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Simultaneous investigation of cardiac pyruvate dehydrogenase flux, Krebs cycle metabolism and pH using hyperpolarized [1,2-¹³C₂]pyruvate *in vivo*

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Abstract

¹³C MR spectroscopy studies performed on hearts *ex vivo* and *in vivo* following perfusion of pre-polarized [1-¹³C]pyruvate have shown that changes in pyruvate dehydrogenase (PDH) flux may be monitored non-invasively. However, to allow investigation of Krebs cycle metabolism, the ¹³C label must be placed on the C2 position of pyruvate. Thus the utilization of either C1 or C2 labeled pre-polarized pyruvate as a tracer can only afford a partial view of cardiac pyruvate metabolism in health and disease. If the pre-polarized pyruvate molecules were labeled at both C1 and C2 position, then it would be possible to observe the downstream metabolites that were the results of both PDH flux (¹³CO₂ and H¹³CO₃⁻) and Krebs cycle flux ([5-¹³C]glutamate) with a single dose of the agent. Cardiac pH could also be monitored in the same experiment, but adequate SNR of the ¹³CO₂ resonance may be difficult to obtain *in vivo*. Using an interleaved selective RF pulse acquisition scheme to improve ¹³CO₂ detection, the feasibility of using dual-labeled hyperpolarized [1,2-¹³C₂]pyruvate as a substrate for dynamic cardiac metabolic MRS studies, to allow simultaneous investigation of PDH flux, Krebs cycle flux, and pH was demonstrated *in vivo*.

Keywords

DNP; ¹³C MRS; heart; [1,2-¹³C₂]pyruvate; metabolism; pH

Introduction

Hyperpolarized $[1-^{13}\text{C}]$ pyruvate in solution has been utilized for non-invasive, real time, metabolic assessment in various tissues *in vivo* (1-5). ^{13}C MR spectroscopy (MRS) studies performed on rat hearts *ex vivo* and *in vivo* following infusion of pre-polarized $[1-^{13}\text{C}]$ pyruvate have shown that changes in pyruvate dehydrogenase (PDH) flux due to diabetes or ischemia may be monitored non-invasively (3,5). Myocardial intra-cellular pH (pH_i) may also be monitored by measuring the $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ ratio (3,6). However, pyruvate labeled at the C1 position does not allow investigation of Krebs cycle reactions, since the ^{13}C nucleus is carried by CO_2 following PDH mediated oxidation of pyruvate, and not incorporated into Krebs cycle intermediates. It has recently been shown that pre-polarized $[2-^{13}\text{C}]$ pyruvate can be used to assess Krebs cycle metabolism in normal and ischemic rat hearts (7). In this work, however, the extent to which the $[1-^{13}\text{C}]$ citrate and $[5-^{13}\text{C}]$ glutamate signals were reduced due to changes in Krebs cycle flux, or to reduced upstream ^{13}C flux through PDH, remained unclear. Thus, the utilization of either C1 or C2 labeled pre-polarized pyruvate as a tracer can only afford a partial view of cardiac pyruvate metabolism in health and disease.

If the pre-polarized pyruvate molecules were labeled at both C1 and C2 position, then it would be possible to observe both the down stream metabolites that were the result of PDH flux ($^{13}\text{CO}_2$ and $\text{H}^{13}\text{CO}_3^-$) and Krebs cycle flux ($[5-^{13}\text{C}]$ glutamate) with a single dose of the tracer. Cardiac pH could also be obtained from the same experiment, provided that adequate signal to noise ratio (SNR) of the $^{13}\text{CO}_2$ resonance is obtained. In the normal physiological pH range (7.0 – 7.4), the equilibrium between bicarbonate and CO_2 favors bicarbonate by > 10 to 1. After decarboxylation by PDH, most of the C1 label on pyruvate is rapidly transferred to the $\text{H}^{13}\text{CO}_3^-$ molecule, via the enzyme carbonic anhydrase (CA) and less than 10% of it is observed as $^{13}\text{CO}_2$. Therefore, accurate measurement of the much weaker $^{13}\text{CO}_2$ signal may be a challenge, especially if PDH flux is reduced due to cardiac disease including ischemia or diabetes (6).

