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Resolving Confounding Enrichment Kinetics Due to Overlapping Resonance Signals From ^{13}C -Enriched Long Chain Fatty Acid Oxidation and Uptake Within Intact Hearts

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Abstract

Purpose—Long chain fatty acid (LCFA) oxidation measurements in the intact heart from ^{13}C -NMR rely on detection of ^{13}C -enriched glutamate. However, progressive increases in overlapping resonance signal from LCFA can confound detection of the glutamate 4-carbon (GLU-C4) signal. We evaluated alternative ^{13}C labeling for exogenous LCFA and developed a simple scheme to distinguish kinetics of LCFA uptake and storage from oxidation.

Methods—Sequential ^{13}C -NMR spectra were acquired from isolated rat hearts perfused with ^{13}C LCFA and glucose. Spectra were evaluated from hearts supplied: U ^{13}C LCFA, [2,4,6,8,10,12,14,16- $^{13}\text{C}_8$] palmitate, [2,4,6,8,10,12,14,16,18- $^{13}\text{C}_9$] oleate, [4,6,8,10,12,14,16- $^{13}\text{C}_7$] palmitate, or [4,6,8,10,12, 14,16,18- $^{13}\text{C}_8$] oleate.

Results— ^{13}C signal reflected the progressive enrichment at 34.6 ppm from GLU-C4, confounded by additional signal with distinct kinetics attributed to ^{13}C -enriched LCFA 2-carbon (34.0 ppm). Excluding ^{13}C at the 2-carbon of both palmitate and oleate eliminated signal overlap and enabled detection of the exponential enrichment of GLU-C4 for assessing LCFA oxidation.

Conclusion—Eliminating enrichment at the 2-carbon of ^{13}C LCFA resolved confounding kinetics between GLU-C4 and LCFA 2-carbon signals. With this enrichment scheme, oxidation of LCFA, the primary fuel for cardiac ATP synthesis, can now be more consistently examined in whole organs with dynamic mode, proton-decoupled ^{13}C -NMR.

Keywords

carbon-13; glutamate; oleate; palmitate; dynamic mode spectroscopy

Introduction

Significant changes or defects in fatty acids metabolism have been reported for cardiac hypertrophy, ischemic heart disease, diabetic cardiomyopathy, and congenital heart defects (1–6). Experimental protocols using isotopic-labeled fuels to follow mitochondrial oxidative

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measures and lipid storage continue to provide critical new insight into the underlying mechanisms of these disease states. These approaches have evolved to enable a level of investigation several orders of complexity and physiological relevance above long-chain fatty acid (LCFA) oxidation rates assessed in cardiomyocyte cultures or isolated mitochondria, and enable truly mechanistic protocols to be implemented in an intact, functioning myocardium.

In particular, dynamic mode ^{13}C -NMR provides a particular level of physiological insight that holds distinct advantages over both conventional $^{14}\text{C}/^3\text{H}$ -radiolabeled approaches (7–9), and the more recently developed hyperpolarized ^{13}C -NMR (10–12), for assessing metabolism of the primary energy-yielding fuel for the heart, LCFAs (1,13–18). Dynamic mode, proton-decoupled ^{13}C -NMR enables real-time observation of metabolic rates of LCFA oxidation and the storage and turnover kinetics of the triacylglyceride pool from ^{13}C protocols in beating hearts. In contrast to radiolabeling methods and $^{14}\text{CO}_2$ trapping, the combined use of ^{13}C -NMR and end point mass spectrometry provides direct quantification of flux from labeled and unlabeled fractions of fats, with detection of signals that emanate from within the functioning cell of the whole organ.

Despite the emerging promise of dynamic nuclear polarization for ^{13}C metabolite detection (10–12,19,20), technical and procedure difficulties currently preclude quantitative assessment of LCFA metabolism and tricarboxylic acid cycle flux, including flux through the oxaloacetate-malate carrier protein of the malate-aspartate shuttle on the mitochondrial membrane (21–25). Although proton-decoupled ^{13}C -NMR spectroscopy is currently the only approach to accurately quantify LCFA oxidation and storage directly in the intact beating heart (1,13), the signal dynamics due to progressive, intracellular LCFA storage that may overlap with and confound the use of glutamate resonance signals for assessing oxidative rates must also be recognized and overcome.

