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Assessing inflammatory liver injury in an acute CCl₄ model using dynamic 3D metabolic imaging of hyperpolarized [1-¹³C]pyruvate

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Abstract

To facilitate diagnosis and staging of liver disease, sensitive and non-invasive methods for the measurement of liver metabolism are needed. This study used hyperpolarized ¹³C-pyruvate to assess metabolic parameters in a CCl₄-model of liver damage in rats. Dynamic 3D ¹³C CSI data from a volume covering kidney and liver were acquired from 8 control and 10 CCl₄-treated rats. In 12 time points at 5-s temporal resolution, we quantified the signal intensities and established time courses for pyruvate, alanine and lactate. Those measurements were compared to standard liver histology and an alanine transaminase (ALT) enzyme assay using liver tissue from the same animals. All CCl₄-treated, but none of the control animals showed histological liver damage and elevated ALT enzyme levels. In agreement with those results, metabolic imaging revealed an increased alanine-to-pyruvate ratio in liver of CCl₄-treated rats, which is indicative of elevated ALT activity. Similarly, lactate/pyruvate ratios were higher in CCl₄-treated compared to control animals, demonstrating the presence of inflammation. No significant differences in metabolite ratios were observed in kidney or vasculature. Thus this work shows that metabolic imaging using ¹³C-pyruvate can be a successful tool to non-invasively assess liver damage *in vivo*.

Keywords

hyperpolarized ¹³C; CCl₄; liver; inflammation

Introduction

With the signal amplification of multiple orders of magnitude afforded by dissolution dynamic nuclear polarization [1], hyperpolarized ^{13}C magnetic resonance spectroscopy (MRS) and spectroscopic imaging (MRSI) provide a unique opportunity to measure dynamic metabolic processes *in vivo* under normal and pathologic conditions. Numerous studies have investigated metabolic imaging of hyperpolarized pyruvate (Pyr) in cancer and heart disease (see references in [2] and [3]), and it has recently been applied in the first clinical trial of this methodology in prostate cancer patients [4]. However, other than liver cancer [5–10], few studies have reported applications of hyperpolarized ^{13}C compounds in the diseased liver. Using a fatty-liver mouse model of type 2 diabetes, Lee et al. [11] found increased hyperpolarized $[1-^{13}\text{C}]\text{Pyr}$ to $[1-^{13}\text{C}]\text{alanine}$ (Ala) labeling, which also correlated with *ex vivo* hepatic alanine transaminase (ALT) activity. Zandt et al. [12] demonstrated detection of hepatocyte necrosis in a carbon tetrachloride (CCl_4) injured rat liver model using hyperpolarized $[1,4-^{13}\text{C}_2]\text{fumarate}$. Given the current epidemic of obesity and type 2 diabetes mellitus [13], the diagnosis and treatment of associated liver pathologies such as nonalcoholic fatty liver disease (NAFLD) emerged as a major clinical challenge with significant public health consequences [14–17]. NAFLD is broadly categorized into simple hepatic steatosis, a relatively benign condition, and nonalcoholic steatohepatitis (NASH), which occurs in approximately 20% of patients with NAFLD. NASH can have deleterious consequences including cirrhosis, liver failure, and hepatocellular carcinoma and is also associated with cardiovascular related mortality [13, 14, 18]. While hepatic steatosis can be detected by routine ultrasonography, computed tomography, and magnetic resonance imaging (MRI), definitive diagnosis of NASH can currently only be obtained through liver biopsy – an invasive procedure associated with complications in up to 5% of patients and subject to sampling errors [19–22]. Early detection of hepatocyte injury would provide opportunity for treatments aimed at prevention of cirrhosis and the clinical sequelae. The aim of this work was to investigate the use of dynamic metabolic imaging of hyperpolarized $[1-^{13}\text{C}]\text{Pyr}$ for the detection of inflammatory mediated liver damage.

CCl_4 is used widely in experimental models to induce liver injury and can cause liver damage in a dose-dependent manner within hours [23]. In hepatocytes, CCl_4 is metabolized into trichloromethyl radicals (CCl_3^*), which then can react with and inactivate various biomolecules (nucleic acids, proteins, lipids), impairing crucial cellular processes such as lipid metabolism with the potential outcome of fatty degeneration (steatosis) [24]. The damaged hepatocytes generate free radicals, thereby activating Kupffer cells/macrophages to produce pro- and anti-inflammatory cytokines that control development and the progression of liver inflammation and further injury [25].

