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***In vivo* assessment of intracellular redox-state in rat liver using hyperpolarized [1-¹³C]alanine**

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Abstract

Purpose—The intracellular lactate to pyruvate concentration ratio is a commonly used tissue assay biomarker of redox, being proportional to free cytosolic [NADH]/[NAD⁺]. In this work, we assess the utility of hyperpolarized [1-¹³C]alanine, and the subsequent detection of the intracellular products of [1-¹³C]pyruvate and [1-¹³C]lactate, as an useful substrate for assessing *in vivo* redox levels in the liver.

Methods—Animal experiments were conducted to measure *in vivo* metabolism at baseline and following ethanol infusion. A solution of 80-mM hyperpolarized [1-¹³C]alanine was injected intravenously at baseline (n=8) and 45-min post-ethanol infusion (n=4), immediately followed by the dynamic acquisition of ¹³C MRS spectra.

Results—*In vivo* rat liver spectra showed peaks from [1-¹³C]alanine and the products of [1-¹³C]lactate, [1-¹³C]pyruvate, and ¹³C-bicarbonate. A significantly increased ¹³C-lactate/¹³C-pyruvate ratio was observed following ethanol infusion (8.46±0.58 at baseline versus 13.58±0.69 post-ethanol, p<0.001) consistent with the increased NADH produced by liver metabolism of ethanol to acetaldehyde and then acetate. A decrease in ¹³C-bicarbonate production was also noted, potentially reflecting ethanol-induced mitochondrial redox changes.

Conclusion—A method to measure *in vivo* tissue redox using hyperpolarized [1-¹³C]alanine is presented, with the validity of the proposed ¹³C-pyruvate/¹³C-lactate metric tested using an ethanol challenge to alter liver redox state.

Keywords

dynamic nuclear polarization; hyperpolarized; alanine; redox; liver

Introduction

Nicotinamide adenine dinucleotide (NAD⁺) and its reduced form (NADH) are essential coenzymes involved in several vital biochemical reactions controlling cellular redox states in two fundamental cellular compartments: cytoplasm and mitochondria. Unlike the protein-bound NAD⁺ and NADH, the ratio of the concentration of free NAD⁺ and NADH reflects intracellular redox-states. Growing evidence has indicated that altered redox and associated oxidative stress are implicated in the etiology of multiple pathological conditions such as cancer (1,2), diabetes (3,4), neurodegenerative diseases (5) and inflammatory processes (6). Moreover, the redox plays a fundamental role in cell survivals (7,8) and cellular signaling (9), and is closely related to the regulation of embryonic stem cell development (10) and aging (11).

Conventionally, two types of methods are used to measure free cellular [NAD⁺]/[NADH]: optical and chemical. The non-invasive optical methods (12,13) directly estimate the free [NAD⁺]/[NADH] but require genetically encoded autofluorescent cells, not viable for many *in vivo* studies. The chemical methods (14) exploit near-equilibrium between NAD-linked dehydrogenase systems and the nucleotide, and indirectly measures the redox from the ratio of the concentrations of the oxidized and reduced metabolites:

$$\frac{[Oxidized\ substrate] \cdot [NADH][H^+]}{[Reduced\ substrate] \cdot [NAD^+]} = K \quad \text{Eq. [1]}$$

For example, [lactate]:[pyruvate] via lactate dehydrogenase (LDH) and [β-hydroxybutyrate]:[acetoacetate] via β-hydroxybutyrate dehydrogenase have been used to estimate free cytoplasmic and mitochondrial redox states, respectively (14). The accuracy of these measurements depends on the stability of intercellular pH, which, for most case, is a relatively safe assumption (15,16). These chemical methods, however, require cell extraction and, thus, cannot be used for *in vivo* studies. The ratio of carbon-13 (¹³C)-labeled lactate and pyruvate, as measured by magnetic resonance spectroscopy (MRS) (17) after administration of ¹³C-labeled glucose, allows non-invasive *in vivo* assessment of redox state (reflecting the intrinsic pool sizes of endogenous lactate and pyruvate), but suffers from low sensitivity and the long measurement times increase sensitivity to potential metabolic perturbations from the ¹³C-labeled substrate.