To address this problem, we performed dynamic ^{13}C -NMR enrichment studies of the uptake, oxidation, and storage of exogenous ^{13}C -labeled LCFAs in the heart, and identified those carbons from glutamate and triacylglycerol that resonate at similar frequencies and preclude analysis of oxidative measures. From evaluation of multiple ^{13}C -labeling schemes for the exogenous LCFA, a simple scheme is shown that distinguishes oxidative rate components from LCFA storage dynamics within the protocol for ^{13}C -NMR detection of metabolic rates.

Methods

Isolated Retrograde-Perfused Rat Hearts

Hearts were excised and perfused in retrograde mode with modified Krebs-Henseleit buffer (116 mM NaCl, 4 mM KCl, 1.5 mM CaCl_2 , 1.2 mM MgSO_4 , and 1.2 mM NaH_2PO_4 equilibrated with 95% O_2 to 5% CO_2) containing 5 mM glucose with either 0.4 mM palmitate or oleate complex to albumin (3:1 molar ratio) (1,13–17). The hydrostatic perfusion pressure adjusted to 80 mm Hg, and a water-filled latex balloon in the left ventricle was set to a diastolic pressure of 5–10 mm Hg and connected to a pressure transducer for hemodynamic recordings (Powerlab; ADInstruments, Colorado Springs,

Colorado, USA). Left ventricular developed pressure (LVDP), dP/dt , and HR were continuously recorded. Perfused hearts were maintained at 37°C and positioned in an NMR spectrometer for continuous mode ^{13}C -NMR data collection. The protocol was approved by the Animal Care Policies and Procedures Committee at the University of Illinois at Chicago (Institutional Animal Care and Use Committee accredited). Animals were maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* by the US National Institutes of Health (revised 2011).

^{13}C -NMR Spectroscopy from Intact Heart

Perfused hearts were positioned in a 20-mm NMR probe within a vertical 89-mm bore, 9.4T magnet. NMR data from isolated beating hearts were obtained with a Bruker 400 AVANCE NMR spectrometer (Bruker Daltonics, Billerica, Massachusetts, USA). ^{13}C -NMR measurements were acquired as described previously (1,13–17). A ^{13}C -NMR spectrum was collected every 2 s and summed in 2-min time blocks.

The sequential ^{13}C -NMR spectra provide both glutamate and acyl group enrichment kinetics necessary for LCFA measures of uptake, oxidation, and storage. This is illustrated in Figure 1, which depicts the transport of exogenous ^{13}C -labeled long-chain fats across the sarcolemma via CD36 (17) and activated to form long-chain acyl-coenzyme A (CoA) within the cytosol. The acyl group is transported into the mitochondria via the carnitine shuttle (CPT1) (15) and oxidized through β -oxidation and the tricarboxylic acid cycle (TCA) cycle. The intermediary metabolites of the mitochondria are below NMR detection at 9.4T. However, ^{13}C -labeled α -ketoglutarate is transported from the mitochondria via oxoglutarate-malate carrier and converted to the NMR-detectable cytosolic glutamate pool (21–24,26,27). The time course of enrichment data for glutamate carbon-2 (54.6 ppm) and carbon-4 (34.6 ppm) are routinely used to quantify substrate oxidative rates, TCA cycle activity, and transport activity as part of the malate-aspartate shuttle (13,15,16,23,24,27). Alternatively, the cytosolic fatty acyl-CoA is stored via esterification to triacylglycerol. The ^{13}C enrichment data for the various acyl groups and triacylglycerol pool are observed as linear during the first 40 min of enrichment, because this large lipid pool is predicted to take 10–16 hours to reach steady-state enrichment (1,14,17).

