 60° angle, 2 s interpulse delay) were collected throughout the experiment. An initial ATP concentration of 23 μmol/g dry wt, as measured by HPLC in both normal and heat shocked hearts, was used for calibration of N.M.R. data. For calculation of high-energy phosphate levels in saturated spectra, saturation factors obtained from repeated fully relaxed and saturated spectra acquired during baseline conditions were used. Correction of the saturation factor obtained at 37°C versus 4°C was established in a solution of ATP, PCr and Pi with intracellular concentrations of inorganic ions.

2.7. Functional assessment

Assessment of mechanical function was performed using a balloon catheter inserted into the left ventricle to determine systolic and end-diastolic pressures, as previously described [17]. The balloon was inflated with water in 25 μl steps from 0 to 300 μl. Peak-systolic and end-diastolic pressures were recorded at each loading of the balloon and used to calculate developed pressure. Recovery of mechanical function was expressed as relative recovery of post-ischemic versus pre-ischemic developed pressure (RRDP). Pre- and post-ischemic coronary flows were obtained by timed collection of the coronary effluent into a measuring cylinder.

2.8. High performance liquid chromatography

At the end of perfusion hearts were freeze-clamped using aluminium clamps pre-cooled in liquid nitrogen and freeze-dried overnight. About 40 mg of left ventricle was extracted at 4°C with 0.6 M perchloric acid (25 μl/mg dry tissue ratio), centrifuged (13000 × g for 3 min at 4°C) and the supernatant neutralized with 2M KOH. After removal of precipitated potassium perchlorate by centrifugation (13000 × g for 3 min at 4°C) extracts were immediately analysed using HPLC, with application of the reverse phase method as previously described [18], using a Hewlett-Packard series 1100 chromatograph (Hewlett-Packard, UK). The analytical column (150 × 4.6 mm ID) was packed with 3 μm ODS Hypersil (Shandon, UK). Chromatographic conditions were as follows: Buffer A consisted of 150 mM KH₂PO₄ containing 150 mM KCl (pH 6.0); Buffer B consisted of a 15% (v/v) solution of acetonitrile in buffer A. The composition of the mobile phase changed from 0 to 100% of buffer B according to the gradient curve, as previously described [18]. Sample peaks were integrated and quantified using a HP-Chemstation chromatography data system (Hewlett-Packard, UK). Coronary effluent samples collected during reperfusion were also analysed using the above method; samples were injected directly onto the chromatograph without prior extraction.

2.9. Assessment of HSP70 concentration

HSP70 concentration was assessed by SDS-PAGE and Western immunoblotting, as previously described [9]. Whole heart homogenates were solubilised in 1% w/v SDS (sodium dodecyl sulphate), assayed for total protein using the Bradford assay, denatured by heating at 100°C in Laemmli buffer and separated on 10% SDS
gels until the Bromophenol Blue tracking-dye reached the end of the gel. The gels were equilibrated for 30 min in transfer buffer before protein transfer at 500 mA for 1 h. Western blots were blocked for 1 h using 3% w/v skimmed milk powder (Marvel) in phosphate-buffered saline (PBS: 0.15 M NaCl, 0.05 M phosphate buffer, pH 7.2) containing 0.05% w/v Tween-20; this blocks non-specific binding sites. Blots were then probed with monoclonal mouse antibody (SPA-810) specific to inducible HSP70 (Bioquote, York, UK), diluted to a final concentration of 1/1000, for 1 h. Blots were washed three times and incubated with secondary horseradish-peroxidase-conjugated rabbit anti-mouse antibody for 1 h.

Blots were visualized using an enhanced chemiluminescence (ECL) detection system (Amersham, UK). Hyperfilm MP (myoperoxidase) was exposed to blots treated with ECL for 30 s and developed in an automatic film processor; following ECL exposure, antibodies were removed from blots by incubation in a solution of 2% w/v SDS, 6.25% v/v 1 M Tris–HCL, pH 6.8 and 0.7% v/v 2-mercaptoethanol. Proteins were then visualized by staining with 0.01% Amido Black in a solution of methanol, water and acetic acid (45:45:10 v/v ratio). Amido Black-stained blots and ECL films were scanned using a Molecular Dynamics 300A laser densitometer and HSP70 levels determined as a proportion of total protein loaded using Quantity One software (P.D.I., New York, USA).