Hyperpolarized ¹³C-substrate using dynamic nuclear polarization (DNP) in combination with a rapid dissolution technique immediately followed by MRS signal acquisition permits a rapid assessment of enzymatic activities in multiple metabolic pathways *in vivo* with an unprecedented amplification of the MR signals (18). In particular, pyruvate has been the most successful substrate to date for DNP because of its central role connecting multiple cellular metabolic pathways in addition to high polarization and relatively long longitudinal relaxation time (T₁). Although the ¹³C-lactate to ¹³C-pyruvate ratio derived from injected hyperpolarized ¹³C-pyruvate may reflect cellular redox state to some extent (19,20), it does not provide an accurate measure because much of the ¹³C-pyruvate signal comes from the vasculature and a significant lactate is also produced in extracellular space (Figure 1A).

Previously, Keshari *et al* (21) and Bohndiek *et al* (22) assessed cellular redox state by estimating the balance of [NADPH]:[NADP⁺] with intracellular reduction of injected hyperpolarized ¹³C-dehydroascorbic acid (DHA), but the method is also susceptible to the extracellular substrate concentration and can only assess relative changes in redox. A more recent study measured cytosolic [NAD⁺]/[NADH] using hyperpolarized ¹³C-glucose and showed large variations in cytosolic redox in different cancer cells (23). The method provides the quantitative cytosolic redox state, but hyperpolarized ¹³C-glucose suffers a significantly short T₁ even with deuteration and needs to pass through multiple metabolic steps to produce pyruvate and lactate, resulting in low signal-to-noise ratio (SNR) and therefore limitation in *in vivo* studies.

In this work, we propose a quantitative method to measure cytosolic redox state in the rat liver using hyperpolarized [1-¹³C]alanine. Hyperpolarized [1-¹³C]alanine, which is readily transported across the plasma membrane (24), is useful to measure intracellular redox because the level of alanine transaminase (ALT) in the serum is negligible, resulting in almost all ¹³C-pyruvate and ¹³C-lactate labeling occurring in the cytosol, with the ratio of these signals reflective of cytosolic [NAD⁺]/[NADH] (Figure 1B) (25). We demonstrate the feasibility of hyperpolarized [1-¹³C]alanine to measure *in vivo* liver redox in both baseline conditions and following ethanol-induced redox changes.

Methods

Hardware and animal preparation

All MR measurements were performed on a clinical 3T GE Signa PET/MR scanner (GE Healthcare, Waukesha, WI). Eight healthy male Wistar rats (body weight = 365 – 453 g) were prepared for the experiments. Each animal was anesthetized with 2 – 3 % isoflurane in oxygen (~1.5 L/min) and catheterized into the tail vein before placing at the center of the MR bore. A custom-built ¹³C surface coil (single loop, Ø = 28 mm) was placed over the liver for both radiofrequency (RF) excitation and signal acquisition. Animal vital signs were monitored throughout the experiments; respiratory rate was maintained at the levels of 50 – 60 breaths/min by adjusting isoflurane level (26) and body temperature was regulated at 36 – 36.5 °C using a warm water blanket placed underneath the animals. All procedures were approved by the local Institutional Animal Care and Use Committee.

Substrate and dynamic nuclear polarization

Hyperpolarization of the [1-¹³C]alanine was performed using a SPINlab^{TM} clinical DNP polarizer (GE Healthcare, Waukesha, WI) equipped with a 5-T magnet and operated with research fluid paths (GE Healthcare, Waukesha, WI). The optimal microwave irradiation frequency for [1-¹³C]pyruvate (139.93 GHz) was used for [1-¹³C]alanine and the operating temperature was at ~0.8 K. Alanine sample was prepared as described in (25) without gadolinium. For each dissolution, 100 µL of the 6.2-M [1-¹³C]-labeled alanine (Sigma-Aldrich Co., St. Louis, MO) was mixed with 15-mM OX063 and placed in a sample vial, and 16 mL of dissolution media (0.1 g/L of sodium ethylenediaminetetraacetic [EDTA] in water) was injected into the dissolution syringe of the research fluid path. After 7 – 8 h of polarization to ensure the maximum polarization, the sample was dissolved and mixed with

250 μ L of a buffer solution (a mixture of 6.1 mL of 121-mM HCl, 100 mg/L of disodium EDTA, 40-mM sodium phosphate buffer), resulting in a 7.5-mL solution of 80-mM $[1-^{13}\text{C}]$ alanine with pH of 7.5. Independent hyperpolarized phantom experiments were performed to estimate liquid polarization level at the time of dissolution and T_1 of ^{13}C -labeled alanine (27). A dose of 0.8 mmol/kg body weight of the $[1-^{13}\text{C}]$ alanine solution was administered 30 s after the dissolution through the tail vein catheter at a rate of 0.25 mL/s. Up to two alanine solutions were injected per animal.

