Published in final edited form as:

NMR Biomed. 2011 October ; 24(8): 980–987. doi:10.1002/nbm.1668.

Determining *In Vivo* Regulation of Cardiac Pyruvate Dehydrogenase Based on Label Flux from Hyperpolarized [1-¹³C]Pyruvate

Marie A. Schroeder^{#*}, Helen J. Atherton^{#,†}, Lisa C. Heather^{*}, Julian L. Griffin[†], Kieran Clarke^{*}, George K. Radda^{*}, and Damian J. Tyler^{*}

^{*}Cardiac Metabolism Research Group, Department of Physiology, Anatomy & Genetics, University of Oxford

[†]Department of Biochemistry, University of Cambridge

[#] These authors contributed equally to this work.

Abstract

Background—Pyruvate dehydrogenase (PDH) is a key regulator of cardiac substrate selection and is regulated by both pyruvate dehydrogenase kinase (PDK)-mediated phosphorylation and feedback inhibition. The extent to which chronic upregulation of PDK protein levels, acutely increased PDK activity and acute feedback inhibition limit PDH flux remains unclear because existing *in vitro* assessment methods inherently disrupt the enzyme complex. We have previously demonstrated that hyperpolarized ¹³C-labelled metabolic tracers with magnetic resonance spectroscopy (MRS) can monitor flux through PDH *in vivo*. The aim of this study was to determine the relative contributions of acute and chronic changes in PDK and PDH activities to *in vivo* myocardial PDH flux.

Methodology/Principal Findings—We examined both fed and fasted rats with either hyperpolarized [1-¹³C]pyruvate alone or hyperpolarized [1-¹³C]pyruvate co-infused with malate (to modulate mitochondrial NADH/NAD⁺ and acetyl-CoA/CoA ratios, which alter both PDH activity and flux). To confirm the metabolic fate of infused malate, we performed *in vitro* ¹H NMR spectroscopy on cardiac tissue extracts. We observed that in fed rats, where PDH activity was high, the presence of malate increased PDH flux by 27%, whereas in the fasted state, malate infusion had no effect on PDH flux.

Conclusions/Significance—These observations suggest that pyruvate oxidation is limited by feedback inhibition from acetyl-CoA only when PDH activity is high. Therefore, in the case of PDH, and potentially other enzymes, hyperpolarized ¹³C MR can be used to non-invasively assess enzymatic regulation.

Keywords

Cardiac Metabolism; Hyperpolarization; Magnetic Resonance Spectroscopy; Metabolic Regulation; Pyruvate Dehydrogenase

Introduction

The pivotal position of the pyruvate dehydrogenase enzyme complex (PDH) in the glucose-fatty acid cycle makes it a key regulator of cardiac substrate selection [1,2]. PDH catalyzes the irreversible oxidation of pyruvate to form NADH and acetyl-CoA, which may be oxidized to produce ATP. To effectively balance the competitive processes of pyruvate and fatty acid oxidation, PDH is controlled *in vivo* by a reversible phosphorylation cycle [3], partially controlled by pyruvate dehydrogenase kinase (PDK), which phosphorylates and thereby inactivates PDH [4,5,6]. Chronic elevation of plasma free fatty acids has been shown to increase PDK protein expression [7,8], whereas increases in PDK activity are effected by high acetyl-CoA/CoA and NADH/NAD⁺ ratios, typically arising from fatty acid β -oxidation [5]. In addition, PDH flux is thought to be limited directly by feedback inhibition via accumulation of its own end products acetyl-CoA and NADH, and the resultant increases to acetyl-CoA/CoA and NADH/NAD⁺ ratios [1,9,10]. Table 1 summarizes the regulatory processes that can affect flux through PDH.

Despite several studies of chronic high fat feeding and of overexpression of the nuclear receptor peroxisome proliferator activated receptor (PPAR)- α , the extent to which transcriptional changes to PDK expression, acute modulation of PDK activity, and feedback inhibition each regulate *in vivo* cardiac PDH flux remains unclear [10,11,12]. Part of this confusion may originate in the limitations of standard methods for assessing the PDH enzyme complex, which involve measuring PDH activity *in vitro* in tissue samples [11,13] and thus removing plasma substrate and hormone levels that play a fundamental role in determining absolute PDH flux [5,6,14]. A method for *in vivo* study of the enzyme complex may advance understanding as to how both modifications to PDK activity/protein expression and feedback inhibition regulate the glucose-fatty acid cycle, in various physiological and pathological metabolic states.

