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A ^{31}P-NMR STUDY OF THE EFFECTS OF REFLOW ON THE ISCHAEMIC RAT HEART

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(1) The recovery of perfused rat hearts experiencing various lengths of total global ischaemia was studied using ^{31}P-NMR. Mechanical function was monitored by measuring left ventricular pressure. (2) Hearts exposed to a maximum of 14 min total global ischaemia regained stable contractile function on reperfusion. The concentration of phosphocreatine in these hearts rapidly exceeded its pre-ischaemic value while that of ATP rose very slowly. P_i fell on reflow to approximately its original level. These observations are interpreted as being the result of a rapid turnover of ATP stimulating phosphocreatine production by the mitochondrial isozyme of creatine kinase (ATP : creatine *N*-phosphotransferase, EC 2.7.3.2). (3) The recovery of intracellular pH on reperfusion does not depend upon the duration of ischaemia, nor on the pH or the percentage of ATP depletion at the end of the ischaemic period. This indicates that pH recovery is a flow-dependent phenomenon. (4) In non-recovering hearts, multiple P_i resonances are observed which arise from areas of differing myocardial pH. Phosphocreatine levels did not rise above 50% of their pre-ischaemic values. ATP levels remained depressed. This suggests that localized tissue necrosis only characterizes the failing situation.

Introduction

When a heart is subjected to total global ischaemia there is a switch from dependence on the metabolism of fatty acids to produce ATP to that of carbohydrates [1]. Fatty-acyl CoA derivatives build up [2], glycogen depletion occurs [3,4] and the concentrations of phosphocreatine and ATP decline [5]. The intracellular pH also decreases [6,7] and P_i accumulates. The relative importance of these effects to the recovery of cardiac function is unclear.

The relationship between functional recovery, tissue necrosis, pH and cellular energetics are incompletely understood. The recovery of mechanical function in a heart depends on the length of the ischaemic period [8]. Transient recovery followed by a period of depressed contractility normally follows short periods of ischaemia in the dog [9]. Reperfusion of the canine myocardium after periods of ischaemia in excess of 20 min eventually results in histologically detectable necrosis [8]. Heart mitochondria undergo ultrastructural changes during ischaemia and reflow, though the appearance of calcium phosphate particles on reflow characterizes irreversible damage [10,11]. The leakage of enzymes from the ischaemic myocardium also suggests that an amount of cellular disruption occurs even when the ischaemic period is short [12].

^{31}P-NMR can be used to measure non-invasively the intracellular concentrations of phosphocreatine, ATP and P_i in the perfused rat heart [13,14]. From the resonance position of P_i, the intracellular pH can be calculated [15]. This corresponds to the pH of the myocardial cytoplasm [16]. The technique has proved useful in the study of ischaemic acidosis and cardiac glycogen metabolism [16,17]. It can also be applied to the study of recovery from myocardial ischaemia [18].

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The technique has three major advantages in such a study.

(i) Metabolic changes in an individual heart may be followed during ischaemia and reflow.

(ii) Changes in the cytoplasmic pH may be followed during ischaemic acidosis and recovery.

(iii) As it is possible to resolve P_i resonance in environments with different pH values in the same tissue [14,19], it should be possible to detect any differential recovery of pH in different areas of the heart arising, for example, from localised tissue necrosis.

In this paper, we report our observations of cellular bioenergetics in isolated perfused rat hearts recovering from total global ischaemia.

Methods

Hearts from 280–320 g male Wistar rats were perfused in the Langendorff mode [20] with buffer containing 5 mM D-glucose as described elsewhere [17]. After being allowed to stabilise for at least 30 min, six ^{31}P-NMR spectra were obtained as controls before subjecting the hearts to 6, 8, 12, 14, 18 or 24 min total global ischaemia. Spectra were collected continuously during this ischaemic period and during the subsequent 45 min of reflow with the same buffer. Throughout the perfusion, left ventricular pressure was monitored after cannulation using a Bell and Howell 4-327-I pressure transducer and Lectromed 3552 amplifier and thermal recorder. Spectra were obtained by applying 45° pulses to the sample at 1 s intervals and accumulating 120 transients. A 4.2 T superconducting magnet (Oxford Instruments Ltd.) linked to a Nicolet 1180 computer was used for all of the NMR measurements. In order to correct for variations in spectrometer output, resonance amplitudes were measured relative to an external standard (methylene diphosphonate in ^2H_2O). This was placed in the glass annulus surrounding the sample chamber within the radiofrequency coil.

Data are presented as percentage changes based on the average of the first six control measurements as 100%. The mean and standard deviations of six similarly treated hearts are quoted unless otherwise indicated.

