Effect of Ischemia on NMR Detection of Phosphorylated Metabolites in the Intact Rat Heart†

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ABSTRACT: Phosphorus NMR spectroscopy is an important technique for the investigation of metabolism in tissues and intact organisms (including man). However, quantitation of the signals from an NMR experiment is difficult because it is not known from which regions of a cell metabolites are detected. It is generally believed that only metabolites free in the cytosol are observed. In this study a comparison of concentration measurements obtained by NMR and after freeze extraction was made in the normoxic and ischemic rat heart. The influence of ischemia was examined because of its potential effect on the level of phosphate metabolites in various compartments. The same fraction of ATP always appears visible to NMR, whereas inorganic phosphate is largely NMR invisible until after a period of ischemia and the phosphomonoesters are only partially observed early in ischemia.

Phosphorus NMR spectroscopy has been applied to the study of physiology and metabolism in intact organisms, organs, and cells with much success. Quantitation of the signals arising from an NMR experiment has often proved to be a difficult task however, particularly when applied to intact systems. It is known that there are discrepancies between concentration measurements by NMR and by more classical techniques for certain tissue metabolites. In view of the usefulness of NMR in addressing metabolic problems (particularly since it is one of the few techniques that can obtain biochemical information directly and noninvasively from man), this is a matter of some importance.

It is generally thought that NMR signals detected from living tissues arise from metabolites found free in the cytosol only. Compounds bound to large structures are relatively immobile and therefore give rise to broad signals. For example, the phosphorus signal from bone is observed as a large hump in the base line of brain spectra (Ackerman et al., 1980). In addition, materials present in viscous solution or bound to paramagnetic ions are difficult to detect because of extreme line broadening. Both of these conditions may exist in the mitochondrial matrix.

Two well-known metabolites that are incompletely detected by NMR in tissues are ADP and P_i. The concentration of ADP measured in many tissues is lower than that measured after the more conventional approach of freeze clamping and acid extraction. Binding to actin in muscle (Dawson, 1982) and/or localization in the mitochondrial matrix (Wong, 1981) has been implicated. Inorganic phosphate is also measured at a level lower than that estimated by classical biochemical techniques (Ackerman et al., 1980).

Recently a report appeared suggesting that ATP, normally thought to be entirely "NMR visible", is in fact only partially detected in the liver (Murphy et al., 1988). This is of great importance since ATP is often used as an internal standard. In addition, little information is available on a further class of metabolites often observed in tissues: the phosphomonoesters. There is evidence that glucose 6-phosphate may exist in two different compartments in muscle (Busby et al., 1978). In view of these considerations and their importance to the understanding of cardiac metabolism, a study of the relationship between the measurement of various metabolites by NMR and that by classical techniques was undertaken in the normoxic and ischemic heart.

Experimental Procedures

Heart Perfusions. Male Sprague-Dawley rats weighing 350–400 g were injected with 250 units of heparin ip and anesthetized with 50 mg of sodium pentobarbitol. Hearts were rapidly excised and placed briefly in ice-cold arresting medium, and then the aorta was cannulated. Retrograde perfusion was conducted at a pressure of 70 cmH_2O in modified, phosphate-free Krebs Henseleit bicarbonate buffer (all concentrations millimolar): NaCl, 119.2; KCl, 4.7; $CaCl_2$, 3.0; $MgSO_4$, 1.2; $NaHCO_3$, 25; EDTA, 0.5 (free Ca^{2+}, 2.5). The perfusate was equilibrated with 95% O_2/5% CO_2. The substrate was 5 mM glucose with 5 units/L insulin. All hearts were maintained at 37 °C throughout the experiment.