Fortunately, carbonic anhydrase has an extraordinarily high turnover rate, near 10^6 s^{-1} in humans in the direction of hydration (8). Thus, given the nominal 2-4 second TR times used in a typical dynamic MRS study, it should be feasible to selectively excite the $^{13}\text{CO}_2$ resonance with a tip angle even as large as 90-degree to yield higher SNR for $^{13}\text{CO}_2$ and not impact the ratio of $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$. Providing an unaltered and higher sensitivity measurement of this ratio should lead to a more robust pH estimate. This strategy may also be realized without compromising the assessment of PDH flux (measurement of total $^{13}\text{CO}_2$ and $\text{H}^{13}\text{CO}_3^-$ signal) (3).

The goal of this study was to evaluate the feasibility of using dual-labeled hyperpolarized $[1,2-^{13}\text{C}_2]$ pyruvate as a substrate for dynamic cardiac metabolic MRS studies, to allow simultaneous investigation of PDH flux, Krebs cycle flux, and cardiac pH *in vivo*. To achieve this goal, we implemented and tested an interleaved acquisition scheme for improved $^{13}\text{CO}_2$ detection. The scheme utilized a spectrally selective RF pulse to selectively excite the $^{13}\text{CO}_2$ resonance, and a non-selective RF pulse to acquire dynamic MRS data from all other resonances of interest.

Methods

MR Hardware, polarizer and compounds

All studies were performed using a 3 T GE MR750 scanner (GE Healthcare, Waukesha, WI) equipped with the multinuclear spectroscopy (MNS) hardware package. A micro-strip dual-tuned ^1H - ^{13}C volume coil with 8 cm inner diameter was used for the phantom measurements (Magvale, San Francisco, CA). A custom build ^{13}C transmit / receive surface coil with ^1H blocking and a diameter of 5" was used in the animal studies. A HyperSense DNP polarizer (Oxford Instruments, Abingdon, UK) was used to polarize the substrates following previously described methods (9) at 3.35 T and 1.4 K. Sodium ^{13}C -bicarbonate (Isotec, Miamisburg, OH) was prepared in glycerol with OX063 trityl radical (Oxford Instruments) as previously described (10). Neat $[1,2\text{-}^{13}\text{C}_2]$ pyruvic acid (Isotec) was doped with 15 mM of OX063 trityl radical (Oxford Instruments) and 1 mM Gd chelate (Prohance®, Bracco International).

For ^{13}C -bicarbonate phantom measurements, ~30 μl of the ^{13}C sodium bicarbonate/glycerol mixture was polarized for ~ 80 minutes and dissolved using 4 ml of de-ionized water/EDTA (100 mg/L); immediately following dissolution, the ^{13}C bicarbonate solution was mixed with 4 ml of 500 mM sodium phosphate buffer (pH 7.25) that contained 6 μg of carbonic anhydrase enzyme (Isozyme II, from bovine erythrocytes, 3,000 W-A units/mg protein, Sigma Aldridge, St. Louis, MO). ~5 ml of this final mixture was then used in each phantom experiment. For T_1 and polarization measurements of pre-polarized $[1,2\text{-}^{13}\text{C}_2]$ pyruvate, 30 μl $[1,2\text{-}^{13}\text{C}_2]$ pyruvic acid mixture was polarized for ~ 60 minutes prior to dissolution with approximately 5 ml of 40 mM TRIS/80 mM NaOH solution, giving a nominal final pyruvate concentration of 80 mM and pH of 7.4 (4). Approximately 3 ml of this mixture was transferred into a syringe and used in each T_1 /polarization measurement.

For the dynamic ^{13}C MRS studies in pigs, 105 μl of $[1,2\text{-}^{13}\text{C}_2]$ pyruvic acid/trityl mixture was polarized for ~ 60 minutes then dissolved with ~6 ml of 100 mM TRIS/250 mM NaOH solution, giving a nominal pyruvate concentration of 250 mM and pH of 7.4. This pyruvate solution was diluted with normal saline to triple the total volume. 15 ml of diluted pyruvate solution was infused into the animal in each experiment.

