13C Magnetic Resonance Spectroscopic Imaging of Hyperpolarized [1-13C, U-2H5] Ethanol Oxidation can be Used to Assess Aldehyde Dehydrogenase Activity In Vivo

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Purpose: Aldehyde dehydrogenase (ALDH2) is an emerging drug target for the treatment of heart disease, cocaine and alcohol dependence, and conditions caused by genetic polymorphisms in ALDH2. Noninvasive measurement of ALDH2 activity in vivo could inform the development of these drugs and accelerate their translation to the clinic.

Methods: [1-13C, U-2H5] ethanol was hyperpolarized using dynamic nuclear polarization, injected into mice and its oxidation in the liver monitored using 13C MR spectroscopy and spectroscopic imaging.

Results: Oxidation of [1-13C, U-2H5] ethanol to [1-13C] acetate was observed. Saturation of the acetaldehyde resonance, which was below the level of detection in vivo, demonstrated that acetate was produced via acetaldehyde. Irreversible inhibition of ALDH2 activity with disulfiram resulted in a proportional decrease in the amplitude of the acetate resonance.

Conclusion: 13C magnetic resonance spectroscopy measurements of hyperpolarized [1-13C, U-2H5] ethanol oxidation allow real-time assessment of ALDH2 activity in liver in vivo. Magn Reson Med 73:1733–1740, 2015. © The Authors. Magnetic Resonance in Medicine Published by Wiley Periodicals, Inc. on behalf of International Society of Medicine in Resonance. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited.

Key words: hyperpolarized 13C; ethanol; acetate; acetaldehyde; aldehyde dehydrogenase

INTRODUCTION

Ethanol is oxidized by the liver to produce acetate, the majority of which exits via the hepatic vein and is metabolized further in other tissues (1). At low to medium blood concentrations of ethanol (up to 24 mM in humans) the bulk of its oxidation to acetaldehyde and further to acetate is via the coupled reactions catalyzed by the NAD+-dependent dehydrogenases: cytosolic alcohol dehydrogenase (ADH) and mitochondrial aldehyde dehydrogenase (ALDH2) (2, 3). Microsomal NADPH-dependent cytochrome CYP2E1 and peroxisomal H2O2-dependent catalase can also oxidize ethanol to acetaldehyde, their relative contributions increasing, for example, during exposure to high-doses of ethanol and in starvation, respectively (4, 5). Ethanol oxidation by hepatic dehydrogenases causes an acute increase in the NADH/NAD+ ratio, leading to an increase in cytosolic lactate concentrations and disturbances of lipid metabolism, with consequent onset of alcoholic fatty liver disease (2, 6). Adaptive induction of hepatic CYP2E1 activity by chronic ethanol exposure, which increases the liver’s capacity to produce acetaldehyde from ethanol, is accompanied by a decreasing capacity of the liver mitochondria to oxidize this acetaldehyde. This decreased capacity results from functional and structural impairment caused by exposure to the elevated acetaldehyde concentrations (7–9). Inefficient disposal of acetaldehyde and its accumulation, both intrahepatically and extrahepatically, is the direct cause of liver cirrhosis, and is implicated in alcohol-induced cardiomyopathy and pancreatitis, and in some types of cancer, particularly in the upper aerodigestive tract (10, 11).

The capacity to oxidize endogenous and exogenous aldehydes, conferred on extrahepatic tissues by expression...
of ALDH2, is fundamental for their normal function. Pharmacological activation of ALDH2 protects the myocardium from ischemic injury by stimulating the oxidative activity of ALDH2 toward 4-hydroxy-2-nonenal, a toxic aldehyde produced in ischemic heart (12–14). ALDH2 is expressed in brain tissue, where it is essential for the maintenance of dopamine homeostasis and the oxidation of the endoge
nous aldehydes 3,4-dihydroxyphenylacetaldehyde and 3,4-dihydroxyphenylglycolaldehyde. Accumulation of 3,4-
dihydroxyphenylacetaldehyde, 3,4-dihydroxyphenylglyco
aldehyde, and dopamine is neurotoxic and disturbances of their metabolism are implicated in neurodegenerative
disorders such as Parkinson’s disease and in Alzheimer’s
disease (15,16). In addition, dopamine signaling in brain
plays a major role in establishing addictive behaviors and in
triggering relapse (17,18).

The role of ALDH2 in human health is illustrated by the
inability of individuals carrying the ALDH2*2 allele, which
encodes an inactive E487K mutant, to dispose efficiently of
toxic aldehydes. ALDH2*2 allele carriers are at higher risk
of increased morbidity after myocardial infarction and are
more likely to develop late-onset Alzheimer’s disease and
cancers in the upper aerodigestive tract (10,12,19,20). The
number of ALDH2*2 allele carriers worldwide is estimated
to be at least 540 million, comprising approximately 40%
of native East Asian populations (20).

