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In vivo measurement of ethanol metabolism in the rat liver using magnetic resonance spectroscopy of hyperpolarized [1-¹³C]pyruvate

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Abstract

[1-¹³C]pyruvate is readily polarizable substrate that has been the subject of numerous magnetic resonance spectroscopy (MRS) studies of in vivo metabolism. In this work, ¹³C-MRS of hyperpolarized [1-¹³C]pyruvate is used to interrogate a metabolic pathway involved in neither aerobic nor anaerobic metabolism. In particular, ethanol consumption leads to altered liver metabolism, which when excessive is associated with adverse medical conditions including fatty liver disease, hepatitis, cirrhosis, and cancer. Here we present a method for noninvasively monitoring this important process in vivo. Following the bolus injection of hyperpolarized [1-¹³C]pyruvate, we demonstrate a significantly increased rat liver lactate production rate with the co-administration of ethanol ($P = 0.0016$ unpaired t-test). The affect is attributable to increased liver nicotinamide adenine dinucleotide (NADH) associated with ethanol metabolism in combination with NADH's role as a coenzyme in pyruvate to lactate conversion. Beyond studies of liver metabolism, this novel in vivo assay of changes in NADH levels makes hyperpolarized [1-¹³C]pyruvate a potentially viable substrate for studying the multiple in vivo metabolic pathways that use NADH (or NAD⁺) as a coenzyme, thus broadening the range of applications that have been discussed in the literature to date.

Introduction

Ethanol is metabolized in the liver via the breakdown of ethanol to acetaldehyde and acetaldehyde to acetate. These two reactions are catalyzed by the enzymes alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH), with both requiring the reduction of the coenzyme nicotinamide adenine dinucleotide (NAD⁺) to NADH (1). Hence, ethanol consumption leads to an accumulation of excess NADH in the liver and when

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Author Contributions: D.M.S., D.M., and Y-F.F. conducted the MRI and MRS experiments with D.M. operating the MR scanner and Y-F.F. and D.M.S. operating the polarizer. J.T. was responsible for the design and construction of the dual-tuned ¹H/¹³C RF coil(18). R.E.H. prepared the pyruvate and solvent formulations and provided advice on the experimental design. D.M and D.M.S analyzed the data. D.M.S. wrote the paper, and D.M.S and A.P. devised and organized the study.

extreme is associated with adverse medical conditions including fatty liver disease, hepatitis, cirrhosis, and hepatocellular carcinoma (2-4).

To date, direct measurements of liver ethanol metabolism have been largely limited to in vitro and ex vivo studies (5,6). In an ex vivo ^1H -MRS study, Jue et al. (6) reported that, following the infusion of 10-mM pyruvate, the rate of lactate formation in the perfused mouse liver increases by a factor 2.8 with the addition of 17-mM solution of ethanol to the perfusion medium. Under the assumption that NADH levels are rate limiting for liver pyruvate-to-lactate conversion, this dramatic increase in lactate production is roughly consistent with the reported in vitro 2.5-fold increase in NADH concentration when ethanol is added to a perfused rat liver model (5).

Here we present a noninvasive method for monitoring this process in vivo. In particular, magnetic resonance imaging and spectroscopy (MRI/MRS) provide unique quantitative anatomical, functional, and biochemical information in vivo (7). Yet, many applications are severely limited by low sensitivity of the method, a consequence of both low metabolite concentrations and the small magnetic energy differences among nuclei relative to the thermal energies of the molecules. At in vivo temperatures and the magnetic field strengths currently used in clinical and research scanners (0.5T – 11T), the nuclear spin polarization (the preferential alignment of the nuclear spins with respect to the external static magnetic field) is typically on the order of a few parts per million (7). Dynamic MRS measurements of in vivo pyruvate metabolism are made possible by the dramatic signal-to-noise ratio (SNR) enhancements provided by hyperpolarization, the creation of nuclear spin polarization well beyond normal thermal equilibrium levels.