Experimental Protocols and Enrichment Schemes

Natural abundance ^{13}C -NMR spectra were first collected from isolated hearts retrograde perfused with buffer containing unlabeled glucose and LCFA. The perfusate was then switched to buffer containing the LCFAs ^{13}C -labeled according to the six schemes: [2,4,6,8,10,12,14, 16- $^{13}\text{C}_8$] palmitic acid, [2,4,6,8,10,12,14,16,18- $^{13}\text{C}_9$] oleic acid, without 2-carbon enrichment [4,6,8,10,12,14, 16- $^{13}\text{C}_7$] palmitic acid, without 2-carbon enrichment [4,6,8,10,12,14,16,18- $^{13}\text{C}_8$] oleic acid, U- $^{13}\text{C}_{16}$ palmitic acid, and U- $^{13}\text{C}_{18}$ oleic acid. Labeled substrates were purchased through SIGMA-Isotec and Cambridge Isotope Laboratories (Cambridge, Massachusetts, USA). Sequential ^{13}C -NMR spectra were acquired from the functioning hearts for 40 min. Data were analyzed for ^{13}C -NMR resonance assignments and glutamate versus triacylglycerol enrichment profiles (i.e., exponential versus linear enrichment profiles, respectively).

With the need for 1- to 2-min time resolution of spectra to provide for each data point during ^{13}C enrichment of glutamate, signal-to-noise ratio and in particular the vertical amplitude of the resonance peak above baseline are at a premium for these experiments. While use of fully labeled LCFAs ($\text{U-}^{13}\text{C}_{16}$ palmitate or $\text{U-}^{13}\text{C}_{18}$ oleate) enable progressive ^{13}C enrichment of triacylglycerols to be monitored by sequential ^{13}C -NMR spectra, the J-coupling between carbon positions that split the signal reduce detection of the glutamate resonances for measuring oxidative flux in the intact heart. This difficulty results from the reduction of vertical amplitude, as the ^{13}C resonance signal intensities are split into multiplets by $J_{13\text{C}-13\text{C}}$ coupling, thus broadening the ^{13}C signals from each of the enriched glutamate carbon positions and thereby reducing the vertical amplitude of signals above baseline noise. Thus, we used a novel ^{13}C enrichment scheme for ^{13}C LCFA delivery to whole heart for ^{13}C -NMR with isotope labeling at only the even-numbered carbon positions along the LCFA chain. This even-carbon labeling scheme reduced the extent of signal splitting due to $J_{13\text{C}-13\text{C}}$ coupling, which enables maximum vertical scaling for signal detection.

However, analyzing oxidation of LCFA, the preferred fuel for the heart, can be further complicated by overlapping NMR signal from ^{13}C LCFA that may confound detection of the GLU-C4 signal. Eliminating potentially confounding kinetics between glutamate and LCFA signals may be more consistently examined in whole organs with ^1H -decoupled ^{13}C -NMR with increased levels of convergence during kinetic analysis.

Fatty Acid ^{13}C -NMR Assignments

Peak assignments for palmitate and oleate were based on high-resolution, proton-decoupled ^{13}C -NMR analysis of unlabeled standards prepared for each (1.1% natural abundance ^{13}C) and software analysis of the chemical structure (NMR Viewer 3.5, Chemaxon.com). The standards were prepared as follows: 10 mg of unlabeled sodium oleate dissolved in 500 μL of 1 M HCl for 1 h at 50°C ; 10 mg of unlabeled sodium palmitate dissolved in 500 μL of 50:50 chloroform:methanol. ^{13}C -NMR data provided natural abundance ^{13}C signal for each carbon, thereby enabling identification of the center frequency (ppm) for each carbon of the LCFA. For assigning peaks to the carbon spectra, unlabeled substrate was used to avoid the splitting of signal for each carbon into multiplets by $J_{13\text{C}-13\text{C}}$ coupling.

Results

NMR Spectra from Hearts Oxidizing $^{13}\text{C}_8$ -Palmitate or $^{13}\text{C}_9$ -Oleate

Figure 2 illustrates the sequential ^{13}C -NMR spectra from isolated hearts perfused with buffer containing the long chain fat, $^{13}\text{C}_8$ -palmitate (Fig. 2a) and $^{13}\text{C}_9$ -oleate (Fig. 2b). The exogenous fats were synthesized with ^{13}C labeled at every other even numbered carbon. The spectra provide, in part, the enrichment kinetics for the 2-, 3-, and 4-carbons of glutamate (55.8, 28.3, and 34.6 ppm, respectively). For the palmitate group, the enrichment profiles for the 2- and 3-carbon of glutamate are exponential and reach steady state within 20 minutes. The signal at ~ 34 ppm increases throughout the 50-min protocol and includes an exponential profile for GLU-C4 (34.6 ppm), and an additional signal with distinct linear

kinetics attributed to endogenous ^{13}C -enriched palmitate at the 2-carbon (34.0 ppm). The summation of this exponential and linear signal is illustrated in Figure 3c.