2.10. Statistics

Values are presented as means ± standard error of the mean (S.E.M.). Statistical comparison was performed by unpaired Student’s t-test or using analysis of variance (ANOVA) for repeated measures followed by Bonferroni test to indicate individual significant differences. A value of $P < 0.05$ was considered as a significant difference.

3. Results

ATP, PCr and Pi concentrations evaluated using N.M.R. are displayed in Fig. 2. Although baseline levels of ATP and PCr were similar in HS and C groups, the rate of depletion of high-energy phosphates during the initial phase of ischemia was attenuated in the HS versus C groups; the rate of Pi accumulation was also lower in HS versus C groups. On reperfusion, the recovery of ATP was 10–20% greater in HS versus C groups. PCr levels also recovered on reperfusion significantly better in HS versus C groups; at 10–30 min reperfusion, PCr levels were 20–40% higher in HS versus C groups.

The concentrations of ATP, ADP, AMP and NAD in freeze-dried hearts are shown in Table 1. Again, significantly higher levels of ATP and total adenine nucleotides were found in HS versus C groups. Total purine catabolite (inosine, adenosine, hypoxanthine, xanthine and uric acid) release during the first 25 min of reperfusion is shown in Fig. 3. There was 20% less total purine catabolite release from HS versus C groups.

The changes in pre- and post-ischemic peak systolic pressure (LVSP) and end diastolic pressures (LVEDP) are shown in Fig. 4A and B; relative recovery of developed pressure (RRDP) for both groups is shown in Fig. 5. There were no significant differences in LVSP and

Fig. 2. Concentration of (A) ATP, (B) phosphocreatine and (C) intracellular inorganic phosphate levels in control hearts ($n = 6$) and heat-stressed hearts ($n = 6$) subjected to cardioplegic arrest for 4 h at 4°C ischemia evaluated using $^{31}$P N.M.R. Values represent the mean (± S.E.M.). * $P < 0.05$ versus control.
Table 1
Concentrations (μmol/g dry wt.) of adenine nucleotides and NAD in control and heat stressed hearts subjected to cardioplegic arrest and ischemia for 4 h at 4°C

<table>
<thead>
<tr>
<th></th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
<th>TAN</th>
<th>NAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>19.60</td>
<td>3.50</td>
<td>0.37</td>
<td>23.47</td>
<td>5.34</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0.42</td>
<td>0.15</td>
<td>0.01</td>
<td>0.45</td>
<td>0.05</td>
</tr>
<tr>
<td>Heat stressed</td>
<td>21.01*</td>
<td>3.87</td>
<td>0.58</td>
<td>25.46*</td>
<td>5.29</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0.30</td>
<td>0.41</td>
<td>0.21</td>
<td>0.74</td>
<td>0.07</td>
</tr>
</tbody>
</table>

TAN, total adenine nucleotides (ATP + ADP + AMP).
* P < 0.05 versus control.

LVEDP before ischemia. Post-ischemic LVSP and LVEDP in HS were not different from post-ischemic C. Higher post-ischemic values of LVSP in HS versus pre-ischemic and higher values of LVEDP in C versus pre-ischemic were the only significant differences. However, the RRDP was 10–25% greater in HS versus C groups at all balloon loadings, indicating improved mechanical function following heat stress. The induction of HSP70 in HS versus C groups was confirmed by Western immunoblotting and laser densitometry; the relative intensity of HSP70 in HS versus C groups was 13.6 ± 4.9 versus 3.9 ± 0.5 (arbitrary units). Pre- and post-ischemic coronary flow rates are reported in Table 2; the post-ischemic coronary flows were significantly different from pre-ischemic values but there was no difference between HS and C groups. Hearts were not paced during the protocol and pre- and post-ischemic heart rates are reported in Table 3; there were slight decreases in heart rates both in HS and C post-ischemia (approximately 6%).