The recently developed ALDH2 activator, Alda-1, was
shown to confer protection against myocardial ischemic
damage in a rat model of infarction (12,13) and a ALDH2-
specific inhibitor, CVT-10216, inhibited addictive substance
seeking and relapse in rat models of cocaine addiction and
alcoholism (17,18). In addition, the catalytic activity of the
E487K ALDH2 mutant can be partially restored by Alda-1,
which could have important implications for clinical man-
agement of diseases affecting carriers of the ALDH2*2 allele
(20). A noninvasive method for measuring ALDH activity in
vivo would inform preclinical development of these drugs
and could accelerate their translation to the clinic.

Much of what is known about ethanol metabolism
derives from ex vivo studies on perfused organs, isolated
hepatocytes, and tissue samples (6,21,22). Investigation of
ethanol metabolism in vivo has been limited to magnetic
resonance spectroscopy (MRS) studies, which have been
used, for example, to investigate the distribution of 13C
label in rat brain metabolite pools following systemic
administration of [1-13C] ethanol (23). The main limitation
of using 13C MRS in this way is its low sensitivity, which
results in relatively low temporal and spatial resolution.

The recent introduction of dissolusion dynamic nuclear
polarization (24), which can increase the sensitiv
ity of 13C MRS by at least four orders of magnitude,
has made possible measurements of the metabolism of
many 13C-labeled substrates in vivo (25,26). In this pro-
cess, the 13C-labeled substrate is mixed with a stable rad-
cal and rapidly frozen to form a glass. The sample is
then cooled to ~1.2 K, at which temperature the electron
spins on the radical become fully polarized. Microwave
irradiation is then used to transfer this electron spin polariza
tion to the 13C nuclear spins (24), following
which the sample is warmed rapidly to room tempera
ture using superheated water, with substantial retention
of the nuclear spin polarization (24). The increase in
polarization is sufficient to allow imaging, using 13C
magnetic resonance spectroscopic imaging, of the hyper-
polarized tracer and its metabolic products following
injection into a biological system. Despite the relatively
rapid decay of the nuclear spin polarization in the liquid
state, which is the principal limitation of the technique,
dissolution dynamic nuclear polarization has already
translated to the clinic, where hyperpolarized [1-13C]
pyruvate was used to detect tumors in the prostate (27).

The activity of ALDH2 has been estimated indirectly
from measurements of hyperpolarized 13C label exchange
between injected [1-13C] pyruvate and endogenous lac-
tate, in the reaction catalyzed by lactate dehydrogenase.
This exchange was increased in rat liver by ethanol
injection, which increased the NADH/NAD+ ratio, and
was decreased by the ALDH2 inhibitor, disulfiram. An
increase in the NADH/NAD+ ratio strongly stimulates
the lactate dehydrogenase-catalyzed exchange (28). How-
ever, this indirect measurement assumes that during
ethanol metabolism the change in the mitochondrial
NADH/NAD+ ratio mirrors its cytosolic equivalent (29).
This is not the case, for example, in physiological states
where the relative contribution of NAD+ -dependent
pathways of ethanol oxidation to acetaldehyde, such as
that catalyzed by CYP2E1, becomes important (3).

We show here that oxidation of hyperpolarized per
deuterated [1-13C] ethanol ([1-13C, U-2H3] ethanol) to
[1-13C] acetate, in the coupled reactions catalyzed by
cytosolic ADH and mitochondrial ALDH2, can be mea-
ured and imaged directly in mouse liver in vivo using
13C MRS/I. The influence of ALDH2 activity on this label
flux was demonstrated by inhibiting the enzyme with
the irreversible inhibitor, disulfiram (22).

METHODS

All chemicals were purchased from Sigma Aldrich (Gil-
lingham, Dorset, UK), unless stated otherwise.

13C T1, Measurements In Vitro

Measurements were made at 11 T (Bruker Avance II+
500 MHz spectrometer (Bruker Spectrospin Ltd., Coven-
try, UK)) using inversion recovery sequences in samples
containing approximately 10 mM [1-13C, U-2H3] ethanol
(Sigma Aldrich (Isotec), St Louis, MO) or 1 M ethanol, or
approximately 0.2 M [U-2H4] acetaldehyde or 0.2 M acet-
aldehyde, or approximately 0.5 M sodium [U-2H3] acetate
or 0.5 M sodium acetate (both neutralized with hydro-
chloric acid) in 10% D2O and 4% bovine serum albumin,
buffered with phosphate-buffered saline to pH 7.5. A
pulse repetition time (TR) of 300 s and 16 delays
between 1 and 200 s were used for measurements with
[1-13C, U-2H3] ethanol, a TR of 50 s and 16 delays
between 1 and 200 s were used for measurements of the
natural abundance 13C signal from 1 M ethanol, a TR of
120 s and 16 delays between 0.25 and 120 s were used
for [U-2H4] acetaldehyde, a TR of 55 s and 16 delays
between 0.5 and 40 s were used for measurements of the
natural abundance 13C signal from acetate and a TR of 270 s and 16 delays between 1 and
260 s were used for measurements of the natural abundance $^{13}$C signal from [U-$^2$H$_3$] ace tone.

