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Hypothermic perfusion preservation of liver: the role of phosphate in stimulating ATP synthesis studied by ^{31}P NMR

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Abstract Hypothermic perfusion of rat livers was investigated by ^{31}phosphorus nuclear magnetic resonance (^{31}P NMR) spectroscopy using a temperature-controlled module that allowed data acquisition at various time points during a 48-h period. The livers were perfused with an oxygenated lactobionate/raffinose-based solution containing adenosine and inorganic phosphate, and changes in tissue oedema were monitored by direct on-line measurements of liver weight changes. Liver tissue ATP concentrations, determined by fluorimetric assay, were low immediately after organ removal, probably reflecting metabolic stress during the removal period, and these increased slightly during the next 3 h. This was reflected by changes in the ^{31}P NMR spectra. However, by 24 h ATP levels had increased significantly, and these were maintained for up to 48 h, suggesting a shift in the balance between energy production and consumption. When inorganic phosphate was replaced by another anion (citrate), ATP was maintained at a constant lower level during perfusion for 48 h. Tissue weight changes were similar in both groups, suggesting that volume control was not affected by the different ATP contents of the livers. By combining the temperature-controlled module with a separate perfusion circuit, NMR spectroscopy can provide a sensitive method for following energy metabolism in the same organ over long periods during hypothermic perfusion.

Key words Liver preservation, role of phosphate · Phosphate, role in liver preservation · NMR ^{31}P, liver preservation · NMR ^{31}P, ATP synthesis

Introduction

It has long been recognised that continuous hypothermic perfusion, performed under controlled conditions, can provide consistently better preservation of organs for transplantation than can simple cold ischaemic storage, and by using optimised methods successful 5-day preservation of canine kidneys has been reported [17]. Methods have also been devised for hypothermic liver perfusion in the rat [7], pig [19] and dog [18], with viability maintained for up to 72 h. However, the clinical application of machine perfusion for liver preservation has not been widespread, mainly due to considerations of the complexity of transporting equipment and the requirement for skilled supervision of the procedure. More recently, there has been a resurgence of interest in combining hypothermic perfusion with cold storage as a means of improving the quality of organs harvested from non-ideal donors [15]. The simplicity of the combined method would be improved if the same solution could be used for cold storage and continuous perfusion.

We have undertaken studies on continuous hypothermic perfusion of rat livers with a modified lactobionate/raffinose-based solution (LR), similar to the solu-

tion used clinically for flush preservation in our liver transplant programme [11] and derived from the original University of Wisconsin solution. This solution also contains adenosine and inorganic phosphate (P_i) and an inhibitor of adenine nucleotide catabolism (allopurinol). It has been previously shown in studies on renal hypothermic perfusion that these components can stimulate synthesis of high levels of ATP [16]. To investigate whether a similar co-operative effect on metabolism is produced in the liver during continuous hypothermic perfusion, a second series of experiments was performed using the LR solution in which phosphate was replaced by another multivalent anion (citrate). ^{31}Phosphorus nuclear magnetic resonance (^{31}P NMR) spectroscopy was performed on the livers at various time points during 48 h of cold perfusion to investigate changes in hepatic adenine nucleotides (including ATP), and tissue oedema was measured by alterations in liver weight. Results showed that hepatic nucleotide triphosphate (NTP) content (which, in the liver, is predominantly ATP) was low immediately after liver harvesting but increased significantly during 24 and 48 h of hypothermic perfusion with the standard LR solution. When phosphate was replaced by citrate, NTP concentrations stabilised and were maintained at the same values as those measured at the start of cold perfusion. Chemical measurements of adenine nucleotides at the end of perfusion confirmed the differences demonstrated by ^{31}P NMR spectroscopy.

Materials and methods

Organ removal

Livers were removed as previously described [12]. Male Wistar rats (250 g) were anaesthetised with halothane/oxygen. The portal vein was exposed and cannulated. The livers were then flushed with 100 ml of cold (4 °C) hypertonic citrate solution via the portal vein to wash out the blood and cool the organ prior to perfusion.