Hyperpolarized ^{13}C bicarbonate phantom experiments

A pulse-acquire pulse sequence was modified to allow toggling of the excitation RF pulses. A 10 ms spectrally selective RF pulse designed to have 150 Hz pass-band (95%) and 10^{-4} stop-band (400 Hz from the center of the pass-band) was interleaved with a 200 μs hard pulse between transients. Readouts (10,000 Hz / 4096 pts) started immediately after each RF pulse. Dynamic MRS data were acquired using the interleaved RF pulses scheme after the pre-polarized $\text{H}^{13}\text{CO}_3^-$ in solution was placed inside the RF coil ($n = 4$, $\text{TR} = 2$ s, 96 transients). The RF transmitter was centered on the $^{13}\text{CO}_2$ resonance when that the selective RF pulse was used, and it was centered between $\text{H}^{13}\text{CO}_3^-$ and $^{13}\text{CO}_2$ when the hard pulse was used. Nominal tip angles for the selective pulse and the hard pulse were set to 40° and 10° , respectively. MRS data was also acquired using the hard pulse only ($n = 3$, $\text{TR} = 4$ s, 48 transients). The Henderson-Hasselbalch equation was applied to estimate the pH in the

solutions using a pK_a value of 6.15 (11). When $^{13}\text{CO}_2$ signals from the selective RF pulse were used to calculate pH, tip angle and echo time (effective echo time of 5 ms was used) corrections were performed; T_1 corrections were also made for $\text{H}^{13}\text{CO}_3^-$ signals (to account for the polarization decay of $\text{H}^{13}\text{CO}_3^-$ between the transient that $\text{H}^{13}\text{CO}_3^-$ signal was measured and the next transient that selective $^{13}\text{CO}_2$ signal was measured). Echo time corrections were made using T_2^* based on linewidth of $^{13}\text{CO}_2$ measured in the spectra. $\text{H}^{13}\text{CO}_3^- T_1$ was estimated from the experiments in which only the hard pulse was used. Immediately following each MRS experiment, the pH of each solution was also measured using a pH meter (Denver Instruments, Bohemia, NY).

T_1 and polarization measurement of hyperpolarized [1,2- $^{13}\text{C}_2$]pyruvate in solution

The polarization achieved, and the T_1 relaxation time of [1,2- $^{13}\text{C}_2$]pyruvate in solution, were estimated at 3 T by using a pulse-acquire pulse sequence to obtain ^{13}C MRS data from ~3 ml aliquots of pre-polarized substrate immediately following dissolution (5° tip angle, $\text{TR} = 3\text{ s}$, 96 transients, 10,000 Hz / 4096 pts. readout), and also at thermal equilibrium polarization (24 μl of prohance was added to the solution, 90° tip angle, $\text{TR} = 5\text{ s}$, 384 transients). The signal from the first hyperpolarized spectrum and the signal from the thermal equilibrium spectra were used to calculate the polarization enhancement factor. This factor was then multiplied by ^{13}C thermal polarization at 3 T to obtain the percentage polarization (12). The T_1 relaxation time of the substrate was obtained from fitting a mono-exponential function (using Marquardt's non-linear least square routine) to the decay of the hyperpolarized signal observed in the small tip angle acquisition (the T_1 reported was corrected for RF tipping) (13,14).

***In vivo* ^{13}C MRS measurements in pigs**

All animal experiments were carried out under a protocol approved by the institutional animal care and use committee. Animal preparation and handling procedures have been described previously (15). The ^{13}C surface coil was placed over the chest of the pig and its positioning over the heart was confirmed by 3-plane ^1H scout images (acquired using the body coil) that provided visualization of the fiducial markers placed on the coil. Cardiac gated, dynamic MRS data were acquired from 3 animals (~20 kg) using the same pulse sequence and interleaved RF pulse scheme used in the ^{13}C bicarbonate phantom experiments. Data acquisition started at the same time as start of the ~15 s infusion of 15 ml of pre-polarized [1,2- $^{13}\text{C}_2$]pyruvate in solution (a dose of approximately 0.06 mmol/kg). One transient was performed every 2 R-R interval (one complete RF interleave cycle every 4 R-R), resulting in a TR of approximately 1-1.3 s depending on the heart rate. In two of the animal used, MRS data was also acquired using only the hard pulse ($\text{TR} = 4\text{ R-R}$) in a separate study.