NMR Methods. ^{31}P NMR spectra of intact hearts were obtained at 121.5 MHz with a Nicolet NT300 spectrometer and Oxford magnet. Fully relaxed spectra (90° pulses, 12-s delay, 60 transients) were collected at the start of the experiment. [The metabolite with the longest longitudinal relaxation time (T_1) is PCr. Since the total delay between pulses (delay time plus data acquisition time) was 12.2 s and the measured T_1 for PCr in hearts under these conditions was 3.29 ± 0.08 s (n = 3, unpublished results), these spectra were 95% relaxed with respect to PCr.] For the remainder of the experiment more rapidly pulsed spectra of 2-min duration (90° pulses, 1.2-s delay, 92 transients) were obtained. The first few spectra were used to estimate the effect of saturation as a result of rapid pulsing to enable concentration measurements. Each

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Table I: NMR and Classical Biochemical Measurements of Phosphorylated Intermediates in Normoxic and Ischemic Rat Hearts[a]

	technique	n	P_i	PME	PCr	ATP
normoxia	NMR	3	5.2 ± 3.9	6.3 ± 4.7	27.0 ± 3.3	17.8
	classical	4	29.2 ± 2.5[c]	3.8 ± 1.0	27.9 ± 4.5	17.8 ± 1.8
10-min ischemia	NMR	3	24.7 ± 3.9	8.9 ± 4.5		11.8 ± 0.6
	classical	3	33.5 ± 2.8[b]	18.5 ± 4.8[b]	2.8 ± 0.4	12.1 ± 2.8
12-min ischemia	NMR	3	28.7 ± 5.1	21.8 ± 9.3		8.3 ± 1.2
	classical	3	34.2 ± 7.9	19.5 ± 2.8	2.1 ± 0.4	9.9 ± 0.9

[a] Data are the means ± standard deviations (μmol/g dry wt). Estimations of metabolite levels using both techniques were from identical hearts in normoxia; separate hearts were used for the 10- and 12-min ischemic hearts. [b] Statistical probability <0.05. [c] Statistical probability <0.01.

spectrum was collected from 2K data points and zero filled, a 20-Hz exponential filter was applied, and then the spectrum was Fourier transformed. Measurements of the area under each peak were made by nonlinear least-squares curve fitting. Conversion of the calculated areas to concentrations was made by comparison with the measurement of ATP in the normoxic state with the analytical methods outlined below. This is a generally accepted practice and is used as a means to compare the different levels for each metabolite. The T_1's of certain metabolites were measured by inversion transfer. Details of the solutions used are given in Table II.

Biochemical Methods. Hearts were freeze clamped with aluminum tongs cooled in liquid nitrogen. The frozen tissue was pulverized with a mortar and pestle at liquid nitrogen temperatures. A weighed portion of this power (ca. 200 mg) was dried overnight in an oven at 70 °C to determine the wet/dry weight ratio; the remainder was quickly weighed and mixed with 3 mL of ice-cold 6% perchloric acid. The resulting homogenate was centrifuged at 32520*g*, 4 °C for 5 min; the supernatant was removed, neutralized with potassium carbonate, and then recentrifuged. Inorganic phosphate (P_i) was measured with an acid molybdate assay; ATP and PCr were measured spectrophotometrically by NADP-linked reactions. The phosphomonoesters were measured by ion chromatography (Dionex BioLC ion-exchange chromatography system). The major components that comprise the phosphomonoester resonance in the spectrum of an ischemic heart that were measured and summed together are IMP, AMP, glucose 6-phosphate, fructose 6-phosphate, fructose 1-phosphate, fructose 1,6-bisphosphate, and α-glycerol phosphate. The remaining phosphorylated, glycolytic intermediates [glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, 2- and 3-phosphoglycerate, and phospho(enol)pyruvate] are either present at very low levels in normoxic and ischemic heart tissue (Rovetto et al., 1975) or, as in the case of dihydroxyacetone phosphate, could not be detected in this study. It was not possible to identify the presence of other compounds that appear in the phosphomonoester region, such as phosphoethanolamine or phosphocholine.

Experimental Protocol. For direct comparison of metabolite levels measured by NMR and after freeze extraction in normoxic hearts, NMR spectra of intact hearts were first collected and then the hearts removed from the magnet and freeze clamped. For comparison of the levels of metabolites after various durations of ischemia, two series of experiments were conducted. In the first, NMR measurements were continuously made on the intact hearts. A fully relaxed spectrum was first obtained followed by two spectra of 2-min duration (rapid pulsing). The hearts were then made ischemic for 12 min, during which time 2-min spectra were continuously acquired. In the second set of experiments, hearts perfused outside the magnet were made ischemic for either 10 or 12 min and then freeze clamped for analysis.