TABLE I

PERCENTAGE CHANGES IN FUNCTIONAL PARAMETERS OF RECOVERING HEARTS AFTER VARYING PERIODS OF TOTAL GLOBAL ISCHAEMIA

Hearts were perfused with Krebs Henseleit buffer containing 5 mM glucose. They were then subjected to varying periods of total global ischaemia followed by 45 min of reperfusion with the original buffer. Left ventricular pressure was measured using a catheter inserted in the left ventricle connected to a pressure transducer. The developed pressure is defined as systolic pressure minus diastolic pressure. Heart rate was determined as the number of beats per min and coronary flow as the volume of effluent per min. Measurements made during reflow were expressed as a percentage of the pre-ischaemic control levels (taken as 100%).

Duration of ischaemia	% change in heart rate	% change in developed left ventricular pressure	% change in coronary flow
6 min control	100	100	100
4 min post reflow	94.2 ± 6.6	74.6 ± 15.9	86.3 ± 19.8
22 min post reflow	91.7 ± 3.0	97.1 ± 27.4	79.9 ± 49
8 min control	100	100	100
4 min post reflow	98.4 ± 10.1	50.0 ± 16.1	95.5 ± 5.41
22 min post reflow	106.6 ± 10	65.7 ± 23.7	81.3 ± 13.6
12 min control	100	100	100
4 min post reflow	102.8 ± 15.2	64.4 ± 12.5	101.2 ± 19.8
22 min post reflow	100.9 ± 1.3	82.0 ± 15.1	83.5 ± 5.4
14 min * control	100	100	100
4 min post reflow	113.6	64.6	99.7
22 min post reflow	99.4	82.4	92

* Mean value of three hearts (40% recovery).

Results

Hearts experiencing total global ischaemia for 6, 8 or 12 min recover functional stability within 4 min of reflow. Table I shows the percentage recovery in heart rate, developed tension and coronary flow of such hearts at 2 and 22 min after reperfusion. Heart rate returns to pre-ischaemic values fairly rapidly, but the coronary flow rate does not regain its control value during the 45 min reperfusion. During the first 2 min of reflow, cardiac contractility increases and exceeds its pre-ischaemic level, but within 4 min it has fallen again to 90% of the control value (Fig. 1). As the period of ischaemia lengthens, hearts recover a smaller percentage of their pre-ischaemic contractility on reflow. However, there does not seem to be a simple relationship between the duration of ischaemia and the contractility recovered.

Fig. 2 shows typical ^{31}P-NMR spectra obtained prior to, during and after 8 min of total global ischaemia. After about 5 min of ischaemia the level of phosphocreatine is undetectable, ATP is severely depleted and the P_i concentration has risen. In addition, the P_i resonance has moved to lower frequency, reflecting its more acidic environment. On reflow, phosphocreatine rapidly rises whereas the recovery of ATP is much slower. The P_i resonance also returns eventually to its pre-ischaemic position.

The time course of high-energy phosphate recovery is shown in Fig. 3 for hearts subjected to 8 min ischaemia. The phosphocreatine concentration exceeds its pre-ischaemic value within 2–4 min and slowly returns to the control level after about 30 min reperfusion. Similarly, the P_i concentration decreases to below the pre-ischaemic control value in 2 min and then slowly rises above it. The concentration of ATP does not return to that observed before the ischaemic period within 45 min of reperfusion. A similar pattern of recovery occurs following 6 and 12 min ischaemia.

Fig. 4 shows that pH recovers at a similar rate on reflow, irrespective of the duration of and the lowest pH reached in ischaemia. One rather broad intracellular P_i peak is observed throughout, which may be differentiated from buffer P_i.

After 14 min total global ischaemia only 40% of hearts recover stable mechanical function. Hearts which do recover demonstrate characteristics similar to those described above. However, hearts which fail to recover after 14, 18 or 24 min ischaemia show markedly different NMR characteristics.

The concentration of phosphocreatine in hearts failing after 18 min ischaemia increases by only 52% within the first 4 min of reflow and never exceeds its pre-ischaemic value (Fig. 5). However, the decrease in the concentration of P_i remains approximately the same as in recovering hearts. The ATP content slowly rises to about 50–60% of its pre-ischaemic value while greater variability in its recovery in individual hearts is observed.

In addition, the spectra on reperfusing individual hearts show one major P_i resonance with a spread of smaller peaks at more acidic pH (Fig. 6). The recovery of the main peak follows a time course similar to that observed in recovering hearts (Fig. 7). Lengthening the ischaemic period has no obvious effect on the rate of recovery of pH, as measured using the major P_i peak.