In recent years, several hyperpolarization techniques have been developed for in vivo use, the most general of which is dynamic nuclear polarization (DNP) (8). DNP, first discovered in the 1960s, involves mixing the targeted compound (e.g. $[1-^{13}\text{C}]$ pyruvate) with a source of unpaired electrons, placing the mixture in a high magnetic field (e.g. 3T), cooling to 1-2 K, and irradiating with microwaves. By choosing the microwave frequency to coincide with the electron spin resonance, the high electron polarization can be transferred to nearby nuclear spins. The enabling technology for in vivo use, namely the ability to obtain liquid-state hyperpolarization by the rapid heating and removal of the frozen polarized sample via the injection of a hot solvent, is a quite recent development (9). Once ejected from the polarizer, the sample loses polarization at rate given by the nuclear T_1 relaxation time (approximately 30-40s for $[1-^{13}\text{C}]$ pyruvate in vivo), and thus must be quickly injected into the subject and scanned. Nuclear polarization is maintained through chemical reactions, enabling the noninvasive detection of both the injected substrate and downstream metabolic products, and dynamic MRS permits the measurement of both in vivo metabolite concentrations and reaction rates (10).

A bolus injection of $[1-^{13}\text{C}]$ pyruvate, hyperpolarized with DNP to approximately 20%, produces a larger than 10,000 fold increase in SNR over polarization achieved at normal in vivo temperatures. This achievement has been largely used for the in vivo study of both anaerobic and aerobic metabolism by monitoring the metabolic conversion of pyruvate to alanine, lactate, and bicarbonate (10). Enhanced signal for assessment of aerobic and anaerobic metabolism is relevant to improve tumor diagnosis (11) and treatment monitoring (12), and assess cardiovascular pathologies (13), neurovascular and neurological diseases (14,15), and metabolic disorders (16).

Unfortunately, the transient nature of the hyperpolarized signal enhancement can severely hinder the observation of many metabolic pathways. In this report, we circumvent this temporal limitation by focusing on NADH, a coenzyme common to both ethanol and

pyruvate metabolism. Ethanol is used to create an altered rat liver metabolic state characterized by elevated NADH. This skewed metabolic state, which can be generated over an unrestricted time interval, is then interrogated using a bolus injection of hyperpolarized [1-¹³C]pyruvate, a non-toxic easily polarized and rapidly metabolized substrate (9). Using this approach, we report an approximately two-fold increased rate of rat liver pyruvate-to-lactate production in the presence of ethanol. Controls for these experiments included repeated studies using saline injections (same volume and temperature as ethanol boluses) and simultaneous measurements of alanine production in the liver and kidney and of lactate production in the kidney.

Methods

Using dynamic ¹³C MRS following the intravenous bolus injection of 2.5 cc of 100 mM [1-¹³C]pyruvate hyperpolarized to approximately 20% (17), we measured the rates of conversion of pyruvate to lactate and alanine in rat liver (6 animals) and rat kidney (3 of 6 animals) in the presence and absence of ethanol. Four additional control animals received a saline injection in place of the ethanol. The overall timing diagram for the study, showing pyruvate, ethanol, and saline injections in relation to MRS data acquisition windows, is shown in Fig. 1.

Animal Model

Ten male Wistar rats between 370 ± 21 g (mean $\pm$ s.d.) were anesthetized with 1-3% isoflurane in oxygen (~ 1.5 l/min). For each animal, a catheter was inserted into the tail vein for administration of ethanol (or saline) and pyruvate. A rectal probe was used to monitor body temperature, which was in turn controlled using a heated-water blanket placed beneath the animal. Breathing was monitored using a small latex balloon positioned against the animal's chest, and a pulse oximeter recorded heart rate and O₂ saturation. Physiological measurements were recorded every 10 minutes throughout each scanning session. Immediately following the completion of the ¹H-MRI and ¹³C-MRS acquisitions, blood was collected into heparinized Eppendorf tubes for blood alcohol level determination (BAL). After centrifugation, the plasma was extracted and assayed for alcohol content based on direct reaction with the enzyme alcohol oxidase (Analox Instruments, Ltd., U.K.). Following the blood draws, all animals were euthanized in accordance with approved institutional animal care protocols.