This work uses a rat model of acute liver damage induced by administration of CCl_4 , investigating the use of hyperpolarized $[1-^{13}\text{C}]\text{Pyr}$ metabolic imaging for the detection of liver injury and validating the MR metrics with histology and tissue enzyme assay.

Methods

Each polarized sample consisted of 40 μL of a mixture of 14-M $[1-^{13}\text{C}]$ pyruvic acid and 15-mM Ox063 trityl radical, to which 3 μL of a 1:50 dilution of Dotarem (Guerbet, France) was added prior to polarization. The sample was polarized using a HyperSense system (Oxford Instruments Molecular Biotoools, Oxford, UK) to achieve approximately 25% liquid-state polarization at dissolution. The polarized sample was dissolved with a solution of 125-mM NaOH mixed with 40-mM Tris buffer, 50-mM NaCl, 0.1-g/L EDTA- Na_2 , and 40-mM ^{12}C -Ala leading to a 125-mM solution of hyperpolarized pyruvate with a pH of approximately 7.5. The final solution also contained 40 mM unlabeled alanine to reduce the effects of pool size in the label exchange between Pyr and Ala and to increase the observed ^{13}C -Ala signal [26]. The hyperpolarized pyruvate solution (125 mM concentration, target dose of 1.25 mmol/kg) was injected into the tail vein at a rate of about 0.25 mL/s.

Healthy male Sprague Dawley rats ($n=18$, 269 ± 21 g body weight) were divided into two groups: control group ($n=8$) and the CCl_4 -treated group ($n=10$). The treated group received CCl_4 through intraperitoneal (i.p.) injection at a dose of 1 mL/kg body weight (dissolved in olive oil in a ratio of 1:1 v/v), while the control group was administered only oil. Imaging was performed 48-72 h post treatment, with the animals from the two groups imaged in alternating order.

For the imaging session, the rats were anesthetized with 1-3 % isoflurane in oxygen (~ 1.5 L/min) and a catheter was inserted in a tail vein. Respiration, temperature, heart rate, and oxygen saturation were monitored throughout the experiment session, with temperature regulated using a warm water blanket placed underneath the animals. Each rat received one injection of the hyperpolarized pyruvate solution followed by a ^{13}C 3D CSI acquisition. All animal procedures were approved by the local Institutional Animal Care and Use Committee.

Enzyme Analysis and Histopathology

Immediately after the imaging session, the animals were euthanized using an injection of Beuthanasia, and for each animal two liver tissue specimens were harvested from the left lateral lobe. One specimen for measuring tissue ALT activity was flash-frozen by immersion in liquid nitrogen at -196°C and stored in falcon tubes at -70°C until processing. At the time of processing, each specimen ($\sim 1.5 \times 1.0 \times 0.5$ cm³) was further divided into 2 equal parts, with the ALT enzyme analysis performed for each part, and the average value then used for the respective animal. Protein concentrations for the tissue samples were determined via the Bradford assay, and then using a commercially available kit (Sigma-Aldrich, St. Louis, MO), ALT activity was determined by a coupled enzyme assay, which results in a colorimetric (570 nm)/fluorometric ($\lambda_{\text{ex}} = 535/\lambda_{\text{em}} = 587$ nm) product, proportional to the pyruvate generated. The other liver specimen was fixed in 10% neutral buffered formalin. After 48 to 72 h of formalin fixation, the fixed liver was trimmed and processed routinely for light microscopic examination. Specifically, 4-micron thick tissue sections (stained with hematoxylin and eosin, and Masson's trichrome) were evaluated by a board-certified veterinary pathologist with an Olympus BX-41 microscope (Olympus, Center Valley, PA) for the following pathologies: hepatic steatosis (intracellular

accumulation of fat in hepatocytes), hepatitis (hepatocellular swelling and/or necrosis, with or without a neutrophilic inflammatory reaction; presence of Mallory bodies; and presence of sinusoidal and/or portocentric fibrosis), and cirrhosis (hepatocellular parenchymal loss with evidence of nodular hepatocellular regeneration; presence of bridging fibrosis; bile ductular proliferation; and presence of portocentric lymphoplasmacytic inflammation). Representative digital photomicrographs were taken using an Axioscope 2 Plus microscope (Carl Zeiss, Thornwood, NY) with a Nikon DS-Ri1 digital microscope camera (Nikon, Melville, NY) and NIS-Elements imaging software (Nikon, Melville, NY).