Data processing

All MRS data were processed using SAGETM software (GE Healthcare). All hyperpolarized ^{13}C phantom data were apodized by a 5 Hz Gaussian filter in time domain prior to FFT. *In vivo* ^{13}C data were apodized by a 10 Hz Gaussian filter. Peak heights (due to some peak overlaps) were measured from the hyperpolarized $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ phantom

spectra and *in vivo* spectra, while peak integrals were measured from the spectra of pre-polarized [1,2- $^{13}\text{C}_2$]pyruvate in solution for T_1 /polarization calculations.

Results

Representative spectra from experiments with pre-polarized ^{13}C bicarbonate in solution, and the pulse sequence that interleaved excitation RF pulses between a spectrally selective pulse on $^{13}\text{CO}_2$ and a non-selective pulse, are shown in Figure 1 (top). In the transients that the selective excitation pulse was used, only the $^{13}\text{CO}_2$ resonance was detected. Stable and almost identical pH measurement were obtained using $^{13}\text{CO}_2$ signal from either the non-localized RF pulse or the spectrally selective RF pulse (Fig. 1, bottom). The average pH obtained using the interleaved pulse sequence (using $^{13}\text{CO}_2$ signal from the selective pulse, and data taken from 0-60 s of each experiment) was 7.42 ($n = 4$, stdev. = 0.02), and it differed from the pH meter by an average value of 0.07 (pH meter values were lower in all runs, Student's t-test: $p = 0.008$, stdev. = 0.02). The average pH measured in the experiments using only the hard pulse was 7.42 ($n = 3$, stdev. = 0.02) and it differed from the pH meter by a value of 0.06 (pH meter values were lower in all runs, $p = 0.10$, stdev. = 0.04). The deviations of the MRS measurement from the pH meter between the two cohorts (interleaved scheme vs. hard pulse only) were not significant ($p = 0.32$). The ^{13}C bicarbonate T_1 , estimated from the hard pulse experiments, was 34.1 s (stdev. = 1.1, average R^2 from fit = 0.9984).

Polarization build up time constant of [1,2- $^{13}\text{C}_2$]pyruvic acid was approximately 900 s, similar to that of pyruvic acid labeled at either the C1 or C2 position alone and doped with the same concentration of OX63 trityl (7). Representative spectra from the pre-polarized [1,2- $^{13}\text{C}_2$]pyruvate phantom studies are presented in Figure 2. The $J_{\text{C-C}}$ coupling measured was approximately 2.0 ppm. The T_1 values measured for [1,2- $^{13}\text{C}_2$]pyruvate in solution were 56 s (stdev. = 0.7 s) for the C1 label and 44 s (stdev. = 0.7 s) for the C2 label. The polarization levels estimated in these runs (at the start of the acquisition, ~15 s after dissolution) were 14.6 % (stdev. = 1.7 %) for the C1 label and 15.5 % (stdev. = 1.7 %) for the C2 label. The integral sum of each doublet was used for these calculations. The line-shapes of the C1 peaks were narrower in these studies by 30-40%, making the C1 peaks appear much larger in amplitude despite the similar polarization levels achieved for both labels. This difference in line-shape was presumably due to stronger ^{13}C - ^1H coupling experienced by the C2 label.

Figure 3 (top) shows a representative spectrum from the *in vivo* cardiac MRS studies that used [1,2- $^{13}\text{C}_2$]pyruvate and the interleaved RF pulse acquisition scheme. The spectrum shown was acquired ~26 s after the start of the tracer infusion during a transient in which the hard pulse was used; the $^{13}\text{CO}_2$ peak acquired from the next transient using the spectral selective RF pulse is also shown. The [5- ^{13}C]glutamate peak overlapped with the up-field peak of [1- ^{13}C]lactate doublet, while the [1- ^{13}C]acetylcarnitine peak overlapped partially with the down-field peak of the [1- ^{13}C]pyruvate doublet.