We have previously demonstrated qualitative monitoring of *in vivo* real time flux through the PDH enzyme complex [15], using hyperpolarized ¹³C-labelled metabolic tracers coupled with magnetic resonance spectroscopy (MRS) [16,17]. [1-¹³C]pyruvate was intravenously infused into living animals and ¹³C cardiac spectra were acquired to detect hyperpolarized H¹³CO₃⁻ produced directly from pyruvate oxidation, thus enabling qualitative non-invasive measurement of instantaneous cardiac PDH flux [15,18]. However, results from our own group and from others [19,20,21] have been limited by an inability to ascertain the mechanisms responsible for observed alterations in PDH flux. Development of a non-invasive method to understand PDH flux modulation could help to identify early stages of cardiac metabolic dysfunction, such as in heart disease, insulin resistance and ischemia [12,22].

Our aims in this study were two-fold. Firstly, we aimed to adapt our hyperpolarized MR protocol to demonstrate that the technique is not limited to simple direct assessment of *in vivo* enzymatic fluxes, but that it can report on the regulation of enzymatic flux. Secondly, we aimed to distinguish the relative contributions of chronic and acute mechanisms to the regulation of myocardial PDH flux.

To determine the extent that chronic PDK-mediated inhibition of PDH activity specifically affects cardiac PDH flux *in vivo*, we examined both fed and fasted rats [23,24]. To determine the extent to which acute mechanisms of PDH inhibition limits PDH flux *in vivo*, we intravenously infused malate to modulate both mitochondrial NADH/NAD⁺ and acetyl-CoA/CoA ratios. This approach was used based on *in vitro* evidence that extra-mitochondrial malate increases mitochondrial uptake of malate via the malate-aspartate shuttle, increasing the level of mitochondrial reducing equivalents [25]. Also, there is evidence that extra-mitochondrial malate increases mitochondrial oxaloacetate availability, subsequently increasing the capacity for acetyl-CoA to form citrate, thus reducing mitochondrial acetyl-CoA/CoA [26].

To validate our hypothesis regarding the metabolic fate of malate, we performed *in vitro* ¹H NMR spectroscopy to assess the effects of malate infusion on total metabolite pool sizes within the myocytes. The dominant mechanism of PDH inhibition was determined based on *in vivo* and *in vitro* results, and comparisons with other studies. We showed that *in vivo*, a high acetyl-CoA/CoA ratio directly limits flux through PDH in rats in which PDH activity is high, a conclusion that was reached using the novel ¹³C method that allowed enzymatic control to be ascertained in real-time and non-invasively.

Materials and Methods

The [1-¹³C]pyruvic acid and trityl radical were obtained from GE-Healthcare (Amersham, UK). The gadolinium compound ((1,3,5-tris-(N-(DO3A-acetamido)-N-methyl-4-amino-2-methylphenyl)-[1,3,5]triazine-2,4,6-trione), referred to here as 3-Gd) was obtained from Imagina AB (Malmö, Sweden). All investigations conformed to Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act, 1986 (HMSO) and to institutional guidelines.

In vivo hyperpolarized ¹³C MRS protocol

Six male Wistar rats (~350 g) were studied using hyperpolarized [1-¹³C]pyruvate with the MR protocol described below. Each rat was examined using hyperpolarized MR on four separate occasions and in four distinct metabolic states. On the first occasion, rats were examined in the fed state with [1-¹³C]pyruvate plus non-hyperpolarized, unlabelled malate (solutions described below). On the second occasion, rats were examined in the fed state with hyperpolarized [1-¹³C]pyruvate alone. On the third occasion, rats were examined in the fasted state, with hyperpolarized [1-¹³C]pyruvate alone, and on the fourth occasion, rats were examined in the fasted state, with [1-¹³C]pyruvate plus non-hyperpolarized, unlabelled malate. Fed rats were examined between 7 AM and 12 PM after feeding on standard laboratory chow *ad libitum*, while fasted rats were examined between 7 AM and 12 PM following the withdrawal of food >14 h before examination.