It should be noted that the slope for the linear component of the resonance at 31 ppm is five times the slope of the linear component of the profile at ~ 34 ppm. This is consistent with five carbons contributing to the signal at ~ 31 ppm from palmitate compared with one carbon from palmitate at 34.0 ppm.

For the oleate group, the sequential ^{13}C -NMR enrichment data at 55.8 and 34 ppm were similar to the palmitate group, whereas the signal at 28 ppm represents the summation of the 3-carbon from glutamate (28.3 ppm) with the 6- and 8-carbon of oleate (28.2 and 28.7 ppm, respectively).

Distinguishing GLU-C4 from Acyl Group 2-Carbon ^{13}C Enrichment

Sequential ^{13}C -NMR enrichment profile data for the 2- and 4-carbon of glutamate are required for quantitative measures of LCFA oxidation (13). While the enrichment profile for the 2-carbon of glutamate (55.8 ppm) is resolved, the enrichment data for GLU-C4 (34.6 ppm) is confounded with overlapping signal from the 2-carbon of the acyl groups (34.0 ppm), as described above. To remedy this “overlap”, both long chain fats were synthesized without the 2-carbon labeled: [4,6,8,10,12,14,16- $^{13}\text{C}_7$] palmitic acid and [4,6,8,10,12,14,16,18- $^{13}\text{C}_8$] oleic acid. The sequential ^{13}C -NMR spectra from hearts oxidizing either substrate are illustrated in Figure 4. Figure 3b illustrates the enrichment profiles for signal detected at both 54 and 34 ppm. Importantly, both profiles are exponential, consistent with glutamate enrichment at the 2- and 4-carbon (55.8 and 34.6 ppm, respectively). Therefore, eliminating the ^{13}C label at the 2-carbon of either LCFA eliminated the linear component of the overlapping signal at 34 ppm.

^{13}C -NMR from Hearts Oxidizing U- ^{13}C -Labeled Palmitate or Oleate

Sequential ^{13}C -NMR spectra (data not shown) were acquired from hearts oxidizing $^{13}\text{C}_{16}$ -palmitic or $^{13}\text{C}_{18}$ -oleate acid. For hearts provided $^{13}\text{C}_{16}$ -palmitate, the enrichment profiles for glutamate carbons C1, C2, and C3 were exponential, reaching steady state within 10–20 min, as expected. Enrichment profiles for glutamate carbons C4 and C5 were linear throughout the 40-min protocol. The linear profile was consistent with signal overlap with endogenous long chain fats, labeled in carbon positions C1 and C2. The profile was similar for $^{13}\text{C}_{18}$ -oleate except for glutamate carbon C3, which was linear throughout. This is consistent with signal overlap with oleate carbons C5, C6, and C8. Interestingly, no signal was observed at 25 and 23 ppm for either palmitate (C3, C15) or oleate (C3, C17) protocols in a beating heart, even though this signal was observed in the ^{13}C -NMR spectra acquired from the LCFA standards.

^{13}C -NMR Assignments for Palmitic and Oleic Acid Standards

High-resolution ^{13}C -NMR spectra for unlabeled palmitic and oleic acid standards were acquired, and the peaks were assigned based on software analysis of the chemical structure. The assignments for both palmitate and oleate are listed in Figure 5. Carbon-1 of oleate and palmitate correspond to the methyl end of the LCFA ($-\text{CH}_3$), whereas the oxygenated end of

the LCFA would form the ester with cytosolic CoA, carnitine, or glycerol (i.e., TAG, DAG). Several carbons for oleic and palmitic acid resonated near frequencies established for glutamate carbons C3, C4, and C5 (see bracketed data in Fig. 5).