Perfusion

A continuous perfusion circuit based on that developed in our previous study was used [12]. Briefly, the circuit was constructed inside a glass-fronted commercial refrigerator (set at 5 °C) and consisted of disposable nylon tubing (internal diameter 4 mm). The main reservoir was a perspex pot (diameter 9 cm, height 20 cm) containing 300 ml of perfusate, which was gassed with 100% oxygen at a flow rate of 1 l/min. Perfusate was drawn through a Millipore filter (8-μm pore size) in the bottom of the reservoir by a Watson Marlow pump (type LRHE) and passed via a bubble trap to a pressure head, open to the atmosphere, at a height of 10 cm above the liver. The liver was attached via a portal vein cannula (nylon tubing, internal diameter 0.8 mm) and suspended from a digital electronic balance placed above the circuit. Before perfusion commenced, the liver was weighed and the balance was tared (compensating for the weights of cannula, tubing etc). Thereafter, changes in weight reflected only changes in fluid content of the organs on perfusion. Once perfusion was started, the first 50 ml of venous effluent (which contained residual blood) was collected and discarded. After this, the venous outflow returned directly into the reservoir for recirculation, and perfusate flow rates were commonly between 5 and 8 ml/min at this hydrostatic pressure. At the chosen time points weight changes were noted, the portal vein cannula was clamped and the liver was removed and suspended in fresh cold perfusate in a perspex chamber in the pre-cooled, temperature-controlled NMR probe for collection of spectra before being returned to the perfusion circuit. Two groups of experiments were performed for evaluation by ^{31}P NMR spectroscopy; one group ($n = 6$) was perfused with standard LR, while the second group ($n = 6$) was perfused with LR in which phosphate was replaced by citrate (LRC). In three livers from each group, biopsies were taken at the end of perfusion for adenine nucleotide determinations by chemical means.

^{31}P nuclear magnetic resonance spectroscopy

^{31}P NMR spectra were obtained using a vertical 8.5 tesla magnet attached to a Bruker AM360 spectrometer. The NMR probe contained a perspex chamber to house the rat liver, mounted within a 7.3-cm diameter NMR probe body. The liver chamber was surrounded by a saddle coil tuned to 145.8 MHz (the ^{31}P NMR frequency). Immediately after removal and cooling, the rat liver was attached to the NMR probe and suspended by the cannula in the portal vein into the chamber containing the cold LR solution. The temperature was maintained at 5 °–7 °C using an additional perspex module containing ice, placed in the probe above the liver. ^{31}P spectra were obtained in 200 scans, using 60-μs pulses (approximate 60° flip angle) repeated at 1.7-s intervals. The liver was then taken out of the probe and attached to the continuous perfusion circuit. Collection of spectra was repeated at 3, 24 and 48 h, by removing each liver from the perfusion circuit and resuspending it in the probe. This interruption involved a maximum period off perfusion of 20 min at 5 °–7 °C, which was necessary for tuning the probe, shimming the magnet (i.e. optimising the field homogeneity) and for data collection. During this time and at this temperature, the liver energy status does not change significantly [8].

Peak area measurement

Peak areas were determined by integration using the standard spectrometer software. The β-NTP peak area was compared to that of the signal arising from a capillary containing a solution of 1.0 M methylene diphosphonate (MDP), positioned within the perspex liver chamber. Absolute quantification of NTP concentrations from NMR spectra was not attempted.

Composition of the LR solutions

The solutions contained lactobionate as the major anion and, in addition, the following ingredients (in mmol/l): K^+ 125; Na^+ 20; Mg^{2+} 5; Ca^{2+} 1.5; lactobionate-100; SO_4^{2-} 5; PO_4^{3-} 25; raffinose 30; adenosine 5; glutathione 3; allopurinol 1; insulin 100 IU; pH at 4 °C 7.3. In the second series of experiments, phosphate was replaced by citrate (LRC) and the solution adjusted to the same pH. In the present studies colloid was not added because clinically it is not present in the storage solution [11], and because liver perfusion is a low-pressure system for which the role of colloid is uncertain.