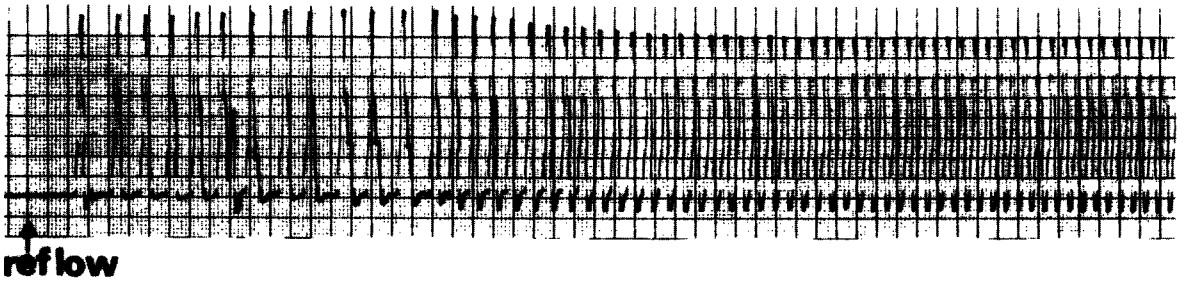

Fig. 1. The recovery of left ventricular pressure after 8 min total global ischaemia. The pressure trace was obtained by cannulation of the left ventricle. The recovery of function after 8 min ischaemia is shown.

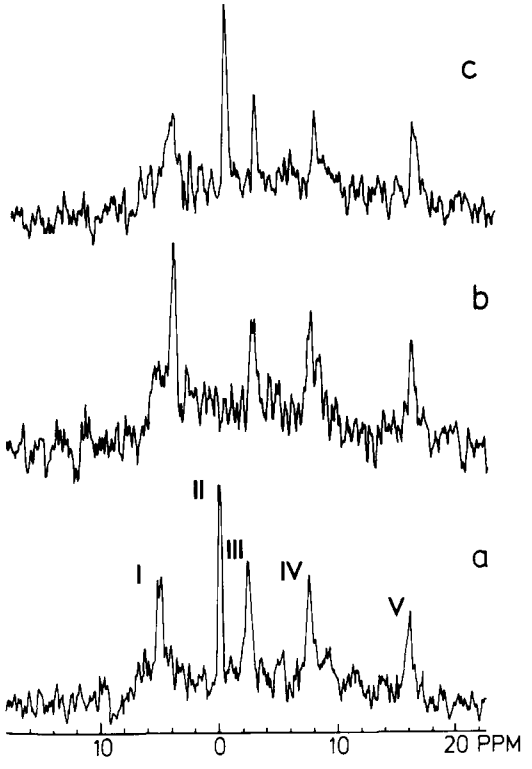

Fig. 2. ^{31}P-NMR spectral changes during ischaemia and reperfusion. Spectra were obtained by accumulating 120 transients over 2 min using a 45° pulse (see text). Spectra are shown before ischaemia (a), between 4 and 6 min total global ischaemia (b), and after 8–10 min of reflow (c). Assignments: I, P_i; II, phosphocreatine; III, γ phosphate (ATP), β phosphate (ADP); IV, α phosphate (ATP), α phosphate (ADP); V, β phosphate (ATP).

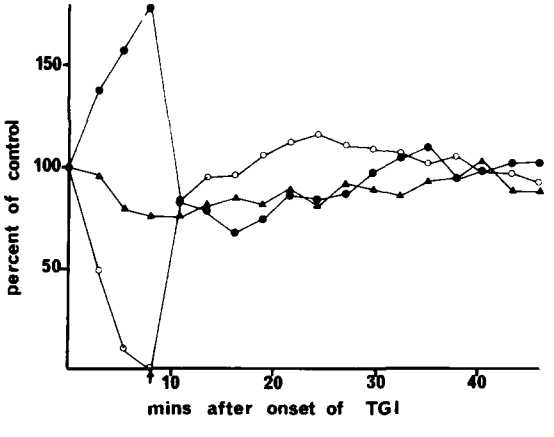

Fig. 3. Time course of high-energy phosphate recovery in hearts regaining function after 8 min ischaemia. The effects of reperfusion after 8 min total global ischaemia (TGI) on the levels of ATP (▲), phosphocreatine (○) and P_i (●) in perfused rat hearts are shown. The mean value of data from six hearts is shown in each case. Standard deviations were less than 10% of the mean in all cases. The start of reflow is indicated by the arrow.

