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DEUTERIUM NUCLEAR MAGNETIC RESONANCE FOR EVALUATING THE METABOLIC STATUS OF LIVERS SUBJECTED TO WARM ISCHEMIA[1]

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Background. **The development of reliable methods for assessing the viability of currently available livers is expected to increase the number of successful transplantations.**

Methods. **^{2}H nuclear magnetic resonance (NMR) was used to search for metabolic markers of ischemia in explanted rat livers. Deuterium oxide (^2H_2O) was used as a source of ^{2}H. A total of 10–80% v/v ^2H_2O was added to homogenates obtained from a liver biopsy and the formation of ^{2}H-labeled metabolites was monitored.**

Results. **Some well-resolved ^{2}H resonances were found in the homogenates from biopsies of warm ischemic liver. Two of these were identified as [3-^{2}H] lactate and [2-^{2}H] lactate, and a linear relationship was found between the ratio of [[2-^{2}H] lactate] to [[3-^{2}H] lactate] and the warm ischemia time. The deuterium incorporation into lactate was explained on the basis of the metabolic events occurring under hypoxic conditions.**

Conclusions. **The experimental results support the application of ^{2}H NMR for a reliable evaluation of the metabolic status of a liver harvested from non-heart-beating donors.**

INTRODUCTION

Accurate methods for assessing organ viability are needed to extend the use of organs from marginal donors in transplantation. The viability of a liver and its postoperative function depend mainly on its metabolic status. Currently available methods for obtaining information on liver function are based on enzymatic analysis (*1*) or high-pressure liquid chromatography (*2*). However, these methods are time-consuming or provide only limited information on organ energetics. ^{31}P-NMR is the most promising technique at present, because it allows for the measurement of phosphorus-containing metabolites by a noninvasive and relatively fast procedure. Many papers report on the use of this technique to assess organ preservation and viability (*3*, *4*). However, several broad resonances occur in ^{31}P-NMR spectra of liver and their interpretation is still a matter of debate (*5*, *6*); in addition, conclusive evidence that high-energy phosphates are a direct indication of organ viability has yet to be reported (*7*). The advantages of monitoring the metabolic status of an organ by NMR has stimulated the development of alternative and more direct spectroscopic methods based on labeled compounds. Among the labeled tracers, deuterated water (^2H_2O) appears very promising due to its fast and free diffusion in tissues and its low cost. Moreover, ^2H_2O offers the advantages of ^{2}H NMR, i.e., minimal interference from resonances of endogenous compounds, a high real-time sensitivity due to fast deuterium relaxation, and a relatively high frequency of resonance (46 MHz at 7 T). ^2H_2O has been used in metabolic studies (*8*, *9*) and we recently demonstrated that deuterium incorporation in lactate in the brain of living rats supplied with ^2H_2O is dependent on O_2 concentration in the air for breathing (*10*). ^2H_2O has also been found to improve the preservation quality of organs for transplantation (*11*). In our study we show that the [3-^{2}H lactate]/[2-^{2}H lactate] ratio produced in liver biopsies incubated in the presence of ^2H_2O is a very sensitive indicator of warm ischemia times before liver explantation and we propose a novel method for establishing the viability of organs for transplantation.

MATERIALS AND METHODS

Deuterium oxide was provided by Sigma Chemical Company (St. Louis, LO). Eurocollins buffer came from Monico (Venice, Italy). Wistar rats (235±10 g) were anesthetized using ethyl ether. Warm ischemia was caused in situ by inducing cardiac arrest with superior and inferior vena cava occlusion. These operations took about 5 min. After a variable warm ischemia time, 1 g liver biopsies were collected and homogenated in 2 ml Eurocollins buffer with a Polytron apparatus (Heidolph, Diax 900) at 12,000 rpm. This step lasted 1 min. After the addition of ^2H_2O (10 or 80% v/v), the homogenate was incubated at 37°C for a certain time (10–30 min), then extracted by perchloric acid. The extract was neutralized by 1 M KOH and centrifuged, and the supernatant was collected. This step took about 20 min. The neutralized extract was frozen in liquid nitrogen and lyophilized. Lyophilization was necessary to remove the excess ^2H_2O because the available NMR instrument did not possess the hardware for suppressing the ^2H_2O signal.