4. Discussion

This study demonstrated significant changes in high-energy phosphate metabolism in heat stressed hearts which accompanies improved preservation of cardiac mechanical function following prolonged hypothermic cardioplegic arrest. A decreased rate of high-energy phosphate depletion during ischemia and an increased rate of recovery during reperfusion, were observed in hearts subjected to heat stress. These changes may play a role in the mechanism of cardioprotection by heat stress during preservation for cardiac transplantation.

A significant preservation of myocardial ATP and PCr in HS versus C groups, as assessed by N.M.R., was observed during the early phase of ischemia; after 3–4 h ischemia, PCr was slightly higher in HS versus C groups. A notable finding was that the PCr signal was still visible after 4 h ischemia, even in the control group, indicating excellent protection of high-energy phosphates by cardioplegia; however this protection was further enhanced in the heat stressed group.
Several mechanisms may be responsible for better preservation of high-energy phosphates in HS versus C groups. One possibility is improved regulation of mitochondrial H(+)-Transporting-ATP-Synthase (F1F0-ATPase). Under conditions of oxygen deficiency, delayed or inefficient inhibition of this enzyme by ATPase inhibitor (IF-1) may result in considerable ATP breakdown [19,20]. It is possible that heat stress may interfere with ATPase activity under ischemia, via either HSP induction or another mechanism. A further possibility is optimized high-energy phosphate utilization for residual membrane transport activity, which is supported by recent studies indicating changes in calcium handling in heat stressed myocytes [10,21].

On reperfusion, the enhanced overshoot of PCr in HS versus C groups was observed together with better recovery of ATP concentration. However, the initial rate of high energy phosphate restoration in the early period of reperfusion was similar. Results of adenine nucleotide levels in heart extracts are in agreement with the N.M.R. data: significantly higher levels of ATP and total adenine nucleotides were found in HS versus C groups.

Table 2
Pre- and post-ischemic coronary flow rates (ml/min) in control and heat stressed hearts subjected to cardioplegic arrest and ischemia for 4 h at 4°C

<table>
<thead>
<tr>
<th></th>
<th>Perfusion (min)</th>
<th>Re-perfusion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30</td>
<td>45</td>
</tr>
<tr>
<td>Control</td>
<td>14.5</td>
<td>15.0</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>HS</td>
<td>15.0</td>
<td>15.7</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>0.4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* P < 0.001 versus all pre-ischemic values.

Analysis of the coronary effluent confirms the decline in ATP catabolism following heat stress; 20% less total purine catabolites was released in HS versus C groups. Increased nucleotide catabolism is a consequence of an imbalance between energy production and energy utilization and is considered to be a sensitive marker of myocardial ischemia [22–25] and previous studies have shown a close association between purine concentration in coronary effluent and preservation of ATP levels in the heart after ischemia [26]. As we were investigating the changes in energy metabolism after heat stress, we did not study markers of necrosis. However, previous studies have shown reduced creatine kinase release after heat stress [27].

Other studies concerning changes in high-energy phosphate metabolism after heat stress report conflicting results. Some studies show better preservation of ATP levels after heat stress [14,28], while others show no changes in nucleotide levels at different stages of ischemia-reperfusion [29]. These differences may be due to varying experimental conditions, especially to the presence of pyruvate in the perfusion buffer [29]. Replacement or addition of pyruvate as metabolic substrate during perfusion markedly affects energy status in the heart [30]; furthermore, pyruvate metabolism is markedly affected after heat stress [15]. Differences in temperature during ischemia seems a less likely explanation for these differences, since we have observed similar changes in high-energy phosphate metabolism in a normothermic model of myocardial ischemia (unpublished observations).

A recent study concerning the effect of heat stress on cardiac function and metabolism after prolonged hypothermic storage [28] supports the results presented here with regard to better maintenance of high-energy phosphate levels during and after ischemia in hearts subjected to heat stress. However, our study provides detailed evaluation of the timing of the metabolic differences and evaluation of function in the same hearts, due to application of N.M.R. spectroscopy. We were able to additionally show that heat stress leads to a slower rate of decline of ATP levels during ischemia, with a significant PCr overshoot in the reperfusion period. Furthermore, our N.M.R. measurements...
provide more accurate estimation of in vivo concentrations, especially that of unstable PCr.