MR protocol

All experiments were performed on a clinical 3T Signa MR scanner (GE Healthcare, Waukesha, WI) equipped with self-shielded gradients (40 mT/m, 150 mT/m/ms). A custom-built dual-tuned ($^1\text{H}/^{13}\text{C}$) quadrature rat coil (inner diameter=80 mm, length=90 mm), operating at 127.9 MHz and 32.2 MHz, respectively, was used for both RF excitation and signal reception. Single-shot fast spin-echo ^1H MR images with nominal in-plane resolution of 0.47 mm and 2-mm slice thickness were acquired in the axial, sagittal, and coronal planes as anatomical references for prescribing the ^{13}C CSI acquisitions. Additional ^1H 3D spoiled gradient echo (SPGR) images (0.625 mm in-plane resolution, 1.25 mm slice thickness, 96 slices) were also acquired matching the ^{13}C CSI prescription for overlay of the metabolic images.

Dynamic 3D ^{13}C CSI data were acquired from a volume covering both kidney and liver using the 3D spiral CSI sequence described in [27]. Imaging parameters were: FOV=80×80×60 mm³, nominal resolution=5×5×5 mm³, spectral width=280 Hz, 3 x-y interleaves, 12 z-phase encoding steps, 32 echoes, flip angle=6°, TE=2.3 ms, acquisition time=4.5 s, temporal sampling time=5 s, 12 time-frames. The time from dissolution to start of pyruvate injection was 20 s, and the scan was started at the same time as the injection. The CSI data were reconstructed similarly as described in [27], and metabolic maps for pyruvate, lactate and alanine were calculated by integrating the signal within ± 20 Hz around each peak in absorption mode. As a result of spectral undersampling, the metabolites are aliased by different amounts. With the center frequency approximately at Ala, Pyr and Lac were aliased once in opposite directions. Hence, a separate reconstruction was performed for each metabolite, with one of the resonances corrected for the chemical shift phase accrual and reconstructed 'in focus', while the aliased resonances are blurred. The mean time-resolved signal intensities for the metabolites were calculated in ROIs in the liver, kidney and vasculature for each animal. Conversion from pyruvate to alanine and lactate, respectively, were evaluated as the ratio of area under the curve (AUC) of the time courses for the metabolites, which has been shown to be proportional to the respective first-order conversion rates [28]. Statistical significance for the metabolite ratios was assessed using Student's unpaired t-test between the control group and CCl₄-treated group.

Results and Discussion

To assess liver damage in CCl₄-treated rats, liver tissue was examined histologically after the animals had been sacrificed at the conclusion of the experiment. Microscopic examination of liver revealed lesions consistent with CCl₄ exposure. Photomicrographs of

livers from representative control and CCl₄-treated animals are shown in Fig. 1. In the control rat, normal hepatic architecture is noted, with viable hepatocytes between a central vein and a portal triad. In contrast, the liver from a CCl₄-treated rat displays acute coagulation necrosis of hepatocytes surrounding the central vein (centrilobular necrosis), appearing as shrunken, hypereosinophilic cells with loss, condensation and/or fragmentation of their nuclei. Additionally, a thin zone of hepatocytes surrounding the necrotic hepatocytes appears markedly swollen due to accumulation of large, clear, round lipid vacuoles (hepatic steatosis/lipidosis). There is also proliferation of reticuloendothelial cells (including resident Kupffer cells) in the affected centrilobular area. Overall, control animals displayed normal liver histology with no evidence of necrosis and minimal or no background inflammation whereas CCl₄-treated rat livers consistently presented with steatosis (10/10) and acute necrosis (8/10) of centrilobular hepatocytes diffusely, sometimes with neutrophilic inflammation (8/10). The severity of steatosis, inflammation, and necrosis ranged from mild to moderate. The respective scores for the individual animals are given in Table 1. Liver tissue ALT activity (shown in Fig 1c) was also elevated in treated animals compared to control animals (mean±standard error, control: 2.70±0.49; treated: 4.89±0.65, P<0.01). This demonstrates that the CCl₄ model indeed reliably and effectively induced liver damage.