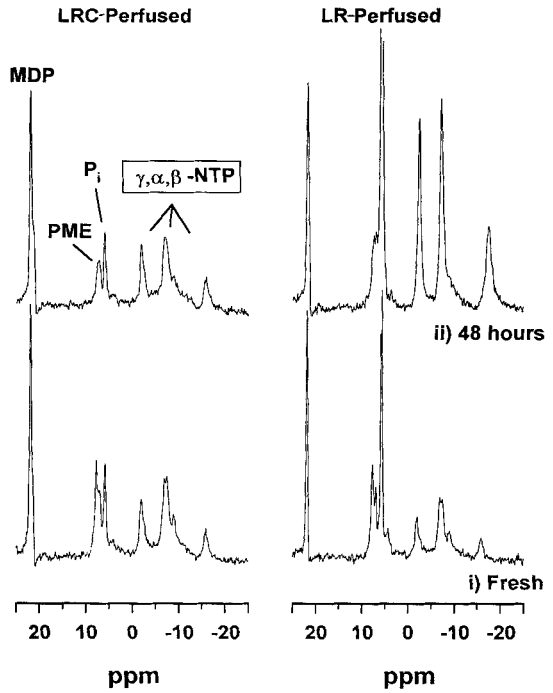

Fig. 1A,B ^{31}P NMR spectra of livers immediately upon cold perfusion (i) and after 48 h of continuous perfusion (ii): **A** liver perfused with LRC solution; **B** liver perfused with LR solution. Signals can be identified for the internal standard *MDP*, phosphomonoesters (*PME*), inorganic phosphate (P_i), and the γ, α and β phosphates of NTP. It can be seen that the signals for the three phosphate groups of NTP increased during the 48 h in the LR-perfused liver, whilst there was little change in these signals in the LRC-perfused liver

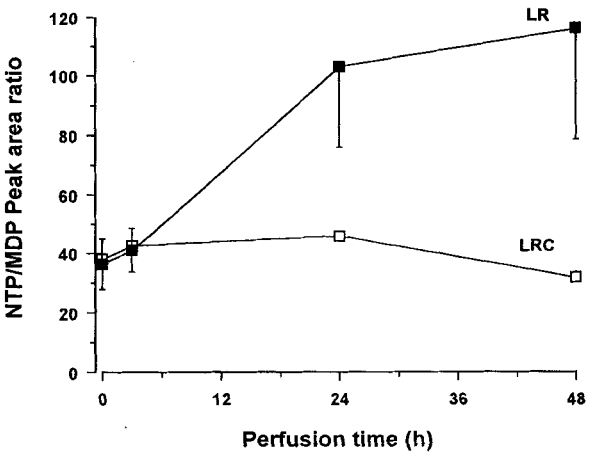

Fig. 2 Values for peak areas of β-NTP signals, measured as ratios of the standard MDP peak areas, in livers perfused with LR and LRC solutions. Values are shown as means ± SEM; for the LRC group, the SEMs at 24 and 48 h were too small to be depicted at this scale. In the LR group there were significant increases in β-NTP after 24 and 48 h ($P < 0.01$ in both cases) compared with initial values. No such increases were seen in the LRC group

Chemical measurements of adenine nucleotides

Chemical measurements of adenine nucleotides were made in livers at the end of perfusion for correlation with the ^{31}P NMR data. In three livers from each group, the left lobe was clamped in tongs pre-cooled in liquid nitrogen. The frozen tissue was ground to a powder under liquid nitrogen, extracted with perchloric acid, and the supernatant was neutralised with potassium hydroxide. Adenine nucleotides were measured by enzyme-linked fluorometric assays as described elsewhere [13]. Energy charge was expressed as [ATP + 1/2 ADP] / [total adenine nucleotides].

Statistical methods

Data from livers perfused for 48 h were collected. Comparisons of NTP contents and liver weight changes at different time points were made using ANOVA and Dunnett's test. Significance was assumed at values of $P < 0.05$.

Results

In Fig. 1 are shown spectra acquired from two livers immediately upon start of perfusion and after 48 h of hypothermic perfusion, in one case with LR and in the other with LRC solution. Peaks can be identified for the MDP standard, the three phosphate groups of NTP (predominantly ATP), for phosphodiesters, inorganic phosphate (P_i) and phosphomonoesters. A large peak for P_i can be seen in the LR-perfused liver, reflecting a significant contribution from the phosphate contained in the solution. On the other hand, the smaller P_i signals in the LRC-perfused liver resulted only from the liver tissue since this solution is phosphate-free. After 48 h of perfusion in the LR liver, there was a clear increase in the peak area for β-NTP relative to MDP, while in contrast in the liver perfused with LRC, the peaks for β-NTP were similar in area at the initial perfusion period and after 48 h.