Hyperpolarization of [1-$^{13}$C, U-$^2$H$_5$] Ethanol

A 2:3 glycerol: [1-$^{13}$C, U-$^2$H$_5$] ethanol mixture, containing 15 mM trityl radical (OX063; tris[8-carboxy-2,2,6,6-tetra-(hydroxyethyl)-benzo-[1,2-4,5]-bis(1,3)-dithiole-4-yl)-methyl, sodium salt; GE Healthcare, Little Chalfont, UK) and 1.5 mM gadoteric acid (DOTAREM; Guebert, Roissy, France), was polarized for approximately 120 min in a 3.35 T Hypersense polarizer (Oxford Instruments, Abingdon, Oxfordshire, UK) using irradiation at 94.124 GHz. The dissolution buffer (PBS, pH 7.5, 100 mg/L EDTA) was heated to 180°C and pressurized to 10 bar before dissolving the frozen polarized samples. The dissolved sample contained 110 mM [1-$^{13}$C, U-$^2$H$_5$] ethanol. For measurements of the liquid-state polarization, a sample of [1-$^{13}$C, U-$^2$H$_5$] ethanol was polarized and dissolved as described above. Two milliliters of the sample, containing approximately 60 mM [1-$^{13}$C, U-$^2$H$_5$] ethanol were injected into 2 mL of PBS buffer, pH 7.4 in a 10-mm NMR tube placed inside a vertical bore 9.4 T NMR spectrometer with a broadband probe tuned to $^{13}$C (Varian NMR Instruments, Palo Alto, CA). Immediately following injection, and approximately 12 s after dissolution, 180 single transient spectra, with a pulse flip angle of 6° and 8-kHz spectral width, were acquired with a TR of 1 s. Three 90° pulses were then applied to eliminate the remaining hyperpolarization. A $^{13}$C spectrum (the sum of 128 transients) was then acquired using a 6° pulse, a spectral width of 8 kHz, and a TR of 300 s. The temperature of the sample was kept at 37°C. The liquid-state polarization was calculated from the ratio of the maximal integrated intensity of the hyperpolarized [1-$^{13}$C, U-$^2$H$_5$] ethanol signal to the integrated intensity of the thermal equilibrium signal corrected for the number of transients.

$^{13}$C MRS Measurements of Ethanol Oxidation In Vitro

Approximately 300 units (2 mg) of ADH from Saccharomyces cerevisiae were dissolved in 100 µL of 0.05 M sodium phosphate buffer, pH 7.5 and mixed with 1.9 mL of 0.05 M pyrophosphate buffer, pH 9.0, containing 1 mg/mL of bovine serum albumin and 15 mM NAD$^+$ (Roche Applied Science, Basel, Switzerland). The sample was then placed immediately in a 9.4 T NMR spectrometer with a 10-mm broadband probe tuned to $^{13}$C (Varian NMR Instruments, Palo Alto, CA). Within approximately 3 min from placing the tube inside the magnet, and approximately 12 s after dissolution, 2 mL of the dissolved polarized sample containing 110 mM [1-$^{13}$C, U-$^2$H$_5$] ethanol was injected into the tube. A series of four transient spectra (sweep width 16 kHz), which were centered either at 240 ppm (the acetaldehyde region of the spectrum) or at 0 ppm (the ethanol region of the spectrum), were then acquired with a flip angle of 12°. Four acetaldehyde spectra were acquired followed by a single ethanol spectrum (TR 1.0 s) and this sequence was repeated over a period of 320 s. The sample temperature was maintained at 37°C.