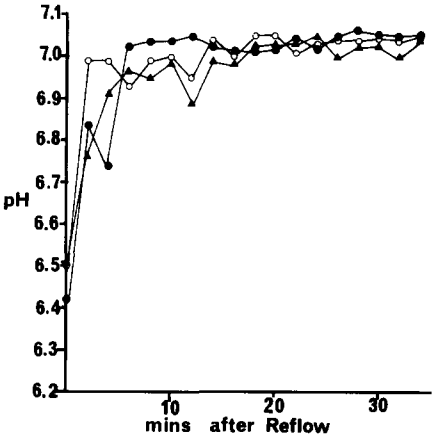

Fig. 4. Changes in pH in hearts regaining function after ischaemia. The mean values for intracellular pH of six hearts are shown following 6 (▲), 8 (○) and 12 (●) min ischaemia. pH was calculated from the resonance position of intracellular P_i [15].

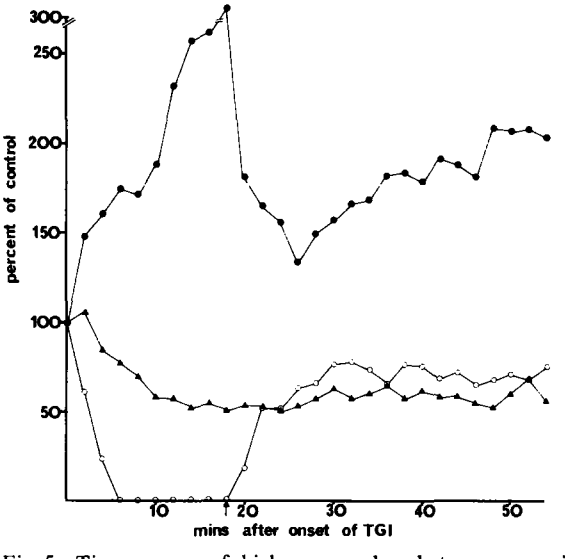

Fig. 5. Time course of high-energy phosphate recovery in hearts failing after 18 min total global ischaemia (TGI). The effects of reperfusion after 18 min ischaemia on the levels of ATP (▲), phosphocreatine (○) and P_i (●) in the perfused rat heart are shown. The mean value of data from six hearts is shown in each case. Standard deviations were less than 10% of the mean. The start of the reperfusion is indicated by the arrow.

Fig. 6. Variations in the P_i resonance of the ^{31}P-NMR spectrum of a reperfused rat heart. Changes in the P_i region of the spectrum of a typical heart failing on reperfusion after 18 min total global ischaemia are shown. Spectra represent accumulations in the last 2 min of ischaemia (a), 0–2 min reflow (b), 2–4 min reflow (c), 10–12 min reflow (d) and 20–22 min reflow (e). The collection parameters used are described in the text.

Occasionally, on reperfusion after 14 or 18 min total global ischaemia, a heart recovered functional stability after about 30 min quiescence. Contraction usually began with a period of fibrillation or unstable arrhythmia which gradually gave way to stable contractile function. In these cases, the onset of beating tended to coincide with the recovery of phosphocreatine to its pre-ischaemic level, but the sample size of the hearts behaving in this manner is too low to analyse further.

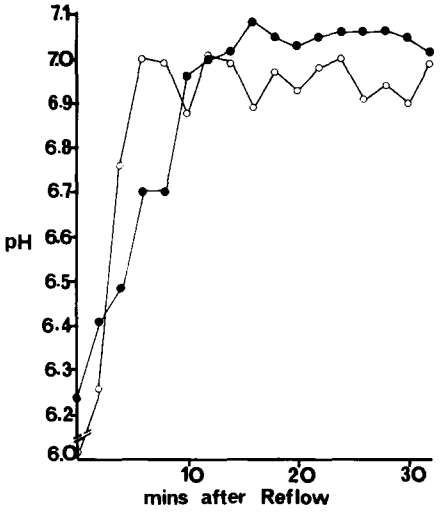

Fig. 7. Changes in pH in hearts failing on reperfusion after ischaemia. The mean values of intracellular pH of six hearts subjected to 18 (•) and 24 (○) min total global ischaemia are shown. pH is determined from the intracellular P_i resonance [15].

Discussion

Recovery of function and pH

Hearts which recover mechanical function after a period of total global ischaemia show reduced ventricular pressure without alteration in heart rate (Table I). This may be explained either by a reduction in the efficiency with which metabolism is coupled to mechanical function or by localized tissue necrosis.

Reibel and Rovetto [21] have demonstrated a direct correlation between the concentration of ATP on reflow and the ventricular power ((mean aortic pressure – left atrial pressure) × aortic flow) in the working rat heart. This would support the first possible explanation. Our data do not show a linear relationship between the percentage recovery of ventricular pressure and that of myocardial ATP in the Langendorff preparation. However, we do observe a depression both of contractility and of ATP on reflow.