Data acquisition and reconstruction

All ^{13}C MRS data were acquired immediately following the injection of the hyperpolarized compounds using the dynamic free induction decay sequence with a 10° hard RF pulse (pulse width = 40 μ s, spectral width = 5,000 Hz, 2,048 spectral points, acquisition time = 4 min, temporal resolution = 3 s). Data from the time-resolved spectroscopy were apodized by a 10-Hz Gaussian filter followed by a zero filling by a factor of four and a fast Fourier transform using MATLAB (Mathworks, Natick, MA, USA). Metabolites were quantified by integrating the respective peak in the absorption mode after zero-order phase correction. For spectral display, the baseline roll was subtracted by fitting a spline to the signal-free regions of the smoothed spectrum.

Tissue redox state was calculated from the measured ratio of ^{13}C -lactate and ^{13}C -pyruvate signals using Eq. 2, where the cytosolic pH was assumed as 7.0 and K_{eq} is 1.11×10^{-11} M (14).

$$K_{eq} = \frac{[pyruvate] \cdot [NADH] \cdot [H^+]}{[lactate] \cdot [NAD^+]} \quad \text{Eq. [2]}$$

Intracellular redox status modulation using ethanol

Hepatic metabolism of ethanol results in the production of large amount of cytosolic and mitochondrial NADH leading to alternations in the normal hepatic redox state. To induce a hepatic redox change (28), 1 mL/kg of 20% ethanol was intravenously infused at a rate of 1 mL/min to achieve a targeted steady-state blood alcohol level of approximately 100 mg/dL at the time of the second acquisition using hyperpolarized $[1-^{13}\text{C}]$ alanine (45 min after ethanol infusion) (20). The altered redox was also calculated using Eq. 2 with an assumption of re-establishing equilibrium following the ethanol infusion (29).

Quantification of ^{13}C -enriched lactate in blood by high resolution proton NMR spectroscopy

1 mL of blood sample was collected from separate control (body weight = 509 – 513 g, n = 3) and ethanol-treated (429 – 508 g, n = 3) rats before and 1-min after a bolus injection of 80-mM $[1-^{13}\text{C}]$ alanine. Injection speed, volume, and dosages of alanine solution and ethanol were identical to the imaging studies. For the ethanol-treated group, the second blood samples were collected 45 min after the start of ethanol infusion. The whole blood samples were extracted with 5% perchloric acid. The extracts were then neutralized with potassium

hydroxide, freeze-dried, and reconstituted in D₂O containing 1-mM EDTA and 0.5-mM DSS-D₆ (Chenomx, Canada) for ¹H NMR. High-resolution NMR spectra were acquired at 600 MHz (Bruker BioSpin, Billerica, MA) equipped with a cryoprobe operating at 15 K. NMR spectra were processed using ACD/NMR Processor (ACD/Labs, Toronto, Canada).

Data analysis

A paired Student's t-test with two-tailed analysis ($\alpha = 0.05$) was used for the rats ($n = 4$) measured both at baseline and post-ethanol infusion to assess significant difference in the metabolite ratios with and without ethanol. Additional two-sample t-test with unequal sample sizes and variances (Welch's test) was performed for all measurements ($\alpha = 0.05$, two-tailed analysis, $n = 8$ for baseline, $n = 4$ for post-ethanol). All values are reported as mean $\pm$ standard error. SNR of each metabolite peak was calculated from maximum of the peak divided by the standard deviation of the spectral noise.

Results

In vitro polarization and T₁

Polarization of the [1-¹³C]alanine samples was measured as 13.9 ± 1.1 ($n = 3$) at the beginning of MR measurement (40 – 42 s after dissolution) and estimated as 24.8 ± 1.6 % at the time of dissolution. *In vitro* T₁ of the hyperpolarized alanine was measured as 66.4 ± 1.6 s at 3T, which is comparable to that of [1-¹³C]pyruvate. Due to the small sample volume and the large noise in the polarization monitoring from the SPINlab, the build-up time constant could not be estimated.