[1-¹³C]Pyruvate Hyperpolarization

Using an Oxford Instruments HyperSense DNP polarizer, we polarized multiple samples of a 32 μ l formulation of 14 M [1-¹³C]pyruvate (Isotex Diagnostics Inc, Friendswood TX), 15 mM OX063 trityl radical (Oxford Instruments, Tubney Woods, Abingdon UK), and 3 μ l of a 0.01 M solution of gadoteridol (ProHance, Bracco Diagnostics Inc., Princeton, NJ). The microwave irradiation frequency was 94 GHz, in accordance with the electron resonance frequency for the 3.35 T main magnetic field. The sample temperature was kept at 1.4 K using a vacuum-pumped liquid helium system. After polarization, the frozen sample was rapidly dissolved by injecting a 4 cc solution of 185°C 100 mM NaOH, 40 mM Trizma Pre-set crystals pH 7.6 (Sigma-Aldrich, St. Louis, MO), and 100 mg/l disodium EDTA buffer. The final concentration of the injected pyruvate solution was 100 mM with a pH of 7.4 ± 0.34 (mean $\pm$ s.d.).

MR Imaging and Spectroscopy

Each rat was placed in a custom-build dual-tuned ¹³C-¹H quadrature volume RF coil(18) (80-mm diameter) centered in the bore of a 3T GE Signa scanner (GE Healthcare, Waukesha WI). After the acquisition of conventional ¹H-MRI for anatomical reference (single-shot fast

spin echo, TR/TE=1492/38.6 ms, 256×192 matrix, 2mm slices, 0.47 mm in-plane resolution), 2.5 cc of 100mM of [1-¹³C]pyruvate hyperpolarized to approximately 20% was injected into the tail vein catheter at a rate of 0.25 ml/s. The pyruvate dose was chosen to be approximately three times the dose needed to observe saturated lactate dehydrogenase (LDH) enzyme kinetics in normal rat liver (17). ¹³C spectra (FID acquisition, flip angle = 5°, 5kHz spectral width, 2048 points) were then acquired every 3 s over a 4-minute period from a single 15-mm axial slice through the liver (3 animals) or one 15-mm axial slice through the liver and one 15-mm axial slice through kidneys (3 animals). Following the acquisition of the baseline pyruvate measurements, 1.0 gm/kg of a 20% ethanol solution was injected into the tail vein in order to achieve a targeted steady-state BAL of 100 mg/dl at the time of the second ¹³C MRS acquisition. As a point of reference, BALs in the range of 80-100 mg/dl correspond to the maximum intoxication levels for driving throughout most of the United States. A second bolus of hyperpolarized pyruvate was injected into the animal 45 min after the administration of the ethanol, and ¹³C spectra were again acquired every 3 s over the next 4 minutes. Control animals (N = 4) were scanned using the same procedures with the exception that the ethanol injection was replaced by an injection of normal saline (same volume and temperature as the ethanol injection).

Data and Statistical Analysis

Spectra from each acquisition were phased, corrected for B₀ inhomogeneities, and Pyr, Lac, and Ala levels were then calculated using peak integration. The resulting in vivo time-resolved metabolic signal intensities from all slices following each Pyr bolus injection were then fit using a three-site exchange model in order to estimate the Pyr-to-Lac (k_{pl}) and Pyr-to-Ala (k_{pa}) rate constants. In particular, we used a state-space systems identification formulation (19) parameterized by the metabolic rate constants and T₁ relaxation times. This approach is particularly useful when an explicit set of first-order differential equations representing the system dynamics can be expressed in terms of the unknown parameters. Assuming additive white Gaussian measurement noise, linear grey-box modelling (idgrey, Matlab, Mathworks Inc, Natick, MA) provides optimal estimates of the parameters by minimizing the sum of squares of the prediction error (difference between measured and modelled output) at each time point.