In the above report, baseline levels of ATP and especially of PCr were very low, which may be a result of different collection/storage/extraction procedures before HPLC analysis; high baseline levels of purine catabolites observed in analysed cardiac samples indicates a considerable breakdown of metabolites between collection and freezing of specimens. However, results of functional evaluation presented in this study are in agreement with our results, with regard to the degree of improvement of developed pressure in heat stressed hearts and with regard to the predominant effect of heat stress on systolic function as opposed to diastolic compliance.

An alternative metabolic mechanism by which heat stress may protect the myocardium is by attenuating intracellular acidosis. However, it was difficult to quantitate changes in intracellular pH in the present study because the intracellular inorganic phosphate NMR signals were very broad during ischemia; this complicates the accurate definition of the position of these peaks. Secondly, the relationship between pH and the chemical shift of inorganic phosphate at 4°C is not well established. Consequently, we are unable to present reliable measurements of intracellular pH in the present study. However, in another set of experiments which involved a protocol of 25 min of normothermic ischemia we have shown that pH is indeed higher during ischemia in hearts subjected to heat stress compared to controls (unpublished observations). Further limitations of our results in terms of clinical application are possible species differences in cardiac energy metabolism and the use of a crystallloid-perfused model.

In conclusion, this study demonstrates improved preservation of myocardial high-energy phosphates in heat stressed hearts subjected to cardioplegic arrest and prolonged hypothermic ischemia. The decreased rate of high-energy phosphate depletion and increased recovery of ATP and PCr levels during reperfusion in heat stressed hearts was associated with improved mechanical recovery. Changes in energy metabolism may thus play an important role in the mechanism of cardioprotection by heat stress during preservation for cardiac transplantation. Future investigations of these metabolic pathways may lead to techniques which can enhance current methods of prolonged donor heart storage.

Acknowledgements

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References


Appendix A. Conference discussions

Dr D. Taggart (Oxford, UK) I have one comment and a question at the end. I did a small series of patients in Oxford looking for heat shock protein by Northern and Western-blotting techniques and I used for coronary bypass grafting an intermittent ischemic protocol, so 10 min to do a distal graft, reperfuse for 10 min, 10 min to do another graft. I compared five patients undergoing coronary artery bypass grafting, with one patient in whom I did an aortic valve replacement using cold crystalloid cardioplegia and we biopsied the left ventricle before and serially throughout the operation until about 40 min after the termination of bypass. In the patients who had the intermittent ischemic protocol we detected very significant increased levels of both heat shock protein itself and indeed marked upregulation of mRNA for that, but what was interesting was that in the one patient undergoing aortic valve replacement submitted to cold crystalloid cardioplegia at the temperature of 4°C we couldn’t find any evidence of upregulation. Could you comment on that.

Dr J. Jayakumar: That is quite an interesting study. Your protocol seems to be a preconditioning protocol; however, you are looking for heat shock proteins. Recent studies have shown that preconditioning can not only lead to the classical preconditioning effect but also lead to a heat shock protein induction effect as well and so what you might be observing is actually the initial stages of the heat stress response induced by your pre-conditioning protocol; the link between the classical and the delayed protection is now being elucidated.

Dr P. Taggart: If I may just pursue this a little bit, how quickly do you think you can induce heat shock protein in the human setting? Animal models would suggest you can detect upregulation of mRNA within about half an hour of ischemia. Do you think that is relevant to clinical practice?

Secondly, you mentioned that in your own study you used cold crystalloid cardioplegia, I think you said for 4 h and the point I was trying to make is do you think that degree of hypothermia may in fact switch off these potentially protective mechanisms.

Dr J. Jayakumar: In answer to the latter point, we don’t know the answer. We do know in animal models that HSP mRNA is increased very early after the ischemic period and this may occur in humans as well. The other point is that patients undergoing cardiac surgery and who have had previous ischemic events may also have increased baseline levels of HSP 70, so therefore we have to look at whether the HSP levels are induced prior to surgery or due to your intermittent ischemic protocol.