To determine the effect of CCl₄-induced liver damage on metabolic parameters *in vivo*, we acquired metabolic maps after injection of hyperpolarized Pyr. The metabolic maps of Pyr, Ala, and Lac from a representative CCl₄-treated animal in Fig. 2 show the spatial distribution of the metabolites. The maps show a slice through the liver (Fig. 2a) and a slice through the kidney (Fig. 2b) from the acquired 3D volume, and were averages of datasets acquired between 30 and 45 s after start of the injection. Given the high metabolic rate of the liver, the substrate (Pyr) is quickly converted into Lac and Ala. Therefore, Pyr was predominantly detected in the vasculature even at these relatively late time frames. Consequently, a large amount of product signal, in particular for Ala, came from the liver. The spectra for Pyr, Lac and Ala from the liver ROI for the same animal are also shown in Fig. 2c. The metabolite time courses from ROIs in the liver, kidney and vasculature are shown in Fig. 3. The time-courses are averaged over all animals for the treated and the control groups. They illustrate the comparatively lower levels of Pyr in the liver, due to its rapid metabolism, and a very brief but high Pyr peak in the vasculature indicating its rapid delivery and subsequent uptake into surrounding tissue.

The ratio of the AUCs for the conversion of Pyr to Ala (rAUC_{PA}) and to Lac (rAUC_{PL}) from ROIs in liver, kidney and the vasculature for the two groups are shown in Fig. 4. Consistent with the results from the liver tissue ALT activity measurements, rAUC_{PA} in the liver was higher for the treated group compared to the control group (P<0.02): 0.38±0.03 (mean±standard error) vs. 0.28±0.02. In addition, rAUC_{PL} was 0.31±0.03 for the control group and 0.41±0.02 for the treated group. An increased Lac/Pyr ratio is consistent with metabolic changes due to inflammation detected with hyperpolarized Pyr in other animal models [29, 30]. Thus, we were able to detect two parameters of liver tissue damage in CCl₄-treated rats: elevated ALT activity and presence of inflammation. In contrast, there were no significant differences in rAUC_{PA} or rAUC_{PL} between control and treated groups for an ROI in the kidney as no metabolic changes due to CCl₄-treatment are expected in the

kidney ($rAUC_{PA}$: 0.10 ± 0.01 control, 0.08 ± 0.01 treated, $P=0.09$; $rAUC_{PL}$: 0.12 ± 0.01 control, 0.12 ± 0.01 treated, $P=0.87$). Similarly, there were also no significant differences in the vasculature $rAUC_{PA}$ and $rAUC_{PL}$ values between the control and treated groups ($rAUC_{PA}$: 0.024 ± 0.002 control, 0.0021 ± 0.001 treated, $P=0.07$; $rAUC_{PL}$: 0.034 ± 0.004 control, 0.033 ± 0.003 treated, $P=0.80$).

40 mM unlabeled Ala was added to the dissolution buffer based on preliminary, unpublished studies that showed a 20–30% increase in Pyr-to-Ala conversion in healthy rat liver *in vivo* (similar to ref. 26, which presents results of unlabeled Lac added to the dissolution buffer). Co-administration of the unlabeled Ala is likely the reason for the high Ala seen in vasculature in Fig. 2a, arising from the conversion of Pyr to Ala in blood. Hyperpolarized ^{13}C -Ala generated in the blood could also be detected in the liver and potentially adding to the variability in the data. The rationale of using the unlabeled Ala was to remove the effect of pool size differences [26]. However, its application to the use of hyperpolarized Pyr for the detection of liver damage needs further investigation.

While there were differences between the control and treated groups for $rAUC_{PA}$ and tissue ALT enzyme activities in the liver for, there was no significant correlation between the metabolite ratios and ALT enzyme activities ($P=0.38$). A source of variability in the MR metric could be the variation in the prandial state of the animals as it has been shown in both rats *in vivo* [31] and perfused mouse liver [32] that the label exchange between hyperpolarized ^{13}C -Pyr and Ala is reduced in fasted versus non-fasted states. The metric $rAUC$ used to evaluate the conversion of Pyr into Lac and Ala is proportional to the respective first-order conversion rates, but also depends on the product relaxation rate and the respective conversion rate in the reverse direction [28]. Hence, variations in the relaxation rates could contribute to the variability in $rAUC$. The variation in time between CCl_4 administration and the MR imaging session, could potentially result in some variability in the severity of liver injury across the treated animals. However, this variation in treatment time does not explain the lack of significant correlation between the MR metrics and the measured tissue ALT levels. Another potential complicating factor is the method of metabolic arrest, i.e., immersion of the liver samples in liquid nitrogen after euthanizing the animal. A better approach to preserve liver tissues for enzymatic analysis would be using the freeze-clamping technique.