Metabolic tracer preparation

Approximately 40 mg of [1-¹³C]pyruvic acid, doped with 15 mM trityl radical and 0.8 μl 3-Gd (14.6 mM), was hyperpolarized via the technique of dynamic nuclear polarization (DNP) using a prototype polarizer system, as previously described [16]. The sample was subsequently dissolved by a pressurized and heated alkaline solution, such that the solution effluent from the polarizer contained 80 mM of hyperpolarized sodium ¹³C-pyruvate with a polarization of 15-30%, and was at physiological temperature and pH [17]. The 80 mM hyperpolarized [1-¹³C]pyruvate solution was collected from the polarizer in a flask, which contained approximately 6 ml of Krebs-Henseleit buffer to dilute the pyruvate concentration to 40 mM. For [1-¹³C]pyruvate plus malate infusions, 80 mM malic acid was added to the Krebs-Henseleit buffer and neutralized to pH 7.3 with concentrated sodium hydroxide, prior to pyruvate dissolution. The resultant diluted pyruvate-in-buffer solution was rapidly transported to the MR scanner (~5 s), and one milliliter of the solution, containing either 40 mM hyperpolarized [1-¹³C]pyruvate and 40 mM malate, or 40 mM hyperpolarized [1-¹³C]pyruvate alone, was then infused into a rat.

Hyperpolarized MR protocol

Rats were anesthetized with isoflurane and a 28G catheter was introduced into the tail vein for intravenous administration of the hyperpolarized solution. A 5 cm by 3 cm surface coil was placed over the chest to localize MR signal from the heart. Rats were positioned in a 7T Varian (Yarnton, UK) horizontal bore MR scanner and, immediately prior to injection, an ECG-gated ¹³C-MR pulse-acquire spectroscopy sequence was initiated [15,18]. One ml of hyperpolarized pyruvate was injected into the rat over 10 s and 60 individual cardiac spectra were acquired over 1 minute.

Analysis of in vivo data

In vivo ¹³C MR spectra, resulting from [1-¹³C]pyruvate infusion, were analyzed using the AMARES algorithm, as implemented in the jMRUI software package [27]. Spectra were DC offset corrected based on the last half of acquired points and peaks corresponding with ¹³C-pyruvate and all metabolic derivatives were fitted assuming a Lorentzian line shape and initial peak frequencies, relative phases and linewidths.

The peak areas of [1-¹³C]pyruvate and ¹³C-bicarbonate at each time point were quantified and used as input data for a kinetic model, as developed by Zierhut *et al* and extended by Atherton *et al*, specifically for the analysis of hyperpolarized MRS data[28,29]. Firstly the change in [1-¹³C]pyruvate signal over the 60 s acquisition time was fit to the integrated [1-¹³C]pyruvate peak area data using equation 1:

$$M_{pyr}(t) = \begin{cases} \frac{rate_{inj}}{k_{pyr}} \left(1 - e^{-k_{pyr}(t-t_{arrival})} \right) & t_{arrival} \leq t < t_{end} \\ M_{pyr}(t_{end}) e^{-k_{pyr}(t-t_{end})} & t \geq t_{end} \end{cases} \quad [1]$$

In this equation, $M_{pyr}(t)$ represents the [1-¹³C]pyruvate peak area as a function of time. This equation fits the parameters k_{pyr} , the rate constant for pyruvate signal decay (s^{-1}), $rate_{inj}$, the pyruvate arrival rate (a.u. s^{-1}), $t_{arrival}$, the pyruvate arrival time (s) and t_{end} , the time correlating with the end of the injection (s). These parameters were then used to fit the

following equation (equation 2) which uses the dynamic ^{13}C -bicarbonate data to calculate $k_{\text{pyr} \rightarrow \text{bic}}$, the rate constant for pyruvate to bicarbonate exchange (s^{-1}), and k_{bic} , the rate constant for bicarbonate signal decay (s^{-1}) which was assumed to consist of metabolite $T1$ decay and signal loss from the 5° RF flip angle pulses. In equation [2], $t' = t - t_{\text{delay}}$, where t_{delay} represents the delay between pyruvate arrival and metabolite appearance caused by the traversal of the pyruvate through the cardiopulmonary circulation before arrival at the coronary arteries.

$$M_x(t') = \begin{cases} \frac{k_{\text{pyr} \rightarrow \text{x}} \text{rate}_{\text{inj}}}{k_{\text{pyr}} - k_x} \left(\frac{1 - e^{-k_x(t' - t_{\text{arrival}})}}{k_x} - \frac{1 - e^{-k_{\text{pyr}}(t' - t_{\text{arrival}})}}{k_{\text{pyr}}} \right) & t_{\text{arrival}} \leq t' < t_{\text{end}} \\ \frac{M_{\text{pyr}}(t_{\text{end}})k_{\text{pyr} \rightarrow \text{x}}}{k_{\text{pyr}} - k_x} \left(e^{-k_x(t' - t_{\text{end}})} - e^{-k_{\text{pyr}}(t' - t_{\text{end}})} \right) + M_x(t_{\text{end}}) e^{-k_x(t' - t_{\text{end}})} & t' \geq t_{\text{end}} \end{cases} \quad [2]$$