In vivo hyperpolarized [1-¹³C]alanine metabolism in liver

As shown in a representative time-averaged (0 – 120 s) spectrum (Figure 2A, B), *in vivo* studies detected [1-¹³C]lactate (185 ppm), [1-¹³C]pyruvate (173 ppm), and ¹³C-bicarbonate (H¹³CO₃⁻, 163 ppm) peaks in rat liver following bolus an intravenous injection of hyperpolarized [1-¹³C]alanine (179 ppm). When normalized by total ¹³C-labeled signals (tC), alanine was the dominant source of the signal, with smaller contributions from the lactate, pyruvate, and bicarbonate peaks (Table 1). The SNRs of metabolite peaks in the time-averaged spectra were 229.2 ± 22.0 for lactate, 28.7 ± 3.1 for pyruvate, and 20.2 ± 2.2 for bicarbonate. The large inter-subject difference in the product-to-tC ratios and the SNRs is probably due to the variations in the peripheral vascular contribution to the measured signal and the relative positions of the surface coil to the liver. ¹³C-lactate/¹³C-pyruvate ratio was measured as 8.46 ± 0.58 for the baseline (no ethanol) case. Although the temporal dynamics of lactate labeling was clearly observed, similar kinetics of pyruvate and bicarbonate were not reliably detected due to the SNR limitation of the time-resolved spectra (Figure 2C), and only the time-averaged spectra were used for further analysis.

Metabolic changes due to ethanol infusion

Figure 3 summarizes the changes in metabolic profile of hyperpolarized [1-¹³C]alanine in rat liver due to the additional metabolism of ethanol. As shown in representative time-averaged spectra (Figure 3A, B) and time-courses of ¹³C-labeled metabolites (Figure 3C, D), lactate labeling moderately increased ($p = 0.16$, paired t-test, $n = 4$) while pyruvate ($p =$

0.05) and bicarbonate ($p = 0.01$) signals significantly decreased 45-min post-ethanol as compared to baseline. In particular, the ^{13}C -lactate/ ^{13}C -pyruvate ratio significantly increased to 13.58 ± 0.69 after ethanol infusion ($p = 0.0008$ for paired t-test, $n = 4$; $p = 0.0007$ for Welch's test, $n = 8$ for baseline and $n = 4$ for post-ethanol groups). ^{13}C -bicarbonate/ ^{13}C -pyruvate was significantly dropped ($p = 0.005$ for paired t-test, $p = 0.007$ for Welch's test). The results are summarized in Table 1, and individual measurements of ^{13}C -lactate/ ^{13}C -pyruvate and ^{13}C -bicarbonate/tC are shown in Figure 3E and 3F.

^1H NMR analysis of alanine and lactate in the blood

High resolution ^1H NMR of the rat blood collected before and after hyperpolarized $[1-^{13}\text{C}]$ alanine injection were analyzed to investigate whether injected $[1-^{13}\text{C}]$ alanine is metabolized extracellularly and in plasma. Representative ^1H NMR spectra of blood are shown in Figure 4. Methyl proton resonances at 1.32 ppm and 1.47 ppm reflecting the J_{HH} spin-spin coupling in non-labeled, endogenous lactate and alanine, respectively. After a bolus injection of $[1-^{13}\text{C}]$ alanine, two sets of outer doublets were observed in the alanine resonance region reflecting the three-bond J_{CH} spin-spin coupling between methyl protons and the ^{13}C at the carboxylate carbon. This confirms the dilution of the injected alanine into the circulating metabolite pool, as expected, in both control and ethanol-treated animals after the bolus injection. Similar outer doublets were not observed in the lactate resonance region confirming that no circulating ^{13}C -enriched alanine was metabolized to pyruvate and subsequently lactate in the blood (Supporting Table S1).