The lyophilized extract was resuspended in 0.5 ml of 0.1 M phosphate buffer at pH 7.4 and transferred to an NMR sample tube. Pyridine-d_5 was added both as chemical shift reference and as an internal concentration standard. Alternatively, the resonance intensities were normalized with respect to ^2H_2O, having previously verified that phosphate solutions of the lyophilized livers have the background ^2H_2O content (8 mM).

^{2}H spectroscopy of the neutralized extracts was performed at 46 MHz in a Bruker MSL 300 wide bore spectrometer run in unlocked

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mode. Shimming was performed on the $^{1}H_2O$ signal. ^{2}H spectra were taken using $\pi/2$ pulses, 800 Hz sweep width, 1 s recycle time. The FID data were processed on an INDY workstation (Silicon) using Felix (MSI) software. Assignments were based on the internal standard and on the values given in the literature (*9*, *10*).

RESULTS AND DISCUSSION

Rat liver homogenates incubated at 37°C in a $^{2}H_2O$-containing medium for a time ranging between 10 and 30 min appeared to be able to generate metabolites containing deuterium nuclei. Figure 1 compares the ^{2}H-NMR spectra of neutralized perchloric extracts of rat livers after 30 min warm ischemia and 30 min incubation in a medium containing 80% $^{2}H_2O$ (spectrum A) or 10% $^{2}H_2O$ (spectrum B). Figure 2 shows the uptake kinetics (uptake index) of ^{2}H in metabolites present in homogenates obtained from livers after various warm ischemia times (0, 10, 30 min warm ischemia) and incubated at 37°C in the presence of 10% $^{2}H_2O$. As a significant ^{2}H uptake index we considered the ratio:

$$R = (\sum A_{metabolites})/A_{2_{H_2O}}$$

Where: $\Sigma\ A_{metabolites}$ is the summation of the resonance areas in the region between −3.2 and −6.8 ppm (taking the up-field resonance of pyridine-d_5 as the chemical shift reference) and $A_{2_{H_2O}}$ is the area of the deuterated water resonance. This last resonance was used to normalize the metabolite areas, since control experiments indicated that all the lyophilized extracts have the background content of deuterated water. According to Figure 2, ^{2}H uptake is rapid and, after a 10-min incubation, a significant concentration of ^{2}H-containing metabolites is detectable. Moreover, despite the decrease in ^{2}H uptake capability with increasing warm ischemia times, a valid metabolic activity is still seen even after 120 min warm ischemia.

The spectra shown in Figure 1 appear well resolved and show some intense resonances. The assignment of these resonances was performed on the basis of ^{1}H-NMR spectra of rat liver perchloric extracts or ^{2}H-NMR spectra previously acquired in brain extracts (*10*) and liver extracts (*9*). Further confirmation of the assignments was obtained from the ^{2}H-NMR spectra of a liver extract diluted 1/1 (v/v) with a concentrated solution (2 M) of the various metabolites, containing ^{2}H in natural abundance and buffered at pH 7.4. From Figure 1 it appears that raising the $^{2}H_2O$ content in the incubation medium to 80% increases the deuterated metabolite concentration and consequently improves the signal-to-noise ratio. In particular, after incubation with 80% $^{2}H_2O$, the S/N for [3-^{2}H] alanine was about 80 (see Fig. 1A). It also appears that, after 30 min of warm ischemia, the resonances due to ([2-^{2}H]lactate (at −3.40 ppm) and ([3-^{2}H] lactate (at −6.19 ppm) are detectable. The deuterium uptake was different in the two lactate positions, but independent of the $^{2}H_2O$ concentration in the incubation medium, the ratio being [3-^{2}H]lactate/[2-^{2}H]lactate=4.38±0.22 (n=3) at 10% $^{2}H_2O$ and 4.31±0.08 (n=3) at 80% $^{2}H_2O$.