The time courses of selected metabolites from this study were plotted (Fig 3. bottom left). The [5- ^{13}C]glutamate signal was calculated as the difference in peak amplitude between the

resonance at 183.2 ppm ($[5-^{13}\text{C}]\text{glutamate}$ + up-field $[1-^{13}\text{C}]\text{lactate}$ doublet) and the resonance at 185.2 (down-field $[1-^{13}\text{C}]\text{lactate}$ doublet). The maximum $\text{H}^{13}\text{CO}_3^-$ to maximum $[1-^{13}\text{C}]\text{pyruvate}$ ratios observed in the three animal studies were 0.057 ($n = 3$ stdev. = 0.009), while the maximum $[5-^{13}\text{C}]\text{glutamate}/[2-^{13}\text{C}]\text{pyruvate}$ ratios were 0.040 (stdev. = 0.004). The time between the peak of the pyruvate substrate signal (the C1 and C2 labels of pyruvate peaked at the same time) to the peak of the metabolite signals were 13 s (stdev. = 1) for $\text{H}^{13}\text{CO}_3^-$ and 14 s (stdev. = 1) for $[5-^{13}\text{C}]\text{glutamate}$. These results were similar to prior studies using pyruvate labeled only at C1 or C2 position in this model and the same tracer dose (15,16). After tip angle and echo time correction, the $^{13}\text{CO}_2$ resonance acquired from the different RF pulse interleaves were very similar both in signal amplitude and time course (Fig 3. bottom right).

To calculate the cardiac pH *in vivo* using the $^{13}\text{CO}_2$ signals acquired with the 40° selective RF pulse, the mean value of $\text{H}^{13}\text{CO}_3^-$ signals from consecutive time points were used to account for the temporal offset between the two interleaves. The resultant pH measurements were plotted in Figure 4. In the initial times points after both $\text{H}^{13}\text{CO}_3^-$ and $^{13}\text{CO}_2$ appeared in the spectra, the pH values were approximately 7.1 – 7.2, agreeing with the published data acquired using the $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ equilibrium (3,6). However, the estimated values gradually increased to 7.5 – 7.6 before polarization decay prevented measurement of the signals. A similar trend was observed in separate studies in the same animals that the interleaved acquisition scheme was not used (pig 1* open diamond and pig 2* open circle).

Discussion

In this study, we demonstrated the feasibility of using hyperpolarized $[1,2-^{13}\text{C}_2]\text{pyruvate}$ as a substrate for dynamic cardiac MRS studies *in vivo*. Both the T_1 relaxation times and polarization levels achieved in solution for this doubly labeled molecule were similar, but slightly lower, to those labeled at either the C1 or the C2 position alone. While it was not the aim of this study to demonstrate the clinical relevance of these measurements, undoubtedly the use of $[1,2-^{13}\text{C}_2]\text{pyruvate}$ to characterize the role of metabolism in heart disease will be advantageous: markers for both substrate selection via the glucose-fatty acid cycle ($\text{H}^{13}\text{CO}_3^-$ and $^{13}\text{CO}_2$) and for incorporation of carbohydrate into the Krebs cycle ($[5-^{13}\text{C}]\text{glutamate}$) will be accessible in a single study, rather than requiring infusion of both $[1-^{13}\text{C}]\text{pyruvate}$ and $[2-^{13}\text{C}]\text{pyruvate}$ in separate experiments. For example, when ^{13}C pyruvate metabolism was examined following 10 min of global ischemia in the isolated perfused heart, separate studies using either $[1-^{13}\text{C}]$ or $[2-^{13}\text{C}]\text{pyruvate}$ were required to determine that under these conditions, PDH flux was maintained and that ischemia had caused a metabolic defect within the Krebs cycle itself (6,7). The same information, requiring only one cohort of animals, could have been obtained from only one study with dual-labeled $[1,2-^{13}\text{C}_2]\text{pyruvate}$. The difference in relaxation times of the two different labels would need to be corrected for if modeling of the data to determine quantitative metabolic fluxes. However, relative changes to metabolic fluxes (i.e. PDH vs. Krebs cycle flux) can be estimated simply by normalizing the metabolite signals to the corresponding substrate ^{13}C label signals ($\text{H}^{13}\text{CO}_3^-/[1-^{13}\text{C}]\text{pyruvate}$ and $[5-^{13}\text{C}]\text{glutamate}/[2-^{13}\text{C}]\text{pyruvate}$).