$^{13}$C MRS Measurements of Ethanol Oxidation In Vivo

Animal experiments were approved by local ethical review committees and performed in accordance with the Animals (Scientific Procedures) Act of 1986. Female CD1 mice (6–8 weeks old) were used in all experiments. Animals were anaesthetized using 3% isoflurane vapor (Isoflo, Abbotts Laboratories Ltd., Maidenhead, UK) in an oxygen/air mixture. Anesthesia was maintained during the MR experiments with 1–2% isoflurane vapor in an oxygen/air mixture delivered via a face mask. A catheter was inserted in a tail vein and the mouse placed in a heated cradle. Throughout the experiment animal breathing rate and core body temperature were monitored using a Biotrig physiological monitor (Small Animal Instruments, Stony Brook, NY). These were within the range of 60–100 bpm and 36–37°C, respectively. A 20-mm-diameter surface coil (Rapid Biomedical GmbH, Rimpar, Germany) was placed over the liver and the entire assembly placed in a $^{13}$C/$^1$H volume coil (Rapid Biomedical, Germany), in a 7 T horizontal bore magnet (Varian, Palo Alto, CA). Livers were localized in transverse $^1$H images, acquired using a spin-echo pulse sequence (TR, 1.5 s; echo time (TE), 10 ms; field of view, 40 × 40 mm$^2$; data matrix, 128 × 128; slice thickness, 2 mm; 15 slices). Immediately after dissolution, $^{13}$C spectroscopic acquisitions were started and 10 µL of the dissolved sample per gram body weight, typically 240 µL in total, were injected via the tail vein over a period of 2 s. Single transient spectra (sweep width 6 kHz; the delay from the middle of the excitation pulse to beginning of data acquisition was 280 µs) from the entire sensitive volume of the surface coil were acquired using a frequency-selective excitation pulse (450 µs three-lobed sinc pulse) centered either at 180 ppm (the acetate region of the spectrum) or at 60 ppm (the ethanol region of the spectrum) with a nominal flip angle of 10°. A series of ethanol and acetate spectra were acquired from one animal, where four acetate spectra were acquired followed by a single ethanol spectrum (TR 1.0 s) and this pattern was repeated over a period of 120 s (Fig. 1). In animals in which ALDH2 was inhibited with disulfiram ($n=7$), spectra were collected using a repeated acquisition pattern, where nine acetate spectra were acquired followed by a single ethanol spectrum (TR 1.1 s). Within each repeat, every spectral acquisition was preceded by a saturation pulse (1 s hard pulse with a B$_1$ field of 100 Hz) at 100 kHz (control saturations 1 and 2). –1840 Hz (control saturation 3), 1840 Hz (saturation at the acetaldehyde resonance frequency), or at 100 kHz (control saturation 4) from the acetate resonance frequency. In three animals, two chemical shift images (FOV 40 × 40 mm, 32 × 32 data matrix, TR 30 ms, TE 0.58 ms) were acquired, starting 15 s after ethanol injection, where the images were acquired first from acetate and then ethanol. All data were phase and baseline corrected and the signal integrals for ethanol and acetate measured.

Disulfiram Dose-Response Experiments

Animals were given 100 or 600 mg/kg body weight of disulfiram (LKT Laboratories, Inc., St. Paul, MN) as a suspension in 5% w/v of gum arabicum, by oral gavage (30). $^{13}$C MRS experiments were performed approximately 24 h after drug administration. In some experiments, shortly after completion of MRS measurements,
animals were killed and their livers rapidly excised, freeze-clamped, and stored at \(-80^\circ C\) before homogenization and enzyme assay.

**Measurement of Blood Ethanol Level**

An appropriate volume of \([2-^{13}C]\) ethanol, polarized and dissolved to the same concentration as \([1-^{13}C, U-^{2}H_{5}]\) ethanol, was injected into the tail vein of animals that had been anesthetized for 30–45 min. Blood was withdrawn by cardiac puncture, which began 75 s after the start of ethanol injection and was completed within 30 s. Individual blood samples were transferred into separate BD Microtainer FE microcollection tubes (BD, Franklin Lakes, NJ), centrifuged at 800g and the plasma removed and frozen in liquid nitrogen. For NMR spectroscopy, plasma samples were thawed and diluted in 200 mM PBS in \(D_{2}O\), pH 7.5. The buffer contained 100 mM 2,2,3,3-D\(_{4}\) sodium-3-trimethylsilylpropionate as an intensity and chemical shift standard (Cambridge Isotope Laboratories Inc., Andover, MA). \(^{13}C\) spectra (the sum of 1024 transients) were acquired under fully relaxed conditions at 125 MHz (Bruker Avance II 500 MHz spectrometer) into 25,294 data points using a 90° pulse, a sweep width of 5 kHz, and a TR of 60 s. Integrated intensities of the \([2-^{13}C]\) ethanol and \([2-^{13}C]\) acetate resonances were normalized to that of 2,2,3,3-D\(_{4}\) sodium-3-trimethylsilylpropionate and multiplied by the methyl \(^{13}C\) concentration in 100 mM 2,2,3,3-D\(_{4}\) sodium-3-trimethylsilylpropionate (3.33 mM) to convert to acetate or ethanol concentrations.

**Liver Tissue Extraction**

Freeze-clamped livers were homogenized using a manual Potter S homogenizer (Sartorius, Epsom, UK) in seven volumes (mL/gram wet weight of liver) of ice-cold extraction buffer (50 mM Tris-HCl, pH 7.4 containing 0.25 M sucrose, 0.1% v/v 2-mercaptoethanol, 1 mM EDTA, and protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland). Ten strokes of 5 s (each followed by a 5 s pause) at 1000 r.p.m. were used. Homogenates were centrifuged at 800g (Eppendorf 5415D benchtop centrifuge (Eppendorf, Hamburg, Germany)) at 4°C. The supernatant was extracted by the addition of 20% v/v of extraction buffer containing 5% v/v of Triton X-100. The extracts were centrifuged at 16,200g at 4°C to remove debris. Supernatants were kept on ice and used for enzyme assays.