Figure 1 (inset) shows the ratio [3-^{2}H]lactate/[2-^{2}H]lactate as a function of warm ischemia time. The variability of these measurements due to sample processing and NMR technique was investigated considering a set of experimental data obtained from the same liver. In this case the organ was divided into five specimens and each of them was processed

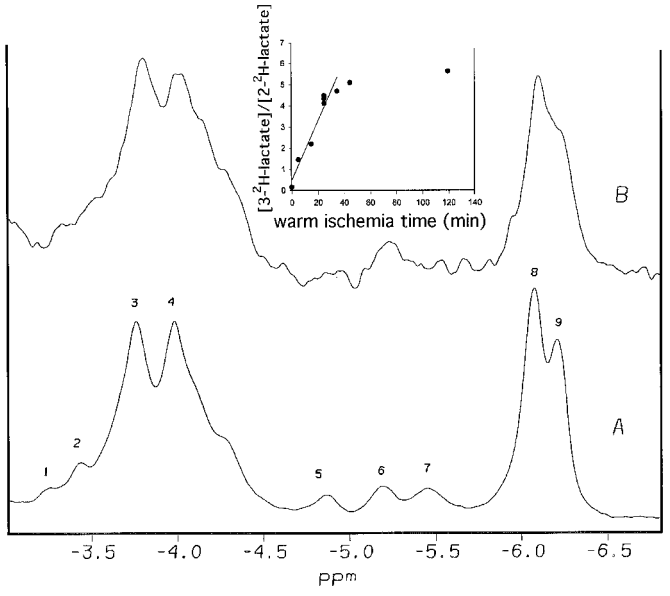

FIGURE 1. ^{2}H-NMR spectra of neutralized and lyophilized extracts of rat liver homogenates. The livers underwent 30 min of warm ischemia followed by 10 min incubation in 80% $^{2}H_2O$ (spectrum A) and 10% $^{2}H_2O$ (spectrum B). Other experimental details and instrumental settings are reported in the text. 1, Unidentified resonance; 2, [2-^{2}H lactate]; 3, [2-^{2}H alanine]; 4, [2-^{2}H glycine]; 5: [3-^{2}H aspartate]; 6, [4-^{2}H glutamate and glutamine]; 7, [3-^{2}H glutamate and glutamine]; 8, [3-^{2}H alanine]; 9, [3-^{2}H lactate]. Inset, [3-^{2}H]lactate/[2-^{2}H]lactate ratio of lyophilized liver extracts obtained under the conditions of spectrum B, as a function of the warm ischemia time induced in the liver.

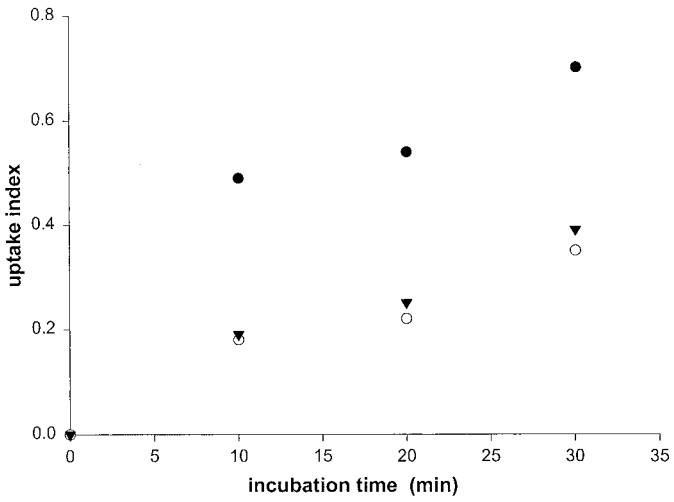

FIGURE 2. Index of ^{2}H uptake by liver homogenates as a function of incubation time in the presence of $^{2}H_2O$. The livers were subjected to different warm ischemia times (10 min [◇], 30 min [▾], and 120 min [○]) before explantation. The homogenates were incubated at 37°C in the presence of 10% $^{2}H_2O$. The spectra were acquired in the lyophilized extracts. The ^{2}H uptake index is calculated as: $(\Sigma\ A_{metabolites})/A_{2_{H_2O}}$ where $\Sigma\ A_{metabolites}$ is the summation of the area of the resonances in the region between −3.2 and −6.8 ppm and A^{2}_{H2O} is the area of the $^{2}H_2O$ resonance.