We have shown previously that the parameter $k_{\text{pyr} \rightarrow \text{bic}}$ correlates significantly with both the maximum ^{13}C -bicarbonate / maximum $[1-^{13}\text{C}]$ pyruvate ratio and PDH activity measured *in vitro* [29]. Therefore, in this study, $k_{\text{pyr} \rightarrow \text{bic}}$ resulting from infusion with $[1-^{13}\text{C}]$ pyruvate and $[1-^{13}\text{C}]$ pyruvate plus malate was compared in both fed and fasted rats, to determine if the presence of malate had a significant effect on PDH flux.

In vitro ^1H NMR protocol

High-resolution ^1H NMR experiments were performed on heart tissue extracts from four separate groups of rats (each group $n=5$). These four groups were prepared identically to the group of rats studied *in vivo* with hyperpolarized $[1-^{13}\text{C}]$ pyruvate. The first group was infused with a 40 mM pyruvate-plus-malate solution that was biochemically identical to the solution infused *in vivo*, while in the fed state. The only difference in the solution was that the included pyruvate was neither labeled with carbon-13, nor hyperpolarized. The second group was infused with a 40 mM pyruvate solution, also identical to the solution infused *in vivo*, while in the fed state. The third and fourth groups were infused with pyruvate plus malate or pyruvate solutions, respectively, while in the fasted state.

The heart from each rat was rapidly removed from the body cavity after the infusion of pyruvate or pyruvate plus malate solutions, at a time point chosen to correspond with the time of maximum $\text{H}^{13}\text{CO}_3^-$ MR signal during *in vivo* data acquisition. Tissue was prepared, as described below, and placed in a high resolution NMR spectrometer to quantify the concentrations of Krebs cycle intermediates in each group.

Tissue dissection

Rats were anaesthetized with 2.5% isoflurane in oxygen, a tail vein catheter was inserted and isoflurane level was then increased to 4%. Upon the loss of corneal and pedal reflexes, a bilateral thoracotomy was performed to expose the heart. At this point, 1 ml of pyruvate or pyruvate plus malate solution was infused into the tail vein over 10 s. Thirty seconds after the beginning of infusion, the heart was rapidly dissected out (< 20 s *post mortem* time prior to freezing), blotted in Krebs-Henseleit buffer, frozen immediately using N_2 cooled aluminium tongs and stored at -80°C until extraction.

Metabolite extraction

Metabolites were extracted using methanol-chloroform-water [30]. Briefly, frozen tissue (~100 mg) was placed in methanol-chloroform (2:1, 600 µl) and homogenized using a tissue homogenizer. Samples were then sonicated for 5 min before chloroform-water (1:1) was added (200 µl of each). Samples were centrifuged (13,500 rpm, 20 min) and the aqueous layer was pipetted off and dried overnight in an evacuated centrifuge (Eppendorf, Hamburg, Germany).

Metabolomic analysis using NMR spectroscopy

The dried extracts were rehydrated in 600 µl of D₂O and buffered in 0.24 M sodium phosphate (pH 7.0) containing 1 mM sodium-3-(tri-methylsilyl)-2,2,3,3-tetraduteriopropionate (TSP; Cambridge Isotope Laboratories, Andover, MA) as an internal standard. The samples were analyzed using an 11.7 T Avance II+ spectrometer (Bruker) operating at 500 MHz for the ¹H frequency equipped with a 5 mm Broadband TXI Automatic Tuning and Matching (ATMA) probe. Spectra were collected using a solvent suppression pulse sequence based on a one-dimensional nuclear Overhauser effect spectroscopy pulse sequence to saturate the residual [¹H] water proton signal (relaxation delay = 2 s, $t_1 = 3 \mu\text{s}$, mixing time = 150 ms, solvent presaturation applied during the relaxation time and the mixing time). One hundred and twenty-eight averages were collected into 16k data points over a spectral width of 12 ppm at 37 °C.