The ability to probe PDH flux and downstream [5-¹³C]glutamate production simultaneously will also enable more thorough investigations of the role of metabolism in clinical cardiovascular disease. For example, in diabetic cardiomyopathy in which PDH activity is likely decreased (17), and pressure-overload hypertrophy in which glycolysis may be increased and the glutamate pool may be affected by increased mitochondrial NADH shuttling (18,19), [1,2-¹³C₂]pyruvate will enable independent assessment of all pathways. Following [1,2-¹³C₂]pyruvate metabolism could improve the sensitivity and specificity of the technique's ability to diagnose cardiovascular disease relative to the use of either singly labeled compound on its own.

In the experiments performed in this study using [1,2-¹³C₂]pyruvate in healthy pigs, the maximum $\text{H}^{13}\text{CO}_3^- / [1-^{13}\text{C}]$ pyruvate and the maximum [5-¹³C]glutamate/[2-¹³C]pyruvate ratios were similar to prior studies using pyruvate labeled only at either the C1 (15) or the C2 (16) position administered at the same dose and in the same animal model. However, the values reported here were somewhat higher compared with *in vivo* studies conducted in normal rat hearts ($\text{H}^{13}\text{CO}_3^- / \text{pyruvate} = 0.02$ in (5), and [5-¹³C]glutamate/pyruvate = 0.009 in (7)). These differences may be partially due to variations between species. In addition, in this study a much lower dose of ¹³C-pyruvate was used in comparison to the previous rat studies (~0.06 mmol/kg vs. ~0.24 mmol/kg). The differences in dose may have contributed to the observed differences in $\text{H}^{13}\text{CO}_3^- / \text{pyruvate}$ and [5-¹³C]glutamate/pyruvate, since these doses of infused ¹³C-pyruvate do not cause proportional increases in PDH flux (measured as $\text{H}^{13}\text{CO}_3^- / \text{pyruvate}$, (20)). The time from the peak of pyruvate signal to the peak of the metabolite signal did not differ significantly for [5-¹³C]glutamate compared with $\text{H}^{13}\text{CO}_3^-$. This was likely due to the relatively low temporal resolution used in this study.

One limitation of using pre-polarized [1,2-¹³C₂]pyruvate as a tracer for MRS experiments at 3 T is that the [5-¹³C]glutamate and [1-¹³C]acetylcarnitine resonances overlap with the [1-¹³C]lactate and [1-¹³C]pyruvate peaks, respectively, making them difficult to measure when B_0 homogeneity is poor. Although the [5-¹³C]glutamate peak overlaps with the up-field [1-¹³C]lactate doublet peak, its amplitude can still be estimated by subtracting the signal amplitude of the down-field [1-¹³C]lactate double peak that is observed unobstructed from the overlapped glutamate peak. [1-¹³C]citrate would have overlapped with the [1-¹³C]pyruvate hydrate doublet; however, as shown in our prior study with pre-polarized [2-¹³C]pyruvate in the same pig model and dose, no [1-¹³C]citrate signal was detected (16). This observation was in contrast to a prior study conducted in rats using a much higher dose of [2-¹³C]pyruvate (7), thus it is likely that the labeling of [1-¹³C]citrate resonance is dose dependent.