Discussion

^{13}C -Lactate-to- ^{13}C -pyruvate ratio as a biomarker for cytosolic redox state

In this study, we assessed the cytosolic redox state in rat liver *in vivo* using the ^{13}C -lactate/ ^{13}C -pyruvate ratio observed immediately following the intravenously injected hyperpolarized $[1-^{13}\text{C}]$ alanine. The calculated $[\text{NAD}^+]/[\text{NADH}]$ ratio using Eq. 2 was $1,103.4 \pm 81.6$, which is higher but close to the expected level of hepatic redox in as reported in the literature (14,30). The possible over-estimation of the ratio could be due to the difference in intracellular T_1 of pyruvate and lactate (likely, $T_{1, \text{pyruvate}} > T_{1, \text{lactate}}$) as the calculation of $[\text{lactate}]/[\text{pyruvate}]$ in this study assumes an equal intracellular T_1 of the metabolites. Fully validating this hyperpolarized ^{13}C MRS metric is difficult due to the lack of an alternative method to measure redox in the living animal. Instead, we measured both absolute and changes in the rat liver ^{13}C -lactate/ ^{13}C -pyruvate ratio under baseline conditions and following ethanol infusion. In particular, our results show an acute increase in the ^{13}C -lactate/ ^{13}C -pyruvate ratio 45-min after an ethanol injection. As shown in Figure 5, we attribute this change to the elevated cytosolic NADH levels generated in the liver from the conversion of ethanol to acetaldehyde via alcohol dehydrogenase (ADH), assuming no other metabolic difference that affects lactate/pyruvate ratio between the baseline and post-ethanol. The acetaldehyde is further oxidized to acetate in both the cytoplasm and mitochondria via aldehyde dehydrogenase (ALDH) 1 and 2, respectively, generating additional NADH in each cellular compartment (31). The observed ^{13}C -lactate/ ^{13}C -pyruvate ratio in the liver was, therefore, acutely increased as previously reported (28,30). The estimated ethanol-induced redox change in this study (post-ethanol lactate/pyruvate = 13.58,

[NAD⁺]/[NADH] = 668.5 ± 34.5) was less dramatic compared to the literature (19.0 – 119.0 of lactate/pyruvate in liver slices incubated with acetaldehyde (28) and 33.9 of lactate/pyruvate in freeze clamped livers), which could be due to the different experimental settings (dosage of ethanol, post-ethanol delay, etc.) or the significantly reduced post-ethanol hyperpolarized [1-¹³C]pyruvate peak, which becomes more susceptible to the natural abundance ¹³C signal of lipids surrounding the liver (25) and the background noise.

The use of hyperpolarized [1-¹³C]alanine to measure intracellular ¹³C-lactate/¹³C-pyruvate can provide a robust non-invasive measure of tissue redox state but only under the following conditions. First, the majority of lactate and pyruvate labeling are produced in intracellular space. Given very low typical ALT activity in plasma (20 – 60 IU/L) (32,33), alanine conversion to pyruvate in extracellular space is expected to be small, barring cellular ALT released to the blood stream due to hepatocellular damage or necrotic cell death. The contribution of ethanol-induced elevation of ALT activity (34) to the hyperpolarized ¹³C-alanine measurement was also negligible as no sign of [1-¹³C]lactate was detected in the blood serum from the high resolution NMR analysis (Figure 4, Supporting Table S1). Second, the chemical exchange between pyruvate and lactate must be sufficiently fast for the measured signals to reflect the endogenous pool sizes. Studies consistently support that the isotopic exchanges between alanine, pyruvate, and lactate are rapid as compared to the time scale of hyperpolarized ¹³C studies (35–37). Finally, the cytosolic pH must remain stable and not perturbed by the excessive alanine infusion.

Effects of ethanol metabolism in ¹³C-bicarbonate production

Another notable ethanol-induced metabolic change was decreased ¹³C-bicarbonate production, which may result from changes in the mitochondrial redox (see Figure 5), assuming that no metabolic disturbance was induced from the baseline alanine injection. In mitochondria, the production of acetate from acetaldehyde leads to increased levels of acetyl-CoA, inhibiting pyruvate dehydrogenase (PDH) (30). Furthermore, increased generation of mitochondrial NADH also reduces the activity of the TCA cycle, and the acetyl-CoA is diverted to fatty acid synthesis, which also leads to a disruption in gluconeogenesis. However, the utility of bicarbonate labeling as a biomarker for mitochondrial redox requires further investigation.

Limitations and potential improvements

Following the injection of hyperpolarized [1-¹³C]alanine, the production of ¹³C-labeled pyruvate, lactate, and bicarbonate is limited by the activities of the alanine-serine-cysteine (ASC) transporter (24) and intracellular enzymes ALT, LDH, and PDH. In the rat studies presented here, the measured pyruvate, lactate, and bicarbonate signals were dominated by the injected alanine (Table 1). Considering the superior ALT activity in liver and kidney as compared to other organs, the feasibility of the proposed method is likely limited to studies of liver and kidney.