**ALDH2 Activity Assay**

The assay mixture contained 0.5 mM NAD\(^{+}\), 0.1% v/v of 2-mercaptoethanol, 200 mM KCl in 100 mM sodium
pyrophosphate buffer, pH 9.0. The sample of liver extract was preincubated at room temperature for 20 min and the reaction initiated by the addition of propionaldehyde to a final concentration of 20 mM. At this low aldehyde concentration, the assay measures predominantly ALDH2 activity (22,31). The formation of NADH was measured for 5 min after substrate addition as an increase in absorbance at 340 nm using a Sunrise 96-well plate reader (Tecan, Mannedorf, Switzerland). One unit of ALDH activity is defined as the amount of enzyme that oxidizes 1 µmol of propionaldehyde (and reduces 1 µmol of NAD\(^+\)) per min. All assays were carried out at 25°C.

RESULTS

The 1\(^{13}\)C T\(_1\) for \([1-13\text{C}, U-2\text{H}_{5}]\) ethanol was 56.0 s (n = 2; 56.6 and 55.3 s) and 9.9 s (n = 2; 9.8 and 10.0 s) for natural abundance 1\(^{13}\)C in the protonated form. For natural abundance 1\(^{13}\)C in \([U-2\text{H}_{4}]\) acetaldehyde, the T\(_1\) was 18.9 s (n = 2, 18.4 and 19.4 s) and 11.5 s (n = 2; 11.2 and 11.7 s) for natural abundance 1\(^{13}\)C in the protonated form. The T\(_1\) for natural abundance 1\(^{13}\)C in \([U-2\text{H}_{3}]\) acetate was 49.2 ± 1.1 s (SD, n = 4) and 41.6 ± 0.8 s (SD, n = 3) for natural abundance 1\(^{13}\)C in the protonated form. All measurements were made in a PBS buffer, pH 7.5, containing 4% bovine serum albumin.

The T\(_1\) calculated from the decay of the polarization in \([1-13\text{C}, U-2\text{H}_{5}]\) ethanol was 49.4 ± 3.0 s in vitro at 9.4 T (SD, n = 3) and 22.0 ± 2.0 s (SD, n = 8) in vivo at 7.0 T. The polarization build up time constant for dynamic nuclear polarization was 41.2 ± 5.5 min (SD, n = 3) and the liquid-state polarization was 14.1% (n = 2; 11.7 and 16.5%) when measured approximately 12 s after dissolution.

Hyperpolarized \([1-13\text{C}, U-2\text{H}_{5}]\) ethanol (110 mM) was injected into mice via a tail vein catheter (10 µL/g body weight) and a series of \(^{13}\)C spectra were acquired using a surface coil placed over the liver (Fig. 1). These showed oxidation of \([1-13\text{C}, U-2\text{H}_{5}]\) ethanol to produce \([1-13\text{C}]\) acetate (245 ppm; Fig. 1a–c). The \([1-13\text{C}]\) acetaldehyde intermediate, at 209.5 ppm, was not observed, even following treatment of the animals with the irreversible ALDH2 inhibitor, disulfiram (Fig. 1d). Acetaldehyde was observed, with a \(^{2}\text{H}\)–\(^{13}\text{C}\) coupling constant of 27 Hz, following oxidation of \([1-13\text{C}, U-2\text{H}_{5}]\) ethanol in vitro, catalyzed by yeast ADH (Fig. 2). The metabolites downstream from \([1-13\text{C}]\) acetate; \([1-13\text{C}]\) acetylcarnitine (at 175 ppm) (32,33), and H\(^{13}\text{CO}_{3}\) (at 162 ppm) were, in general not detected, although in some animals resonances were observed between 175 and 180 ppm (Fig. 1c).

Treatment of the animals with disulfiram decreased the maximum acetate signal (normalized to the maximum ethanol signal) by approximately 43% at a dose of 100 mg/kg (n = 1) and by 79 ± 8% (SD, n = 3) at 600 mg/kg body weight (Fig. 1c,e). The ALDH2 activity in extracts of livers from control animals was 0.37 ± 0.07 units per gram wet weight (U/gww) (SD, n = 3) and 0.12 ± 0.10 U/gww (SD, n = 3) in animals 24 h after treatment with 600 mg/kg body weight disulfiram, a decrease of 68%.

Introduction of a saturation pulse at the resonance frequency of the unobserved \([1-13\text{C}]\) acetaldehyde resulted in a rapid decrease in the acetate signal intensity, when compared with the time course of the acetate signal in the absence of a saturation pulse, and subsequent recovery when saturation was stopped, demonstrating that the acetate was produced via acetaldehyde (Fig. 3). A fit of the acetate signal intensities to the modified Bloch equations for this system is shown in Supporting Information (Fig. S1). Analysis of the slopes on the log plot between 22 and 33 s (control saturation at −1.8 kHz) and between 33 and
44 s (saturation of the acetaldehyde resonance at $+1.8$ kHz) gave values of $0.08 \pm 0.02$ and $0.18 \pm 0.02 \text{ s}^{-1}$, respectively ($P < 0.01, n = 4$), demonstrating that saturation of the acetaldehyde resonance resulted in a significant increase in the rate of decay of the acetate polarization. Chemical shift images showed that the ethanol and acetate signals were mainly from the liver (Fig. 4).