Statistics. Data are expressed as means ± standard deviations. Results were compared by Student's t test for the means of independent or paired groups where appropriate. Significance of difference was considered to be reached at the 0.05 probability level.

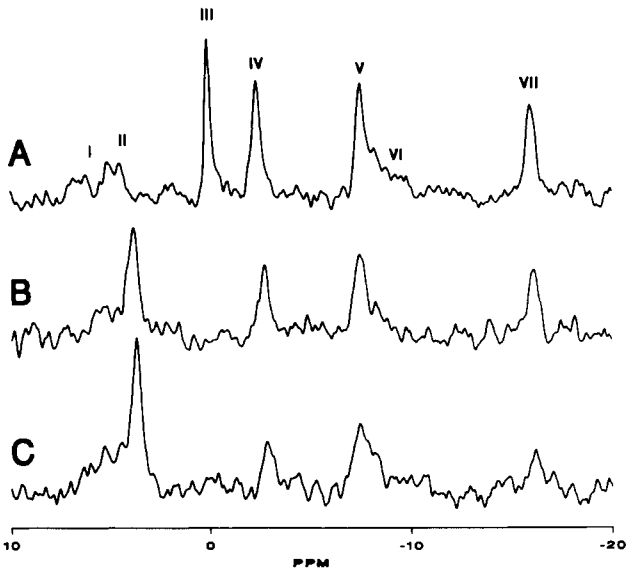

FIGURE 1: Phosphorus NMR spectra of a perfused rat heart acquired (A) before the onset of ischemia and (B) after 10 min and (C) after 12 min of ischemia. Resonance assignments: (I) phosphomonoesters; (II) inorganic phosphate; (III) phosphocreatinine; (IV) γ- and β-phosphates of ATP and ADP, respectively; (V) α-phosphates of ATP and ADP; (VI) NAD(P); (VII) β-phosphate of ATP.

Results

Figure 1 shows spectra from a heart that was made ischemic for 12 min. The spectra shown are prior to the introduction of ischemia and after 8–10 and 10–12 min of ischemia. Note the loss of PCr, the rise in P_i, the decline in ATP, and the appearance of phosphomonoesters.

Table I shows the result of measurements of P_i, phosphomonoesters, PCr, and ATP by NMR and classical biochemical methods in normoxic hearts and at the end of 10 and 12 min of ischemia. Similar to previous results, NMR measurements of P_i in the normoxic heart were only 17% of that obtained after freeze extraction. During ischemia, however, more of the P_i becomes visible: 74% after 10 min and 81% at the end of 12 min (with statistically significant difference apparent for the 10-min group only).

The phosphomonoesters were measurable at low levels in the normoxic hearts by both techniques. Because of their low concentrations (resulting in poor signal to noise) and relatively broad lines (more than one component present), there is much scatter in the NMR determination. During ischemia the phosphomonoesters have reached high levels by 10 min and are still at that level at 12 min as assessed by ion chromatography. The NMR measurements do not agree with this, however: the result gives only 48% of the value determined after freeze extraction at 10 min but a similar value at 12 min.

Table II: T_1 Measurements (s) of Phosphorylated Metabolites[a]

	pH 7.06	pH 5.90
glucose 6-phosphate	7.46 ± 0.11	7.64 ± 0.30
α-glycerol phosphate	9.73 ± 0.04	10.14 ± 0.22
AMP	6.14 ± 0.11	6.14 ± 0.14
P_i	4.81 ± 0.04	5.02 ± 0.06
phosphocreatinine	6.79 ± 0.02	
β-phosphate of ATP	1.67 ± 0.03	1.15 ± 0.06

[a] Metabolites (all 10 mM and added as their sodium salts) were dissolved in a solution containing 100 mM KCl and 12 mM $MgSO_4$ (free Mg^{2+} as calculated from ATP binding 3 mM). Measurements (n = 3 at each pH) were made at 37 °C.