Analysis of in vitro data

NMR spectra were processed using an ACD SpecManager 1D NMR processor (version 8; ACD, Toronto, Canada). Spectra were Fourier transformed after multiplication by a line broadening of 1 Hz and referenced to TSP at 0.0 ppm. Spectra were phased and the baseline was corrected manually. Each spectrum was integrated using 0.04 ppm integral regions between 0.5–4.5 and 4.7–9.5 ppm. To account for any difference in concentration between samples, each spectral region was normalized to the total area under the spectrum, and multiplied to a total integral value of 10,000.

Statistical analysis

Statistical significance among fed, fasted, [1-¹³C]pyruvate alone and [1-¹³C]pyruvate plus malate groups in the *in vivo* hyperpolarized MR experiments was determined using paired one-way ANOVA. Statistical significance was considered at the $p < 0.05$ level.

Metabolomic datasets were imported into the SIMCA-P 11.0 (Umetrics, Umeå, Sweden) for multivariate statistical processing using PCA and PLS-DA (a regression extension of PCA used for classification). Pareto scaling was used, in which each variable was centered and multiplied by $1/(S_k)^{1/2}$, where S_k is the standard deviation of the variable. This scaling increases the importance of low concentration metabolites without significant amplification of noise[30,31]. Identification of major metabolic perturbations within the pattern recognition models was achieved by analysis of corresponding loadings plots. Coefficient scores rank the observations according to their contribution to the model. Any variables with

a statistically significant co-efficient as indicated at the 95 % confidence limit are deemed to be significant in the classification of groups.

Results

Hyperpolarized MR measurements

A representative *in vivo* spectrum of [1-¹³C]pyruvate is shown in Figure 1. Figure 2 shows the $k_{pyr \rightarrow bic}$ calculated for each metabolic condition. In fed rats, the $k_{pyr \rightarrow bic}$ was calculated to be 0.0089 ± 0.0006 a.u. $\cdot$ s⁻¹ following infusion of hyperpolarized [1-¹³C]pyruvate alone. Upon addition of an equivalent concentration of malate, $k_{pyr \rightarrow bic}$ was calculated to be 0.0113 ± 0.0009 a.u. $\cdot$ s⁻¹, a significant increase of 27%. In fasted rats, infusion of hyperpolarized [1-¹³C]pyruvate alone resulted in a $k_{pyr \rightarrow bic}$ of 0.0025 ± 0.0008 a.u. $\cdot$ s⁻¹ and infusion of hyperpolarized [1-¹³C]pyruvate plus malate yielded a similar $k_{pyr \rightarrow bic}$ of 0.0023 ± 0.0011 a.u. $\cdot$ s⁻¹. Therefore, while fasting significantly decreased ¹³C label flux through PDH by 72% in rats infused with pyruvate alone, co-infusion with malate caused no further change to the fasted $k_{pyr \rightarrow bic}$.

¹H NMR measurements

Fed rats co-infused with pyruvate and malate showed a significant, 55% increase in the region of the NMR spectrum that represents malate compared with fed rats infused with pyruvate alone (Figure 3, δ 2.30 – 2.34 ppm). This region of the NMR spectrum signifies the region identified as driving the classification between pyruvate and pyruvate plus malate groups. This result confirmed that malate was taken up into cardiac myocytes. Additionally, the regions of the NMR spectrum associated with citrate (δ 2.66 – 2.70 ppm) and succinate (δ 2.42 – 2.46 ppm) were significantly increased by 25% and 20% respectively. In addition aspartate concentration was increased by 30%, reflecting increased synthesis from oxaloacetate and indirectly indicating increased oxaloacetate availability [32]. Figure 3 shows the percentage change to each metabolite between the fed pyruvate and pyruvate plus malate groups. Multivariate data analysis did not identify any differences between the fasted pyruvate alone and pyruvate plus malate groups.

Discussion

This is the first study in which differences to *in vivo* enzymatic regulation have been observed non-invasively. Hyperpolarized MR has revealed that, in fed rats, co-infusion of hyperpolarized [1-¹³C]pyruvate with malate increased the rate of ¹³C-label flux through PDH by 27% compared with [1-¹³C]pyruvate alone. However, in fasted rats, PDH flux was 72% lower and unaltered by malate addition. The distinct effects of malate in the fed and fasted states suggested that the dominant mechanism regulating the PDH enzyme complex in each state also differed.