$^{13}$C MRS measurements on plasma samples withdrawn 75 s after the start of $[2-^{13}$C] ethanol injection gave an $[2-^{13}$C] ethanol concentration of $2.02 \pm 0.58 \text{ mM} (SD, n = 3)$ and an $[2-^{13}$C] acetate concentration of $1.59 \pm 0.61 \text{ mM} (SD, n = 3)$.

**DISCUSSION**

The disulfiram-sensitive, mitochondrial (low $K_m$) isoform of ALDH (ALDH2) is primarily responsible for acetaldehyde oxidation in the hepatocyte during ethanol metabolism and accounts for approximately 60–80% of acetaldehyde oxidation in the liver in both healthy humans (34) and in rodents (22). The other enzymes, including the cytosolic (high $K_m$) ALDH1 isoform, account for the remaining 20–40% (22,31,34,35). Decreasing ALDH activity in this study by 68%, using the irreversible ALDH2 inhibitor disulfiram (22,36), reduced the acetate signal intensity by $\sim 79\%$, and implies, therefore, that the rate of acetate production from hyperpolarized $[1-^{13}$C, $U-^{2}$H$_5$] ethanol in the liver is determined primarily by ALDH2 activity. This reduction in ALDH2 activity assayed in tissue extracts agrees well with a decrease of approximately 67% measured in rat liver 24 h after administration of 600 mg/kg disulfiram (36). Although the production of acetaldehyde from ethanol was not observed, the level of acetaldehyde being too low to detect, applying a saturation pulse at the acetaldehyde resonance frequency and observing a decrease in the acetate signal intensity confirmed that the production of acetate from $[1-^{13}$C, $U-^{2}$H$_5$] ethanol proceeded via acetaldehyde. The longer $T_1$ in $[1-^{13}$C, $U-^{2}$H$_4$] acetaldehyde, as compared to the protonated form, will contribute to preservation of the hyperpolarization during transfer of the $^{13}$C label from ethanol to acetate. Collapse of the 27 Hz $^{2}$H–$^{13}$C coupling in acetaldehyde (Fig. 2), through deuterium decoupling, may enhance direct detection of the acetaldehyde intermediate in vivo. Detection of label flux from ethanol to acetate was also enhanced by the relatively long $T_1$ of the C1 carbon in acetate, which was $49.2 \pm 1.1 \text{ s} (SD, n = 4)$ in $[U-^{2}$H$_3]$ acetate in vitro.

Although the activity of mitochondrial ALDH has been shown to govern the rate of acetaldehyde oxidation during ethanol metabolism in rat liver slices (35), studies in perfused liver indicate that ADH activity appears to play some role in determining the rate of ethanol oxidation to acetate (21). However, the dependence of the $[1-^{13}$C] acetate signal on ALDH2 activity observed here has shown that the flux between ethanol and acetate is determined primarily by ALDH2 activity. This, further, implies that the delivery of ethanol and its transport into the cell
have little influence on the rate of acetate production. The amphipathic nature of ethanol means that it can diffuse freely from the bloodstream into tissues, and therefore, its utilization is less likely to be limited by transport into cells when compared to other hyperpolarized 13C-labeled tracers that require facilitated membrane transport (28). Assuming that water accounts for approximately 74% of body weight in rodents, injection of 10 μL/g body weight of 110 mM ethanol would be expected to give approximately 1.50 mM of ethanol at equilibrium, with approximately equal concentrations in intracellular and extracellular water (23,37). Consistent with this expectation, we measured a blood concentration of 2.02 mM at 75 s after injection of 10 μL/g body weight of 110 mM ethanol.

Perdeuteration of ethanol, while extending T1 by a factor of ~5, to 56 s, and making possible imaging of the hyperpolarized molecule and its subsequent metabolism in vivo (Fig. 4), could affect the rate of ADH-catalyzed oxidation of ethanol to acetaldehyde through a primary kinetic isotope effect (38). There is no such effect in the subsequent oxidation to acetate, catalyzed by ALDH (39). In stopped-flow pre steady-state kinetic studies on horse liver ADH, a primary kinetic isotope effect was shown to result in an approximately 6.2-fold decrease in the initial rate of oxidation of [U-2H5] ethanol to acetaldehyde when compared with the protonated form, reflecting the fact that hydride transfer is rate-limiting under these conditions (38). However, under steady-state conditions, dissociation of the binary enzyme-NADH product has been shown to be rate limiting, and therefore, under these circumstances the isotope effect will be much smaller.