The PCr/ATP ratio in normoxic hearts is the same whether measured by NMR or spectrophotometric assay (1.51 ± 0.18 and 1.56 ± 0.13, respectively). With ischemia, PCr rapidly declines to a level that cannot be detected by NMR. Because of the low concentrations reached and the insensitivity of the NMR technique, it is not clear whether PCr is NMR visible to the same extent throughout the experiment.

In contrast to the liver study of Murphy et al. (1988), the measured values of ATP decline to the same extent during the ischemic episode whatever the means of measurement, and hence, the same fraction remains NMR visible.

The T_1's of the major components of the phosphomonoester region together with P_i, PCr, and ATP are given in Table II. The T_1 for the β-phosphate of ATP is the shortest in the group (and hence the effect of saturation on decreasing the delay between transients is the smallest). The remaining metabolites have roughly similar T_1's, and the signal intensity per mole arising from each should be affected to a similar degree with different pulsing conditions. No change in the T_1's was found on lowering the pH from 7.1 to 5.9. It was not possible to measure the T_1 of PCr in acid solution because of hydrolysis.

Discussion

A frequently proposed explanation for discrepancies between determinations of the cellular concentrations of low molecular weight metabolites by NMR and after freeze extraction (whole-tissue levels) is that NMR does not detect material inside mitochondria. In studies estimating the distribution of adenylates between mitochondria and cytosol after tissue fractionation, the percentage of ATP present in the cytosol in the perfused heart was 79% in guinea pigs (Bunger & Soboll, 1986) and 85% in rats (Kauppinen et al., 1980). On this basis the practice of equating the NMR-derived value for ATP concentrations with measurements of total ATP by freeze extraction will be in error if the NMR experiment reports the cytosolic concentration only. A comparison can also be made with the distribution of PCr within muscle cells. However, there is some uncertainty in this measurement in the heart: present estimates for the fraction present in the cytosol are 110% (Bunger & Soboll, 1986) and 89% (Kauppinen et al., 1980). Since the ratio of PCr to ATP in the normoxic heart was the same for both types of measurements in this study, this suggests that the fraction of each metabolite that is NMR visible is the same. Thus, it appears that some 10–15% of total ATP and PCr may be present within mitochondria and as a result may be invisible to NMR.

In a recent study, Takami et al. (1988) measured ATP and PCr levels during ischemia by NMR and after tissue extraction. Similar to the present study, they found that the decline in ATP levels was approximately the same for both types of measurements. However, after quantification of the NMR measurement for ATP in normoxic hearts (using ^{23}Na NMR to estimate the intracellular volume), it was found that only 60% of the total ATP content was NMR visible. In contrast, all of the PCr present was found to be fully detectable. This differs from the present study in which the ratio PCr/ATP was found to be the same in normoxic tissue whether measured by NMR or after extraction. The reason for this difference is unclear. Furthermore, Takami et al. were unable to detect ATP in the spectra of intact hearts after 16 min of ischemia when it was still present in the tissue extracts (at 5.7 μmol/g dry wt). This may be due to the limit of detection in intact tissues when NMR is used: in this study PCr was not observed at a level of 2.8 μmol/g dry wt (Table I), and the actual limit may be higher.

In another recent report, the fraction of NMR-observable ATP was found to decrease after ischemia and reperfusion in the heart, but remained constant for PCr (Humphrey et al., 1988). Again, the reason for this difference is not clear. No difference in ATP detection was found during ischemia or after reperfusion in this laboratory (results not shown).

The suggestion that not all of the ATP present is NMR observable is in agreement with the analysis of NMR saturation transfer data, which suggests that ATP is compartmentalized and not fully NMR sensitive (Nunnally & Hollis, 1979; Zuhler et al., 1987).

Measurements of the distribution of P_i after tissue fractionation have shown that the fraction of the total P_i found in the cytosol in 85% in the perfused guinea pig heart (Bunger & Soboll, 1985) and 76% in the perfused rat heart (Kauppinen et al., 1980). If NMR detects cytosolic components only, when the discrepancy reported here is much larger (17%). There are two possible explanations for this difference. First, this may have been due to an overestimation of phosphate levels in this study as the result of hydrolysis of organic phosphates in the acid molybdate assay. Second, the distribution of inorganic phosphate as reported in the above two studies may be in error since P_i was present in the perfusate in both cases. In skeletal muscle of man it has been reported that the rate of decline in P_i following exercise is more rapid than its reappearance as PCr (Taylor et al., 1983), suggesting transportation into a compartment in which it cannot be detected. Since the uptake of P_i into mitochondria is known to be the fastest process involved in oxidative phosphorylation (at least in liver mitochondria; Ligati et al., 1985), uptake into mitochondria could be a plausible explanation for this phenomenon.