Discussion

This study confirmed the potential for confounding ^{13}C -NMR resonance signal overlap between GLU-C4 with the 2-carbon of the LCFAs, palmitate and oleate. Whereas the 2-carbon for glutamate was resolved, the overlapping resonance signals from GLU-C4 and the LCFA 2-carbon precluded measures of mitochondrial activity and fat oxidation in the intact heart. Here, we eliminated the overlapping signal from the LCFA 2-carbon to resolve the independent, kinetic features of GLU-C4 by supplying ^{13}C -enriched LCFA without ^{13}C label at the 2-carbon position. With this modification, both glutamate 2-carbon and GLU-C4 enrichment profiles were exponential, enabling more consistent and accurate measures of LCFA oxidation, parallel to LCFA storage and turnover, in the beating heart. This experimental adjustment increases the number of experiments for which kinetic models of metabolism converge with isotope enrichment data to yield quantitative rates of LCFA oxidation and mitochondrial activity.

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Fig. 1.

Diagram of metabolic pathways for LCFA uptake, oxidation, and storage in the heart. The exogenous ^{13}C -labeled LCFA is transported across the sarcolemma, and activated to form long-chain acyl-CoA. The acyl CoA group is transported into the mitochondria for β -oxidation and TCA cycle metabolism. The metabolic intermediates within the mitochondria are below NMR detection at 14T in real time. However, following efflux of ^{13}C -labeled α -ketoglutarate from the mitochondrial via the oxoglutarate-malate carrier (OMC), α -KG is interconverted to enrich glutamate, which is primarily cytosolic and readily NMR-detected. Alternatively, the acyl-CoA is stored via esterification to triacylglycerol. Kinetic models of metabolism are routinely fit to the enrichment data for glutamate and triacylglycerol to yield rates of fatty acid oxidation and storage. However, NMR signal for GLU-C4 and LCFA carbon-2 resonate near similar frequency (34.6 and 34.0 ppm, respectively), complicating quantification of LCFA oxidative rates.

Fig. 2.

Dynamic mode ^{13}C -NMR spectra acquired from isolated beating hearts perfused with buffer containing unlabeled glucose and $^{13}\text{C}_8$ -palmitate (**a**) or $^{13}\text{C}_9$ -oleate (**b**). ^{13}C signal reflected the progressive enrichment at 34.6 ppm from GLU-C4, confounded by additional signal with distinct kinetics attributed to TAG (i.e., ^{13}C -enriched LCFA 2-carbon at 34.0 ppm).

Fig. 3.

^{13}C enrichment profiles for glutamate carbons C4 and C2 from hearts oxidizing either $^{13}\text{C}_8$ -palmitate (top) or $^{13}\text{C}_7$ -palmitate (middle). **a:** ^{13}C signal for the progressive enrichment of glutamate C4 (34.6 ppm) and C2 (55.8 ppm). **b:** Excluding ^{13}C at the 2-carbon of palmitate eliminated signal overlap at ~34 ppm, enabling detection of the exponential enrichment of GLU-C4 (panel B). **c:** The C4* signal is confounded by additional signal with distinct kinetics attributed to ^{13}C -enriched LCFA 2-carbon (34.0 ppm). The exponential component from glutamate (dashed line) and the linear component from the acyl groups (dotted line) are highlighted.

Fig. 4.

Dynamic mode ^{13}C -NMR spectra from isolated beating hearts perfused with buffer containing unlabeled glucose and $^{13}\text{C}_7$ -palmitate (left) or $^{13}\text{C}_8$ -oleate (right), both labeled at all even numbered carbons with the exclusion of carbon-2. Excluding ^{13}C at the 2-carbon of both palmitate and oleate eliminated signal overlap at 34 ppm and enabled detection of the exponential enrichment of GLU-C4.

Fig. 5.

Top panel: ^{13}C -NMR peak assignments (in ppm) for palmitate, oleate, and glutamate. The signals identified in brackets indicate carbons with near-similar resonant frequencies between glutamate and the LCFA. Middle panel: Orientation of carbon isotope enrichment sites. Bottom panel: ^{13}C resonance signals from a lipid standard of oleate with resonance signals near the chemical shift values for the 4- and 3-carbons of glutamate at 34.6 and 28 ppm, respectively.