For the case of a three-site chemical exchange among Pyr, Lac, and Ala, the state-space systems model is given by

$$\frac{d}{dt} \begin{bmatrix} M_p \\ M_l \\ M_a \end{bmatrix} = \begin{bmatrix} -(1/T_{1p} + k_{pl} + k_{pa}) & k_{lp} & k_{ap} \\ k_{pl} & -(1/T_{1l} + k_{lp}) & 0 \\ k_{pa} & 0 & -(1/T_{1a} + k_{ap}) \end{bmatrix} \begin{bmatrix} M_p \\ M_l \\ M_a \end{bmatrix} + \begin{bmatrix} u \\ 0 \\ 0 \end{bmatrix}$$

where $M_p(t)$, $M_l(t)$, and $M_a(t)$ are the Pyr, Lac, and Ala longitudinal magnetizations, T_{1p} , T_{1l} , and T_{1a} are the Pyr, Lac, and Ala T₁ relaxation times, k_{pl} , k_{lp} , k_{pa} , and k_{ap} are the Pyr-to-Lac, Lac-to-Pyr, Pyr-to-Ala, and Ala-Pyr apparent metabolic rate constants (12). In these experiments, the input pyruvate bolus function, $u(t)$, was modeled as a trapezoid with a 12 s plateau and 24 s total duration, and the measured magnetization after each small flip angle RF excitation pulse, α , is given by $M(t) = (M_p(t) + M_l(t) + M_a(t)) \sin(\alpha)$.

The model was simplified by setting the backward exchange rates, k_{lp} and k_{ap} , to zero based on the roughly 10:1 in vivo Lac:Pyr and Ala:Pyr concentrations found in normal tissues (20). In addition, the 100 mM pyruvate bolus results in a pyruvate concentration arriving at the liver and kidneys well above normal physiologic conditions, further driving the reactions toward lactate and alanine.

We then performed a statistical analysis of the data using a two-factor repeated-measures analysis-of-variance (ANOVA) with the control group (N = 4) receiving saline and the treatment group (N = 6 for liver and N = 3 for kidney) receiving ethanol and the two measures corresponding to the estimated metabolic rate constants at baseline and at 45-min post-injection of saline or ethanol. Group $\times$ time interactions were first identified, and for those interactions found to be statistically significant, follow-up unpaired t-tests and nonparametric Mann-Whitney U tests were performed to identify when the groups differed.

Results

Representative MRI images from a control animal, indicating selected slice locations and corresponding dynamic ^{13}C -MRS data sets (spectra acquired every 3 s over a 4-min period from single 15-mm axial slices), are shown in Fig. 2. Because the ^{13}C MRS data were not spatially localized within a given slice, the liver and kidney slices contained vascular and structures in addition to the targeted organs. Thus, the dynamic MRS spectra should be considered estimates of the true temporal variations of the organ-specific metabolite levels following the pyruvate bolus injection.

Figure 3 shows pyruvate, lactate, and alanine levels versus liver time curves, computed by peak integration of the phased spectra acquired at baseline and 45 min post-ethanol injection from a representative animal. Increased post- versus pre-ethanol injection lactate signal is clearly visible. We then obtained quantitative measurements of this effect for all animals using the three-site exchange model described in Methods to estimate pyruvate-to-lactate and pyruvate-alanine rate constants from the slices through the rat kidneys and liver(20), and estimated apparent pyruvate-to-lactate and pyruvate-to-alanine metabolic rate constants from the liver (N = 6) and kidney (N = 4) slices pre- and post-ethanol injection and pre- and post-saline injection (controls, N = 4) are summarized in Figs. 4 and 5. In particular, the average apparent liver pyruvate-to-lactate rate constants were $0.014 \pm 0.003 \text{ s}^{-1}$ (baseline), $0.029 \pm 0.007 \text{ s}^{-1}$ (post-ethanol), and $0.012 \pm 0.001 \text{ s}^{-1}$ (post-saline). The average apparent liver pyruvate-alanine rate constants were $0.008 \pm 0.002 \text{ s}^{-1}$ (baseline), $0.008 \pm 0.002 \text{ s}^{-1}$ (post-ethanol), and $0.010 \pm 0.001 \text{ s}^{-1}$ (post-saline). For the kidney, the average apparent pyruvate-to-lactate rate constants were $0.012 \pm 0.002 \text{ s}^{-1}$ (baseline), $0.015 \pm 0.003 \text{ s}^{-1}$ (post-ethanol), and $0.011 \pm 0.001 \text{ s}^{-1}$ (post-saline) and average apparent pyruvate-alanine rate constants were $0.010 \pm 0.002 \text{ s}^{-1}$ (baseline), $0.011 \pm 0.002 \text{ s}^{-1}$ (post-ethanol), and $0.008 \pm 0.001 \text{ s}^{-1}$ (post-saline). Blood alcohol levels at the end of those scanning sessions that included ethanol injections ranged from 84.4 to 116.2 mg/dl.