A further common suggestion to explain the inability to detect metabolites by NMR is that they are bound to intracellular macromolecules such that their signals are so broad as to not be detectable. This explanation is commonly applied to ADP (not measured here), which is measured by NMR to be at least an order of magnitude below the whole tissue levels. This has been attributed to binding to actin (Dawson, 1982) and also the many other enzymes involving ADP (Iles et al., 1985).

Using the approach of Murphy et al. (1988) (following the relative changes of tissue metabolites with ischemia) allows one to determine whether or not the extent of NMR visibility of a metabolite differs with changes in cellular integrity. Unlike the liver, however, no difference in the measurement of ATP was observed: the relative change in ATP concentration is the same whatever the means of measurement. Therefore in the heart, either the relative distribution of ATP between the cytosol and mitochondria is different than that found in the liver, or the relative rates of ATP depletion are similar in the two compartments.

P_i is at first only partially detected by NMR but then becomes fully detectable (at least in terms of statistically sig-

nificant differences) by 12 min of ischemia. If the unobservable portion is mitochondrial, this would suggest that the mitochondria are no longer able to maintain a concentration gradient for this metabolite at this stage. In addition, changes in the intracellular environment (for example, pH) may render a larger portion of P_i NMR visible through release from binding sites.

The difference in phosphomonoesters is more difficult to explain. The glycolytic intermediates are not thought to be associated with mitochondria. However, since the glycolytic enzymes are thought to form multienzyme complexes that are bound to membranes and (in muscle) actin (Masters, 1981; Clarke et al., 1985), it is possible that metabolites bound to these are not fully NMR detectable. The effect of ischemia on the phosphomonoesters is somewhat different from that of the other phosphate metabolites: fully observed in the normoxic heart, not fully observed after 10 min of ischemia, but fully observable again by 12 min. In the well-oxygenated heart, the level of glycolytic intermediates is relatively low, and the fraction involved with binding to the enzymes may be small. At the onset of ischemia, the glycolytic flux is known to increase and shortly thereafter become inhibited (Rovetto et al., 1975). Thus, the concentration of the sugar phosphates can be expected to increase, as the freeze-extraction data demonstrates. Initially, a fraction of these phosphates may be bound to the glycolytic enzymes and hence are NMR invisible, but then may be released into the cytosol as glycolysis becomes inhibited. In addition, a small contribution to these resonances is made by AMP and (to an even smaller degree) IMP (11%–18%; data not shown). AMP is thought to be largely mitochondrial (Bunger & Sobell, 1988) and as for P_i may remain so during the initial stages of ischemia when its concentration first increases but then may be redistributed as ischemia progresses.

To determine that the differences in observed phosphates are not just an artifact of the pulsing conditions, the T_1's of various metabolites were measured. In order to collect a reasonable amount of signal within a time suitable to monitor metabolic events, it is necessary to excite nuclei at a rate faster than their rate of relaxation (given by the time constant, T_1). If the T_1's should change to different extents during ischemia, then the ratio of intensities will change during the experiment, and metabolites may appear to become NMR invisible through this mechanism alone. Should the T_1's for the phosphomonoesters become longer during the initial stage of ischemia, then these resonances will suffer a greater degree of saturation and appear smaller in intensity. It is very difficult to mimic the conditions present in vivo, but a comparison can be made between different metabolites in solution. One of the most dramatic events occurring in ischemia is the fall in pH: therefore, measurements of T_1's were made in solution at physiological (7.06) and pathological (5.90) pH. The change in pH did not have an effect on the T_1's. Thus differences in saturation effects resulting from pH changes as ischemia progresses are not a likely explanation for the changes in NMR visibility.

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