In summary, we have measured the oxidation of ethanol to acetate in vivo in real time using hyperpolarized [1-13C, U-2H5] ethanol and demonstrated that in the liver in vivo this flux is determined predominantly by ALDH2 activity. As intravenous ethanol administration is a clinically approved medical procedure in, for example, the treatment of ethylene glycol poisoning (40) hyperpolarized [1-13C, U-2H5] ethanol could be translated to the clinic, where it could be used as a means of assessing the pharmacodynamic properties of ALDH2-targeted therapies. For example, ALDH2 activators, such as Alda-1, have been proposed for the treatment of Fanconi’s anemia, where the accumulation of noxious aldehydes, and specifically acetaldehyde, are particularly toxic (41). A limitation of the method is the requirement that the tissue contain ADH activity, so, for example, it could not be used directly in heart muscle to measure ALDH2 activity as, similarly to most extrahepatic tissues, it lacks ADH (42). Nevertheless, measurements in liver could still be used to assess the efficacy of these drugs. Direct measurement of drug-modulated ALDH2 activity in heart muscle may, however, be possible preclinically as transgenic mouse lines that have high levels of ADH expression in heart muscle have been generated (43). Measurements with hyperpolarized [1-13C] acetaldehyde would remove the requirement for the presence of ADH activity, however, these are likely to be limited by unacceptable toxicity of the aldehyde (44).