The values for hepatic content of β-NTP computed relative to the peak areas of the MDP standard at different time points during hypothermic perfusion in the two groups are shown in Fig. 2. It can be seen that values for β-NTP were maintained similar to the initial, post-harvest values during the first 3 h of hypothermic perfusion, but that by 24 h and 48 h, β-NTP had increased to be significantly higher ($P < 0.01$ in both cases) than the initial concentrations. In contrast, in the LRC group, β-NTP contents were stabilised at the original, lower level and were constant throughout the entire perfusion period.

The chemical measurements of tissue adenine nucleotides reflected patterns similar to those shown by the NMR data. Total adenine nucleotides in the LRC group ($n = 3$) were 5.18 ± 0.27 μmol/g, with ATP concentrations of 2.91 ± 0.14 μmol/g and an energy charge of 0.71 ± 0.03. In the LR group ($n = 3$), total adenine nucleotides and ATP concentrations were higher

(9.87 ± 0.84 μmol/g and 5.02 ± 0.31 μmol/g, respectively), but the overall energy charge was similar at 0.72 ± 0.12.

The weight changes of the perfused livers were recorded as a means of assessing oedema formation, as a percentage of the initial, unperfused organ. In both groups there were small increases in weight over the first 3 h (to 111 % ± 4 % with LR and 109 % ± 6 % with LRC), reflecting to some extent the effects of re-filling the vascular bed during perfusion. After perfusion in the LR group for 24 h, weight had increased to 121 % ± 4 % of original, which largely reflected the effect of re-filling the vascular bed during perfusion. During the following period of perfusion, weight increased further to 133.4 % ± 3.8 % of original ($P < 0.05$). Similar increases were noted in the LRC group at 24 and 48 h (126 % ± 6 % and 132 % ± 5 %, respectively). Perfusate flow rates did not change in response to these small weight changes, and were maintained at 6–7 ml/min in all perfusions at the selected portal pressure of 10 cm H_2O.

Discussion

The results presented in this study demonstrated that by combining the use of a specifically modified MR probe that allowed good temperature control at hypothermia with a separate hypothermic perfusion circuit distant from the NMR magnet, the metabolic status of individual perfused livers could be followed by ^{31}P NMR techniques during prolonged periods (48 h) by taking 'snap-shots' of metabolic status at selected time points. Other groups have studied ^{31}P NMR of livers after prolonged cold ischaemic preservation or during brief periods of perfusion at low temperatures [5, 6]; however, as far as we are aware, this is the only report of a ^{31}P NMR study on continuous hypothermic perfusion of the liver that has followed the same organs over a prolonged time course, presumably because of the problems of monopolising spectrometer time, which this would normally require. Indeed, in our case, the study was only possible by utilising the 'snap-shot' method, which meant that the NMR spectrometer was available for other use during the 48-h study period. In preliminary studies on chemical measurements of ATP (data not shown), it was demonstrated that interruption of perfusion, produced by clamping the inflow for up to 60 min at 6 °–8 °C, did not cause a change in ATP concentrations in liver tissue (initial values of 3.1 ± 0.3 μmol/g versus 3.0 ± 0.2 μmol/g at 60 min) in LRC-perfused livers. This was presumably because the livers were already at hypothermia before the start of the ischaemic period and also because there was likely to be a residual tissue oxygen content (from the perfusate) remaining for a period. Thus, the 20-min period required for the 'snap-shot' NMR measurements would not result in any significant change in the recorded spectra of high-energy phosphates.