In addition to enzyme and the transporter activities, endogenous metabolite pool sizes are also important contributing factors to the generation of ¹³C-labeled products (34). Under typical conditions, the observed ¹³C-labeled pyruvate and bicarbonate peaks are about 1/10

of that from ^{13}C -lactate. While ^{13}C -bicarbonate production is restricted by PDH activity, the small ^{13}C -pyruvate signal is likely limited by the small intrinsic pyruvate pool size in cytoplasm. Such limitations have not been investigated in this study and, additional experiments varying dosages and/or concentrations of hyperpolarized ^{13}C -alanine would be needed to optimize the SNR and reliability of the redox measurements, especially for the localized redox information obtainable with spectroscopic imaging. One potential way to augment the observed pyruvate and lactate signals might be to co-inject unlabeled pyruvate and/or lactate to increase the corresponding metabolic pool sizes (38). Co-injection of a transaminase partner, α -ketoglutarate, may also increase the sensitivity as it can promote the alanine transamination. Moreover, dynamic analysis with a kinetic model that takes the intracellular T_1 's of lactate and pyruvate and their exchange rate into account can improve the quantification accuracy of $[\text{}^{13}\text{C}\text{-lactate}]/[\text{}^{13}\text{C}\text{-pyruvate}]$.

Potential loss of ^{13}C -lactate to the blood stream is another possible limitation to the use of hyperpolarized ^{13}C -alanine as a redox marker since lactate can be readily exported out of the cells (Figure 1). The serum analysis using ^1H NMR spectroscopy, however, suggests that the ^{13}C -lactate export to extracellular space is negligible within the 1-min observation time window, where most hyperpolarized ^{13}C signals are detected.

Higher polarization and/or faster build-up might be achieved by further optimization in alanine polarization. For example, the relatively low polarization (14 % at 40 s post-dissolution) could be due to the multi-step sample insertion procedure of the SPINlab system (14 steps by default), which may induce crystallization in the sample (rather than a glass).

Potential applications

Hepatic redox changes are associated with multiple liver pathologies including hepatocellular carcinoma (39), nonalcoholic steatohepatitis (40,41), viral hepatitis C (42), inflammation (43), fibrosis (44), and hepatic encephalopathy (45). Moreover, hepatic redox measure is a promising indicator of acute hepatotoxicity resulted from drug-induced ROS (46). Acute and chronic ethanol metabolism results in reduced gluconeogenesis and impaired fatty acid oxidation, with the diversion of carbons into fats driving increased triglyceride production, fatty infiltrations, and ultimately liver damage and failure (47). Moreover, increased levels of mitochondrial NADH result in the increased production of reactive oxygen species (ROS), further contributing to liver deterioration. However, applying hyperpolarized $[1\text{-}^{13}\text{C}]\text{alanine}$ to liver diseases with elevated serum ALT require careful interpretation of $^{13}\text{C}\text{-lactate}/^{13}\text{C}\text{-pyruvate}$ ratio due to the increased extracellular alanine conversion to pyruvate, possibly resulting in non-negligible contribution of extracellular balance of pyruvate and lactate to the measured $^{13}\text{C}\text{-lactate}/^{13}\text{C}\text{-pyruvate}$.

Thus, tools to assess cellular redox non-invasively could be very important in early detection of metabolic abnormalities, monitoring the degree of disease progression, and the response of therapeutics in liver, and $[1\text{-}^{13}\text{C}]\text{alanine}$ is a hyperpolarizable substrate that may meet this challenge. Although alanine is known to have positive regulatory roles in energy supplies (e.g. promoting blood glucose regulation and preventing nighttime hypoglycemia for type-2 diabetes), excessive alanine injection may have detrimental effects including strong cellular

depolarization, suppression of insulin secretion and inhibitory effects on gluconeogenesis. The toxicity and allowable dosage of hyperpolarized alanine need further examination.