In theory, peaks representing [5-¹³C]glutamate, $\text{H}^{13}\text{CO}_3^-$ and ¹³CO₂ could also be observed following infusion of a mixture of [1-¹³C] and [2-¹³C]pyruvate, instead of using dual-labeled [1,2-¹³C₂]pyruvate. This could simplify the resultant spectra (fewer doublets) and enable measurement of the [1-¹³C]alanine and [1-¹³C]acetyl-carnitine peaks. However, the SNR of ¹³C-bicarbonate (and most likely, [5-¹³C]glutamate) depends strongly on ¹³C-pyruvate dose in the range of low doses used in this study (20). Therefore, to achieve the same SNR for ¹³C-bicarbonate/¹³CO₂ and [5-¹³C]glutamate from a single bolus injection when using a mixture of [1-¹³C] and [2-¹³C]pyruvate instead of [1,2-¹³C₂]pyruvate, the

same concentrations of $[1-^{13}\text{C}]$ pyruvate plus $[2-^{13}\text{C}]$ pyruvate would be needed. This assumes 15 cc / 83 mM of $[1-^{13}\text{C}]$ pyruvate would result in similar SNR for ^{13}C -bicarbonate/ $^{13}\text{CO}_2$ as 15 cc / 83 mM $[1,2-^{13}\text{C}_2]$ pyruvate; and 15 cc / 83 mM of $[2-^{13}\text{C}]$ pyruvate would result in similar SNR for $[5-^{13}\text{C}]$ glutamate as 15 cc / 83 mM $[1,2-^{13}\text{C}_2]$ pyruvate. Thus twice the total dose of ^{13}C -pyruvate would have to be administered if the mixture of $[1-^{13}\text{C}]$ and $[2-^{13}\text{C}]$ pyruvate is used as compare to $[1,2-^{13}\text{C}_2]$ pyruvate. Although pyruvate is an endogenous substrate and therefore a relatively safe tracer, it has demonstrated inotropic properties (21) and itself stimulates PDH flux by inhibiting pyruvate dehydrogenase kinase (PDK) (22). Therefore, in experimental studies and in future studies in the clinic, minimizing the administered ^{13}C -pyruvate dose will be advantageous to limit both the metabolic perturbation to the organism being studied and the risk to patients with cardiovascular disease. Furthermore, in cardiac studies to date that have utilized hyperpolarized ^{13}C -pyruvate tracers, the alanine and acetylcarnitine resonances appear in a dose-dependent fashion and have not been essential for metabolic assessment of cardiovascular disease. Most importantly, markers including $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ and $[5-^{13}\text{C}]$ glutamate can be measured, in our opinion safely and reliably, when $[1,2-^{13}\text{C}_2]$ pyruvate is used at 3 T. Finally, another advantage of using the doubly labeled pyruvate tracer is the potential to estimate the level of polarization directly *in vivo* by measuring the asymmetry of the pyruvate doublets (9,23,24). With further investigation and validation, this approach could provide more quantitative measurements of cardiac metabolism.

Another limitation of using pre-polarized $[1,2-^{13}\text{C}_2]$ pyruvate as a tracer for MRI experiments is that if spatially resolved data is desired, the large chemical shift range of the spectrum would complicate the design of slice selective RF excitation pulse and fast spectroscopic imaging readout gradient trajectories (such as EPSI or spiral) (25-27). The complexity of the spectrum would likely also prohibit the use of multi-echo methods (28).

An interleaved RF pulse scheme was used in this study to demonstrate improved detection of the $^{13}\text{CO}_2$ resonance, with a view towards measuring $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ equilibrium, and thus cardiac pH, in large animals and in patients. Although $^{13}\text{CO}_2$ resonance was detectable in hearts *in vivo* in our study and a prior study performed in rats (6) by conventional pulse-acquire acquisition following perfusion of pre-polarized pyruvate, reduction in PDH flux occurs in several forms of cardiovascular disease may prevent reliable measurement of this resonance. The proposed method was validated in hyperpolarized $\text{H}^{13}\text{CO}_3^-$ phantom studies and was also tested *in vivo*. In the hyperpolarized ^{13}C -bicarbonate/CA phantom experiments, the pH meter measurements consistently gave lower pH values as compared to the MRS measurements (likely due to a calibration error). More importantly, however, the MRS measurements of pH in the experiments using either the interleaved sequence or the conventional sequence did not differ significantly. Thus the interleaved, selective RF acquisition scheme did not alter the $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ measurements compared with the non-selective RF pulse-acquire method (when $\text{H}^{13}\text{CO}_3^-$ and $^{13}\text{CO}_2$ were in rapid exchange). The *in vivo* measurements showed improvement in $^{13}\text{CO}_2$ signal detection that closely followed the difference in tip angle (Fig 3. bottom right) while observations of all other resonances of interest were not affected (Fig 3. top).