and measured independently. In particular all the samples were incubated with 10% ^2H_2O for 30 min. Under these conditions, the mean value of the ratio [3-^{2}H]lactate/[2-^{2}H]lactate was 4.32±0.14. From these measurements it appears that the SD is quite low, that is about 3%.

From the inset of Figure 1 it appears that the ratio [3-^{2}H]lactate/[2-^{2}H]lactate increases with the warm ischemia time, showing a saturation value of about 6. The first part of the curve (ischemia time <30 min) can be approximated to a straight line, the slope being about 0.12 min^{-1}. The introduction of ^{2}H in some metabolites is easily explained by our present knowledge of liver metabolism. In particular, the tricarboxylic acid cycle and the associated anaplerotic reactions in the presence of ^2H_2O, including transamination reactions, account for the introduction of the deuterium in [3-^{2}H] aspartate, [3-^{2}H] glutamate, [3-^{2}H] glutamine, [4-^{2}H] glutamate, and [4-^{2}H] glutamine (*8*, *9*). Phosphoenolpyruvate is hydrated to [3-^{2}H] pyruvate, from which [3-^{2}H] alanine is generated via transaminases. Our work was aimed more toward finding a relationship between deuterated metabolites and warm ischemia time than to obtaining biochemical information on the flux of deuterium through the metabolic pathways. That is why we tried to identify the ^{2}H-containing metabolites, whose generation during the ^2H_2O incubation depends on the liver's modification due to warm ischemia. In fact lactate is considered a metabolically important marker of tissue ischemia. In this regard Moldes et al. (*9*) were unable to detect ^{2}H lactate resonances in non-ischemic livers. In a previous paper (*10*), we found that the ratio of [3-^{2}H] lactate to [2-^{2}H] lactate in the brain increases at low P_{O_2} because of lactate dehydrogenase stereospecificity. In fact, the introduction of ^{2}H in position 2 of lactate can only occur by pyruvate reduction by NAD^{2}H catalyzed by lactate dehydrogenase, which requires NADH in pro-(R) configuration. Under ischemic conditions, glycolysis becomes the most important energy source, but NAD^{2}H generated by this metabolic pathway does not have the configuration required by lactate dehydrogenase. As a consequence, [2-^{2}H] lactate decreases. Conversely, [3-^{2}H] lactate is generated mainly from hydration of phosphoenolpyruvate by ^2H_2O. The increase in [3-^{2}H] lactate under ischemic conditions therefore appears to be associated with the stimulation of the glycolytic pathway. A more detailed discussion of the biochemistry underlying the production of different amounts of the two lactate peaks was reported elsewhere (*10*). This discussion was based on results obtained on rat brain, however the metabolic pathways involved in brain lactate metabolism should be present also in liver. In ischemic livers the [3-^{2}H] lactate and [2-^{2}H] lactate resonances are well resolved and their ratio is linearly dependent on the warm ischemia time. This dependence, together with the good reproducibility of the data obtained from various experiments (the SD is <5%), suggest that the [3-^{2}H] lactate/[2-^{2}H] lactate ratio can be used to investigate liver preservation and viability. This method offers several advantages over biochemical or ^{31}P-NMR investigations. In fact, in addition to the advantages inherent in the NMR technique, using this ratio as an index of warm ischemia time requires no calibration and the data obtained in different instrumental conditions can be compared directly.

Moreover, the method we are proposing necessitates only a small amount of liver (about 1 g) and we can estimate that, operating under optimized instrumental conditions, the whole procedure for sample preparation and analysis may take less than 45 min. In conclusion, this study suggests that ^2H_2O can be used as an isotopic tracer in liver viability determination for clinical transplantation.

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