Starting with lactate production in the liver slice, a significant group $\times$ time interaction was identified ($P = 0.0027$) using the two-factor repeated-measures ANOVA with both an unpaired t-test ($P = 0.0016$) and Mann-Whitney test ($P = 0.01$) showing the effect was due to the ethanol injection rather than the baseline measurements. Repeating the analysis for alanine production revealed no statistically significant findings. The interaction between lactate and alanine measurements showed significant group interactions ($P = 0.0019$) due to differences in lactate and not alanine ($P = 0.0027$ unpaired t-test, $P = 0.01$ Mann-Whitney).

With respect to the data from the kidney slice, all statistical inferences should be viewed with caution due to the small sample size. Applying the same statistical procedures as used for the liver slice, lactate production in the kidney slice showed a statistically significant group $\times$ time interaction ($P = 0.0075$), with marginal differences in post-ethanol lactate production identified by the unpaired t-test ($P = 0.047$) and the more conservative Mann-Whitney test ($P = 0.077$). Smaller effects are expected for the kidney because ADH levels in the kidney are $1/30^{\text{th}}$ of those in the rat liver(21). However, statistical inferences with respect to the kidney data should be viewed with caution due to the small sample size. No

statistically significant group $\times$ time interactions were found with respect to kidney alanine production.

A final analysis comparing the kidney and liver data showed a significant group $\times$ time interaction ($P = 0.001$) due to the post-ethanol injection measurement of liver lactate production being disproportionately high compared with that in the kidney or controls. The estimated increase in liver lactate production with ethanol was $R_L = (\text{post-ethanol pyruvate-to-lactate rate constant})/(\text{baseline pyruvate-to-lactate rate constant}) = 2.0 (\pm 0.5 \text{ s.e.m})$ compared with $R_L = 1.3 (\pm 0.2 \text{ s.e.m})$ in the kidney slice.

Discussion

This relatively simple rodent model produces a large reproducible change in liver lactate production with minimal changes detected in liver alanine, kidney lactate, and kidney alanine. These results demonstrate that, at least in the case of the liver, the rate-limiting step for the conversion of a pyruvate bolus to lactate is not lactate dehydrogenase (LDH) activity but rather NADH availability. From a technical development perspective, measurement of the conversion of pyruvate to lactate as modulated by ethanol is an ideal animal model to validate new in vivo hyperpolarized ^{13}C -MRS and magnetic resonance spectroscopic imaging (MRSI) pulse sequences (22,23) and associated metabolic modeling algorithms. Dynamic ^{13}C -MRSI will likely provide improved in vivo rate-constant estimates by allowing the analysis of localized spectra from organ-specific regions-of-interest, albeit at a reduced signal-to-noise-ratio (SNR) due to the smaller voxels.

This report further exemplifies an experimental paradigm different from previously published in vivo hyperpolarized ^{13}C -MRS studies in which the short T_1 relaxation time of the labeled substrate was a primary limitation (10). Here, in assessing a non-glycolytic metabolic process, we used a biological manipulation, performed prior to the bolus injection, affecting a coenzyme involved in the metabolism of the hyperpolarized substrate, thus circumventing the critical T_1 time constraint typically limiting which enzymatic pathways can be studied using this technology. The buildup of liver NADH in the presence of ethanol was allowed to occur over 45 minutes. The high NADH levels (and indirectly, ADH and ALDH activities) were then detected via the conversion of pyruvate to lactate, a fast enzymatic reaction.