A gradual increase in measured pH over time, to levels that were higher than expected (pH_i of 7.0 – 7.2 in myocytes (29), and pH of 7.4 in blood and extracellular space), was observed *in vivo*. It is likely that this phenomenon was not caused by the RF saturation of the $^{13}\text{CO}_2$ signal, as the same trend was also observed in studies when only a non-selective small tip pulse was used (Fig. 4). One possible explanation is that a fraction of the labeled $^{13}\text{CO}_2$ generated by PDH flux diffused out of the cardiac myocyte into extracellular space and the vasculature (30). Thus, the total $^{13}\text{CO}_2$ and $\text{H}^{13}\text{CO}_3^-$ signals observed would have included increased weighting from the $^{13}\text{CO}_2$ and $\text{H}^{13}\text{CO}_3^-$ signals in the extracellular pool and the blood pool, which have both a higher pH than myocytes and known CA activities to facilitate $^{13}\text{CO}_2$ and $\text{H}^{13}\text{CO}_3^-$ inter-conversion. Additionally, if the signal decay of $^{13}\text{CO}_2$ was greater than that of $\text{H}^{13}\text{CO}_3^-$ in the blood pool, then the Henderson-Hasselbalch equation could result in apparent pH overestimation and measured pH values beyond 7.4. We speculate that if CO_2 binding to hemoglobin in erythrocytes occurs faster than dehydration of HCO_3^- , apparent $^{13}\text{CO}_2$ signal loss by shortening of T_1 or alteration of chemical shift in its bound form may be observed. These rates have not been determined *in vivo*; however, an *in vitro* study of erythrocytes in resting mammals, in which a greater amount of CO_2 was observed bound to hemoglobin than was dissolved (30), lends support to this hypothesis. Further studies are needed to determine the mechanism of this apparent increase in the measured $\text{H}^{13}\text{CO}_3^-/^{13}\text{CO}_2$ ratio, and if the ability to probe changes in myocardial pH *in vivo* may be affected by this phenomenon.

Conclusions

Methods to investigate cardiac PDH flux, Krebs cycle metabolism and cardiac pH *in vivo* following a single dose of hyperpolarized tracer was described. Application of these methods to assess cardiac disease models may provide more insights on the limitations and potentials of the described techniques for noninvasive cardiac disease diagnostics.

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Abbreviations

PDH	pyruvate dehydrogenase
CA	carbonic anhydrase

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Figure 1.

Hyperpolarized ^{13}C MRS data acquired from pre-polarized bicarbonate using an interleaved RF excitation scheme that alternate between a 40° spectrally selective RF pulse (for CO_2 only) and a 10° non-selective RF pulse. Stable pH values were obtained temporally (range: 7.38 - 7.43) that agrees well with the pH meter measurement (7.35).

Figure 2.
Representative spectra from pre-polarized [1,2- $^{13}\text{C}_2$]pyruvate phantom study at 3T.

Figure 3.

In vivo cardiac MRS data acquired from one of the pigs following infusion of pre-polarized [1,2- $^{13}\text{C}_2$]pyruvate. ^{13}C spectrum from one of the time points is shown above ([2- ^{13}C]pyruvate hydrate at 96.5 ppm is outside the range of display) while the time courses of the substrate and some of the metabolites are plotted below. $^{13}\text{CO}_2$ signal amplitudes (corrected for tip angle and echo time differences) and time courses from the two interleaves (40° selective RF and 10° non-selective RF) were observed to have good agreement (lower right).

Figure 4.

Cardiac pH estimated using hyperpolarized $\text{H}^{13}\text{CO}_3^-$ and $^{13}\text{CO}_2$ signals measured *in vivo* in pig hearts. $^{13}\text{CO}_2$ signals from the 40° selective pulse were used in the data shown (empty circle and solid square). pH measurement from the studies without using the interleaved RF scheme is also shown (pig 1* open diamond and pig 3* open circle).