Two of our observations can be used to argue against the second possibility, that the decrease in contractility on reflow in hearts recovering mechanical function is caused by cellular necrosis. We observe

little change in the concentration of ATP on reflow from that measured at the end of total global ischaemia, while the phosphocreatine concentration dramatically rises. This is difficult to reconcile with a simple model of localized necrosis where one might expect parallel reductions in phosphocreatine and ATP. Secondly, we observe only one P_i peak as the acidosis abates in hearts recovering function on reflow, whereas multiple peaks are observed when the hearts fail. This we interpret as localised necrosis characterising the failing but not the recovering situation.

We observe no dependence of the rate of recovery of intracellular pH in recovering hearts on the duration of the ischaemic insult. Neither do we observe a dependence using the main P_i resonance peak in failing hearts. Were the recovery of pH a metabolic phenomenon, we would expect to see parallel changes in high-energy phosphates and pH. This is not the case and there is also no dependence on the pH value and the ATP depletion at the end of the ischaemic period. We believe that this strongly indicates that the recovery of myocardial pH under these circumstances is a flow-dependent phenomenon.

The role of phosphocreatine

We observe a dramatic rise in the concentration of phosphocreatine on reflow while the concentration of ATP changes little from that measured at the end of the ischaemic period. If creatine kinase were homogeneously distributed in the cytoplasm, the elevated phosphocreatine-to-ATP ratio might be explained by the effect of a low concentration of ADP on the creatine kinase equilibrium.

However, Jacobus and Lehninger [22] and Saks et al. [23,24] have shown that a mitochondrial isozyme of creatine kinase is located on the outer surface of the inner mitochondrial membrane and used preferentially ATP synthesized by oxidative phosphorylation. This may be facilitated by a close coupling of the enzyme with the ATP-ADP translocase in the rat heart [25]. Phosphocreatine could thus be regarded as a means of conveying the energy production of oxidative phosphorylation to the myofibrils, of which the creatine kinase has kinetic properties favouring ATP production [26]. Furthermore, externally added creatine stimulates the ADP-induced respiration of isolated mitochondria by regenerating ADP from mitochondrial ATP via the creatine kinase reaction [22]. The phosphocreatine overshoot which we observe could be explained analogously as the effect of a high cytosolic concentration of ADP.

The first model involves an equilibrium situation, whereas the second is a kinetic treatment based on the rate of ATP production by the mitochondria. In principle, these hypotheses can be differentiated by measurement of the cytosolic ADP concentration.

ADP is visible in the ^{31}P-NMR spectrum of the heart. However, its α and β phosphate resonances overlap those of the α and γ phosphates of ATP [15]. As ATP can be measured from the ATP β phosphate resonance, the ADP concentration can be estimated by subtracting the ATP β phosphate peak from that of the γ phosphate. The error associated with this measurement is relatively high. The ADP concentration has also been calculated using the equilibrium constant of creatine kinase and the concentrations of ATP and phosphocreatine measured by ^{31}P-NMR [27]. This method assumes that cytoplasmic constituents are selectively observed by NMR. As neither the mitochondrial nor the myofibrillar forms of the enzyme appear to be in equilibrium [23,26], the equilibrium constant used must be that for the cytoplasmic isoenzyme. This method cannot be applied throughout the ischaemic period because phosphocreatine rapidly falls below the detection limit of the technique [17].

The ADP concentration at the end of ischaemia estimated by the first method is approx. 3.5 μmol/g wet wt. This is of the same order of magnitude as measurements by freeze-clamping [21] and is higher than the normoxic levels [28]. This correlation between a high concentration of ADP and the phosphocreatine overshoot cannot be reconciled with an equilibrium model of creatine kinase action and favours the kinetic treatment.

On reoxygenation on reperfusion the high concentrations of ADP and P_i lead to a high rate of ATP synthesis in the mitochondrion. The mitochondrial creatine kinase isozyme is stimulated by the high rate of turnover to produce phosphocreatine from mitochondrial ATP and to replenish the ADP and thereby maintain the high respiration rate. Under these circumstances and the lower contractility developed, the level of phosphocreatine exceeds the pre-ischaemic control situation. This correlates with the high rate of

mitochondrial phosphorylation of creatine in the presence of high concentrations of phosphocreatine observed by Saks et al. [23].

Our observation that phosphocreatine recovery greatly exceeds ATP recovery on reflow therefore confirms the physiological importance both of the subcellular location of the isozymes of creatine kinase and of phosphocreatine as an energy-transporting molecule.

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