Day et al. (12) recently reported a study of tumor response to chemotherapy as detected by ^{13}C -MRS of hyperpolarized pyruvate in which decreased lactate production in both cell suspensions and implanted lymphoma tumors in mice occurred following the administration of the chemotherapeutic agent. These findings were explained by the reduction of the entire coenzyme NADH and NAD^+ pool due to PARP inhibition as well as decreases in LDH concentration (12). In contrast, our results suggest that, under the condition of excess pyruvate and LDH, pyruvate-to-lactate conversion rates can also reflect simple changes in the NADH/NAD^+ ratios.

The rodent model presented here also provides initial insights into liver metabolism in vivo at the systems level, potentially applicable to alcoholism-induced conditions including alcoholic fatty liver disease (2,24,25), alcoholic hepatitis, liver cirrhosis (3) and liver cancer (3,4). A related application is the in vivo study of drugs to treat chronic alcoholism including disulfiram, an inhibitor of both the cytosolic form of ALDH (ALDH1) and the mitochondrial form of ALDH (ALDH2) (26,27), and the ALDH2-inhibitor cyanamide(28).

As an indirect assay of changes in ALDH activity, with its concomitant NADH production, hyperpolarized $[1-^{13}\text{C}]$ pyruvate may be applicable for evaluating acetaldehyde's role in oxidative stress (29), with ALDH2 activity essential for cardioprotection from ischemia and

reperfusion-induced damage (30),(31). Nitrate tolerance with chronic nitroglycerin use is linked to downregulation of ALDH2 (32). There are ALDH2-linked cancers (33); and deficiency in ALDH2 both increases the vulnerability of nerve cells to oxidative stress and has been identified as a risk factor for late-onset Alzheimer's disease, synergistically acting with APOE-4 (34).

More generally, the approach of using the bolus injection of hyperpolarized [$1\text{-}^{13}\text{C}$]pyruvate and its subsequent conversion to lactate as an indirect in vivo assay of changes in NADH levels makes pyruvate a potentially viable substrate for studying any of the large number of in vivo metabolic pathways that use NADH (or NAD^+) as a coenzyme.

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Figure 1. Timing diagram for MRI/MRS acquisitions and pyruvate, ethanol, and saline injections.

Figure 2. Magnetic resonance imaging and spectroscopy of a rat model. (a) ^1H -MRI images showing representative coronal (left) and axial slices (right) through rat kidney and liver. (b) Stack plot of dynamic ^{13}C -MRS spectra from a 15 mm axial slice through the rat liver acquired every 3 s following the bolus injection of hyperpolarized $[1-^{13}\text{C}]$ pyruvate.

Figure 3. Ethanol-modulated pyruvate metabolism in rat liver. Representative data, curve fits, and calculated pyruvate-to-lactate (k_{pl}) and pyruvate-to-alanine (k_{pa}) rate constants following the bolus injection of hyperpolarized $[1-^{13}\text{C}]$ pyruvate acquired at (a) baseline and (b) 45 min post ethanol administration.

Figure 4.

Estimated rat liver (a) pyruvate-to-lactate and (b) pyruvate-to-alanine rate constants. A total of 10 animals were studied, $n = 6$ for the ethanol group and $n = 4$ for the control group. An approximately 2-fold increase in lactate production, as compared to the controls, was found following ethanol administration. Blood alcohol levels obtained at the end of each exam are given on (a).

Figure 5. Estimated rat kidney (a) pyruvate-to-lactate and (b) pyruvate-to-alanine rate constants. A total of 7 animals were studied, $n = 3$ for the ethanol group and $n = 4$ for the control group. No statistically significant changes were found, although there is a trend toward increased lactate production with ethanol. Blood alcohol levels obtained at the end of each exam are given on (a).