The ability to non-invasively record changes in metabolism in the same organ increases the sensitivity of the study and provides the possibility that small changes in energy metabolism can be more easily identified. The data show that hepatic energy status, as defined by ATP concentration, is well preserved after perfusion for 48 h with oxygenated LR solution, and in fact that ATP increased significantly over the period of hypothermic perfusion. It has been known for some time that continuous hypothermic perfusion allows maintenance of hepatic ATP levels measured by chemical means [1] and by HPLC [4], whilst ATP disappears within a few hours when the organ is stored in the cold without perfusion [8]. It has also been shown previously in the hypothermic perfused kidney that perfusates containing mixtures of adenine nucleotide precursors, such as adenosine, adenine plus ribose and inorganic phosphate, stimulate the synthesis of increased ATP concentrations to levels higher than measured in the normal tissue at 37 °C [16]. Our results show that the liver appears to respond similarly during hypothermic perfusion, with increasing levels of ATP produced during the 48-h period. The chemical measurements of adenine nucleotide contents fully supported the data derived from ^{31}P NMR spectroscopy and indicated that after perfusion for 48 h with LR, adenine nucleotide and ATP concentrations were higher (1.9 and 1.7 times, respectively) than in the LRC group (which were, in turn, similar after 48-h perfusion to those measured in rat liver freeze-clamped in vivo [1]). However, the benefit of using non-invasive ^{31}P NMR spectroscopy was demonstrated because it allowed monitoring of changes in energy metabolism during the 48-h period, which is not possible for chemical measurements that would require tissue biopsy and thus alter perfusion characteristics within the liver. In the LR group there was a small increase in ATP during the first 3 h of perfusion, probably reflecting recovery from the ischaemia the organs encountered during the initial harvesting and cooling procedures. However, there was a further, more marked increase during the following perfusion to 24 h, and thereafter little change in ATP took place. It may be that the 24-h measurements reflect a more complete recovery from the initial ischaemia; however, this would imply that ATP resynthesis during hypothermic perfusion was a slow process, which would be at variance with other experiments we have reported where ATP resynthesis during cold resuscitation perfusion after ischaemia had reached a maximal plateau by 60–90 min [9]. The mechanisms for synthesis of high concentrations of ATP during hypothermic perfusion may relate to the activity of the enzymes of purine salvage, such as adenosine kinase, which is present in many tissues including the liver and which catalyses the reaction ATP + ade-

nosine ⇌ AMP + ADP. The high levels of ATP produced may reflect a shift in the overall balance between ATP synthesis and utilisation during hypothermic liver perfusion, where ATP synthesis has proceeded at a faster rate than utilisation by anabolic processes such as protein synthesis, which are known to be inhibited at low temperatures. However, it should be pointed out that exogenous adenosine stimulates synthesis of increased ATP concentrations in hepatocytes and kidney tubules at 37 °C [14], so hypothermia cannot be the sole causative factor. The experiments in which phosphate was replaced by citrate (LRC) clearly demonstrated that external inorganic phosphate was essential for the synthesis of high ATP levels. This might be expected on the basis that increased ATP synthesis would require a matching equivalent increase in intracellular phosphate, but it also suggests that transmembrane uptake of phosphate is still sufficiently active at 6 °–8 °C to provide the additional supply of anion.

It has generally been assumed that a colloid would be required during continuous hypothermic perfusion of the liver to prevent oedema formation, and agents such as gelatin polypeptides [7] or plasma proteins [2, 4] have been proposed. However, many of these earlier studies were performed with solutions containing commonly encountered anions such as chloride or citrate. In the present study, colloid was deliberately omitted to investigate an extreme example of the principle of using the same solution for flush storage and perfusion preservation, as might be undertaken in combined flush/perfusion. Oedema, as measured by weight gain on perfusion, was significant by 24 h, although there was little further increase by 48 h and perfusion flow was not compromised. The changes in liver weight may reflect cell swelling, which follows from less efficient ion pumping and compromised cell homeostasis at hypothermia. It is known that cell swelling in the perfused liver (produced by changing the tonicity of the perfusate [3]) will cause an increase in organ weight, although perfusion rates may not be reduced until swelling exceeds a certain threshold beyond which sinusoidal compression occurs. The relatively successful control of cell swelling in our study may partly reflect the fact that liver perfusion via the portal vein is a low pressure system, and lactobionate may control transmembrane water movement at a cellular level. More recently, hydroxyethyl starch has been used as a colloid for canine livers perfused with a gluconate-based solution [10], which allowed successful storage for up to 72 h, and our data would reinforce the use of such agents. Preliminary studies in four livers perfused with LR solution to which 3 % hydroxyethyl starch was added indicated that weight gain over the 48-h period was similar to that reported in the present study in the absence of colloid (mean values of 138 % versus 133 %), and chemical measurements of total adenine nucleotides and energy charge (10.6 ± 1.6 μmol/g and 0.8 ± 0.1, respectively) were also similar. However, the viscosity of the solution was noticeably increased by the colloid addition, which may change pressure/flow relationships within the perfused organ, making direct comparisons difficult. Further studies will be necessary to define the exact role of added colloid. For brief, hypothermic resuscitation perfusion (of the order of 60–90 min), colloids may not be an absolute requirement, although again this will need to be investigated in other models. The ability to use NMR as a non-invasive measure of metabolism, coupled with on-line assessments of oedema, will make a useful contribution to such studies.

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