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REFERENCES

1. Lundquist F. Production and utilization of free acetate in man. Nature 1962;193:579–580.
2. Lieber CS. Ethanol metabolism, cirrhosis and alcoholism. Clin Chim Acta 1997;257:59–44.
3. Korsten MA, Matsuzaki S, Feinman L, Lieber CS. High blood acetaldehyde levels after ethanol administration: Difference between alcoholic and nonalcoholic subjects. N Engl J Med 1975;292:386–389.
4. Lieber CS. Microsomal ethanol-oxidizing system (MEOS): the first 30 years (1968–1998)—a review. Alcohol Clin Exp Res 1999;23:991–1007.
5. Handler JA, Thurman RG. Catalase-dependent ethanol oxidation in perfused rat liver. Requirement for fatty-acid-stimulated H2O2 production by peroxisomes. Eur J Biochem 1986;176:477–484.
6. Krebs HA. Freedland RA, Hems R, Stubbs M. Inhibition of hepatic gluconeogenesis by ethanol. Biochem J 1969;112:117–124.
7. Lieber CS, DeCarli LM. Ethanol oxidation by hepatic microsomes: adaptive increase after ethanol feeding. Science 1968;162:917–918.
8. Hasumura Y, Teschke R, Lieber CS. Aldehyde oxidation by hepatic mitochondrial decrease after chronic ethanol consumption. Science 1975;192:727–729.
9. Hoek JB, Cahill A, Pastorino JG. Alcohol and mitochondria: a dysfunctional relationship. Gastroenterology 2002;122:2049–2063.
10. Yokoyama A, Omori T, Yokoyama T. Alcohol and aldehyde dehydrogenase polymorphisms and a new strategy for prevention and screening for cancer in the upper aerodigestive tract in East Asians. Keio J Med 2010;59:115–130.
11. Poschl G, Seitz HK. Alcohol and cancer. Alcohol Alcohol 2004:39:155–165.
12. Chen CH, Budas GR, Churchill EN, Dosanjh MH, Hurley TD, Mochly-Rosen D. Activation of aldehyde dehydrogenase-2 reduces ischaemic damage to the heart. Science 2008;321:1493–1495.
13. Sun L, Ferreira JC, Mochly-Rosen D. ALDH2 activator inhibits human mitochondrial aldehyde dehydrogenase. J Biol Chem 1997;272:18823–18826.
14. Chen CH, Gray MO, Mochly-Rosen D. Cardioprotection from ischemia-reperfusion injury by nitroglycerin tolerance. Sci Transl Med 2013;5:1–10.
15. Trippodo NC, Walsh GM, Frohlich ED. Fluid volumes during onset of spontaneous hypertension in rats. Am J Physiol 1978;235:H52–H55.
16. Trippodo NC, Walsh GM, Frohlich ED. Fluid volumes during onset of spontaneous hypertension in rats. Am J Physiol 1978;235:H52–H55.
17. Tottmar O, Marchner H. Disulfiram as a tool in the studies on the mechanism of human liver mitochondrial aldehyde dehydrogenase. J Biol Chem 1997;272:18823–18826.
18. Trippodo NC, Walsh GM, Frohlich ED. Fluid volumes during onset of spontaneous hypertension in rats. Am J Physiol 1978;235:H52–H55.
19. Trippodo NC, Walsh GM, Frohlich ED. Fluid volumes during onset of spontaneous hypertension in rats. Am J Physiol 1978;235:H52–H55.
20. Trippodo NC, Walsh GM, Frohlich ED. Fluid volumes during onset of spontaneous hypertension in rats. Am J Physiol 1978;235:H52–H55.
21. Yao CT, Lai CL, Hsiieh HS, Chi CW, Yin SJ. Establishment of steady-state metabolism of ethanol in perfused rat liver: the quantitative analysis using kinetic mechanism-based rate equations of alcohol dehydrogenase. Alcohol 2010;44:541–551.
22. Klyosov AA, Rashkovetsky LG, Tahir MK, Keung WM. Possible role of liver cytosolic and mitochondrial aldehyde dehydrogenases in acetaldehyde metabolism. Biochemistry 1996;35:4445–4456.
23. Xiang Y, Shen J. In vivo detection of intermediate metabolic products of [1-13C] ethanol in the brain using 13C MRS. NMR Biomed 2011;24:1054–1062.
24. Ardenkjaer-Larsen JH, Friidlund B, Gram A, Hansson G, Hansson L, Lerche MH, Servin K, Thanning M, Golan K. Increase in signal-to-noise ratio of >10,000fold in liquid-state NMR. Proc Natl Acad Sci USA 2003;100:10158–10163.
25. Gallagher FA, Kettunen MJ, Brindle KM. Biomedical applications of hyperpolarized 13C magnetic resonance imaging. Proc Natl Acad Sci USA 2009;5:285–295.
26. Kurhanewicz J, Vigneron DB, Brindle KM, et al. Analysis of cancer metabolism by imaging hyperpolarized nuclei: prospects for translation to clinical research. Neoplasia 2011;13:81–97.
27. Nelson S, Kurhanewicz J, Vigneron D, et al. Metabolic imaging of patients with prostate cancer using hyperpolarized [1-13C] pyruvate. Proc Natl Acad Sci USA 2013;110:607–612.
28. Spielman DM, Meyer D, Year YF, Tropp J, Hurd RE, Pfefferbaum A. In vivo measurement of aldehyde dehydrogenase-2 activity in rat liver ethanol model using dynamic MRS of hyperpolarized [1-13C] pyruvate. NMR Biomed 2013;26:607–612.
29. Josan S, Xu T, Yen YF, Hurd R, Ferreira J, Chen CH, Mochly-Rosen D, Pfefferbaum A, Mayer D, Spielman D. In vivo measurement of aldehyde dehydrogenase-2 activity in rat liver ethanol model using dynamic MRS of hyperpolarized [1-13C] pyruvate. NMR Biomed 2013;26:607–612.
30. Spielman DM, Mayer D, Year YF, Tropp J, Hurd RE, Pfefferbaum A. In vivo measurement of ethanol metabolism in the rat liver using magnetic resonance spectroscopy of hyperpolarized [1-13C] pyruvate. Magn Reson Med 2009;62:307–313.
31. Eriksson CJ, Marselos M, Koivula T. Role of cytosolic rat liver aldehyde dehydrogenase in the oxidation of acetaldehyde during ethanol metabolism in vivo. Biochem J 1975;152:709–712.
32. Jensen PR, Peitersen T, Karlsson M, In ’t Zandt R, Gisselsson A, Gisselsson A, Hansson G, Meier S, Lerche MH. Tissue-specific short chain fatty acid metabolism and slow metabolic recovery after ischaemia from hyperpolarized NMR in vivo. J Biol Chem 2009;284:36077–36082.
33. Bastiaansen JAM, Cheng T, Mishkovich M, Duarte JMN, Comment A, Gruetter R. In vivo enzymatic activity of acetylCoA synthetase in skeletal muscle revealed by 13C turnover from hyperpolarized [1-13C] acetate to [1-13C] acetyl carnitine. Biochim Biophys Acta 2013;1830:4171–4178.
34. Forte-McRobbie CM, Pietruszko R. Aldehyde dehydrogenase content and composition of human liver. Alcohol 1985;2:375–381.
35. Svanas GW, Weiner H. Aldehyde dehydrogenase activity as the rate-limiting factor for acetaldehyde metabolism in rat liver. Arch Biochem Biophys 1985;236:36–46.
36. Tottmar O, Marchner H. Disulfiram as a tool in the studies on the metabolism of acetaldehyde in rats. Acta Pharmacol Toxicol (Copenh) 1976;38:366–375.
37. Tripoddo NC, Walsh GM, Frohlich ED. Fluid volumes during onset of spontaneous hypertension in rats. Am J Physiol 1978;235:H52–H55.
38. Brooks RI, Shore JD. Effect of substrate structure on the rate of the catalytic step in the liver alcohol dehydrogenase mechanism. Biochemistry 1971;10:3855–3858.
39. Ni L, Shrikh S, Weiner H. Involvement of glutamate 399 and lysine 192 in the mechanism of human liver mitochondrial aldehyde dehydrogenase. J Biol Chem 1997;272:18923–18926.
40. Scalley RD, Ferguson DR, Piccaro JC, Smart ML, Archie TE. Treatment of ethylene glycol poisoning. Am Fam Physician 2002;66:807–812.
41. Langevin F, Crossan GP, Rosado IV, Arends MJ, Patel KJ. Fancd2 of [1-13C] ethanol in the brain using13C MRS. NMR Biomed 2011;24:1054–1062.