Another limitation of the study is that due to the applied acquisition scheme (constant small excitation flip angle for both substrate and products) together with the analysis assuming a constant metabolic exchange rate throughout the observation window, saturation effects cannot be taken into account. Using a different excitation scheme, by applying effective 90° pulses for the excitation of the metabolic products at each time point, that allows the measurement of saturable kinetics characterized by apparent maximum reaction velocity and Michaelis-Menten constant [33, 34] would improve the metabolic quantitation and could lead to a better correlation between MR metrics and tissue ALT activity.

Conclusion

These results demonstrate that ^{13}C metabolic imaging with hyperpolarized $[1-^{13}\text{C}]$ pyruvate is sensitive to inflammation mediated liver injury. The higher rAUC_{PA} in the CCl_4 -treated group reflected the elevated tissue ALT activity, while the higher rAUC_{PL} indicated inflammation confirmed by histology. Therefore, these MR metrics can potentially serve as non-invasive biomarkers for assessment of liver injury, e.g., identifying the more severe stages of NAFLD. Future work will involve optimization of the imaging to reduce measurement variability and validating the correlation of metabolite ratios with tissue enzyme activity. These optimized methods will then be applied to animal models of NAFLD/NASH that more closely mimic the human disease [35] and, if proven successful, ultimately translated into the clinic.

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Abbreviations used

ALT	alanine transaminase
AUC	area under the curve
CCl_4	carbon tetrachloride
CSI	chemical shift imaging
EDTA	ethylene-diaminetetraacetic acid
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
ROI	region of interest

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Figure 1.

H&E stained photomicrographs of liver (200x magnification) from (a) control and (b) CCl₄-treated rat. Normal liver microarchitecture is seen in a control rat with hepatocytes of normal appearance between portal triads (p.t.) and central veins (c.v.). Note the absence of any inflammatory cells and vacuolar change in hepatocytes. (b) In the liver of a CCl₄-treated rat, there is steatosis (black arrow) and acute necrosis (white arrow) of centrilobular hepatocytes, with neutrophilic inflammation. (c) Liver tissue ALT activity was higher in CCl₄-treated rats (red) than in control animals (blue, * unpaired t-test, $P < 0.02$).

Figure 2.

^{13}C metabolic maps of Pyr, Lac, and Ala superimposed onto corresponding ^1H MRI from a slice through the liver (a) and kidney (b) of a CCl_4 -treated rat (display threshold for the metabolic maps is 15%). The ^{13}C images were averaged over the 3 time frames acquired between 30 and 45 s after start of injection. Representative ROIs for the organs are outlined on the ^1H images. (c) Spectra from the liver ROI. The spectra for Pyr, Lac and Ala are shown separately as each metabolite was aliased differently and reconstructed independently.

Figure 3.

^{13}C metabolite time courses for the CCl₄-treated group (top row) and control group (bottom row) after bolus injection of hyperpolarized [1- ^{13}C]pyruvate, from ROIs in the liver, kidney and vasculature. The time-courses are plotted as mean $\pm$ standard error over all rats in each group.

Figure 4.

Ratios of AUCs for metabolite time courses from ROIs in liver, kidney and vasculature of control (blue) and CCl₄-reated (red) rats. Conversion of hyperpolarized [1-¹³C]pyruvate to both alanine and lactate was higher in the liver of CCl₄-treated animals (* unpaired t-test, $P < 0.02$). There was no statistically significant difference between control and treated groups in kidney or in vasculature ($P > 0.09$).

Grading of the severity of steatosis/lipidosis, necrosis and inflammation for all animals (C:control, T:treated) based on the H&E stained sections. Scoring system: 0=Absent, 1=Minimal, 2=Mild, 3=Moderate, 4=Severe.

Table 1

ID	Lipidosis		Hepatocyte necrosis		Inflammation	
	Centrilobular swelling		Subcapsular	Centrilobular	Neutrophilic Parenchymal	Lymphohistiocytic Portocentric
C1	0		0	0	0	0
C2	0		0	0	0	1
C3	0		0	0	0	1
C4	0		0	0	0	0
C5	0		0	0	0	0
C6	0		0	0	0	0
C7	0		0	0	0	0
C8	0		0	0	0	0
T1	2		1	1	0	1
T2	3		2	3	2	1
T3	2		1	0	0	0
T4	2		1	1	1	1
T5	2		3	2	3	0
T6	1		1	0	1	1
T7	2		0	0	1	1
T8	1		2	1	2	1
T9	1		0	0	1	0
T10	1		2	1	2	1