Conclusion

We showed the feasibility of using hyperpolarized [1-¹³C]alanine as a substrate to measure *in vivo* cytosolic redox in rat liver, and demonstrated a significant increase of the ¹³C-lactate/¹³C-pyruvate ratio after liver redox modulation using ethanol. The coincident reduction of ¹³C-bicarbonate with ethanol may reflect changes in mitochondrial redox state.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cellular metabolic pathways of hyperpolarized substrates

The ratio of the concentration of intracellular lactate to pyruvate ($[\text{lactate}]/[\text{pyruvate}]$) reflects the cytosolic redox state. (A) When hyperpolarized ^{13}C -pyruvate is infused, the measured ^{13}C -labeled signal (marked in blue) cannot differentiate intracellular lactate (lac^{int}) and pyruvate (pyr^{int}) from those in plasma (lac^{ext} , pyr^{ext}). (B) On the other hand, when hyperpolarized $[1-^{13}\text{C}]$ alanine is injected, pyruvate and lactate labeling (marked in red) reflect only the intracellular compartment.

Figure 2. *In vivo* spectrum of hyperpolarized $[1-^{13}\text{C}]\text{alanine}$ and the products in rat liver
 (A) Dynamic change of the spectrum acquired from a representative rat liver with a temporal resolution of 3 s after an injection of 80-mM hyperpolarized $[1-^{13}\text{C}]\text{alanine}$, (B) time-averaged (0 – 120 s) spectrum clearly shows $[1-^{13}\text{C}]\text{lactate}$, $[1-^{13}\text{C}]\text{pyruvate}$ and ^{13}C -bicarbonate ($\text{H}^{13}\text{CO}_3^-$) produced from the infused $[1-^{13}\text{C}]\text{alanine}$. (C) The time-courses of alanine, pyruvate and lactate were obtained by integrating the peak values of the spectrum at each time point. The SNR of $\text{H}^{13}\text{CO}_3^-$ was not sufficient to acquire a reliable time curve.

Figure 3. Effects of ethanol metabolism in rat liver

Time-averaged (0 – 120 s) spectra at (A) baseline and (B) 45-min post ethanol infusion and the corresponding time-courses (C, D) from a representative rat liver. While the ^{13}C -lactate increase was not significant ($p = 0.16$, paired t-test, $n = 4$), ^{13}C -pyruvate decreased significantly ($p = 0.05$). The changes in (E) the ^{13}C -lactate to ^{13}C -pyruvate ratio and (F) ^{13}C -bicarbonate/tC were significant with both the paired t-test ($n = 4$) and the Welch's t-test ($n = 8$ for baseline, $n = 4$ post-ethanol).

Figure 4. High resolution ^1H NMR spectra in plasma

Blood serum was collected from control and ethanol-treated (45-min post) rats at baseline and 1-min after 80-mM $[1-^{13}\text{C}]$ alanine injection. Whereas the alanine peaks showed additional quartets after the alanine injection due to the J_{CH} coupling, the lactate peaks were comparable to the baseline measurement in both groups, suggesting that the increase of ^{13}C -lactate concentration in plasma at the time of measurement was not significant. All spectra were plotted at the same vertical scale using the peak intensity of DSS internal standard (0.5-mM DSS at 0 ppm) as a reference.

Figure 5. Ethanol-induced redox change in hepatocytes

The oxidation of ethanol via ADH and ALDH1 in the liver induces the elevation of cytosolic NADH, leading an increase in pyruvate to lactate ratio. Further oxidation of acetaldehyde to acetate in mitochondria generates additional NADH, suppressing PDH activity and reducing the TCA cycling and gluconeogenesis. As a result, lactate labeling from hyperpolarized $[1-^{13}\text{C}]$ alanine increases and both pyruvate and bicarbonate signals decrease. The metabolites in green and red indicate the metabolites induced from exogenous ethanol and $[1-^{13}\text{C}]$ alanine, respectively. The green arrows are for the ethanol-induced metabolic changes.

Table 1

Ethanol-induced changes in metabolite ratios.

	Baseline (n = 8)	Post-EtOH (n = 4)
Alanine/tC	0.9907 ± 0.0023	0.9894 ± 0.0041
Lactate/tC	0.0078 ± 0.0020	0.0097 ± 0.0038
Pyruvate/tC	0.0010 ± 0.0002	0.0007 ± 0.0005
Bicarbonate/tC	0.00051 ± 0.00007	0.00014 ± 0.00005
Lactate/Pyruvate	8.46 ± 0.58	13.58 ± 0.69
Bicarbonate/Pyruvate	0.6569 ± 0.1047	0.2228 ± 0.0753