Effects of *in vivo* malate infusion

In this study, we hypothesized that mitochondrial acetyl-CoA/CoA and NADH/NAD⁺ ratios could be modulated in the heart, *in vivo*, via systemic malate infusion and the malate-aspartate shuttle (Figure 4). Therefore, confirming that malate was taken up into the heart

and had these effects was fundamental to the primary aim of this study. To achieve this we used high-resolution ^1H MRS on heart tissue extracts to examine the effects of malate infusion on Krebs cycle intermediates.

Hardin *et al.* have reported evidence of a sarcolemmal dicarboxylate transporter, which facilitated malate and fumarate uptake and efflux in the isolated perfused heart [33]. *In vivo*, plasma malate could also enter cardiac myocytes rapidly via this transporter. The 55% increase in the levels of malate seen with high-resolution ^1H MRS performed on cardiac tissue extracts taken 30 s following malate infusion, alongside the 25% increase in intracellular citrate levels, the 20% increase in succinate levels, and the 30% increase to aspartate levels, confirm uptake of intravenous malate into the cytosol in our experiments.

Increased extra-mitochondrial malate is known to engage the malate-aspartate shuttle to promote mitochondrial malate uptake via a one-to-one exchange for α -ketoglutarate along the oxoglutarate-malate carrier (OMC, Figure 4) [25,34]. Increased mitochondrial levels of malate are incorporated into oxaloacetate [26,34], increasing the mitochondrial NADH/NAD $^+$ ratio [25,34,35]. Further, oxaloacetate availability limits the rate of citrate formation from oxaloacetate and acetyl-CoA via citrate synthase [25]. Therefore increased oxaloacetate availability induced by malate infusion enables increased citrate synthase flux [25,26] within a time frame of less than a minute [35], which consumes mitochondrial acetyl-CoA and thus decreases the acetyl-CoA/CoA ratio [25]. Consistent with this was the observation by Lysiak *et al.* that malate reduced the amount of acetylcarnitine produced from pyruvate in isolated mitochondria [36]. As acetylcarnitine levels reflect acetyl CoA/CoA ratio [32], these authors attributed the lowering of acetylcarnitine production by malate to low mitochondrial acetyl CoA/CoA due to competition from citrate synthase [36].

The 25% increase in the myocardial citrate pool that we observed provides additional evidence of this increased citrate production via citrate synthase. Citrate exists both within the mitochondrial Krebs cycle, and in the cytosol, implying that altered citrate levels do not implicitly imply increased citrate synthase flux. However, increased myocardial citrate is consistent with malate stimulating citrate synthase flux by increasing oxaloacetate availability. Further, in isolated mitochondria, elevated malate levels have been demonstrated to increase intra- and extra-mitochondrial citrate [35], and in the perfused heart, the rate of citrate release paralleled total tissue levels of citrate plus malate [37]. Given the elevated citrate and malate levels we observed using ^1H MRS, it is also likely that citrate release was substantially elevated in the malate infused rats compared with rats receiving pyruvate alone.

The observations made using high-resolution ^1H MRS, in combination with references to numerous studies performed in isolated mitochondria [25,26,35,36], have indicated that systemic malate infusion affected mitochondrial metabolism in the heart by decreasing the ratio of mitochondrial acetyl-CoA/CoA and increasing mitochondrial NADH/NAD $^+$. Therefore, our original hypothesis that co-infusing malate with hyperpolarized $[1-^{13}\text{C}]$ pyruvate would isolate acute mechanisms of PDH regulation appears to be valid, allowing us to fulfill our original study aim of assessing the mechanisms of PDH regulation using hyperpolarized ^{13}C MR.

Mechanisms of pyruvate dehydrogenase regulation

The decreased $\text{H}^{13}\text{CO}_3^-$ production observed in the fasted state agrees with numerous *in vivo* and biochemical studies from our group and others [1,15,24,38,39]. In the fasted state, both expression and activity of PDK is increased [24], in turn phosphorylating the PDH enzyme complex, such that less than 15% of the enzyme is in an active form (compared with the 30%-50% of active PDH in fed rats [1,13,23,24,39]). Therefore, in the system examined here, it is to be expected that long term, PDK-mediated phosphorylation-inactivation dominated PDH regulation and set an absolute upper limit on pyruvate flux.

In the fed state, however, a much larger proportion of the PDH enzyme complex exists in the active form able to catalyze pyruvate oxidation [23]. The exact amount of PDH flux cannot exceed the maximum capacity of the active fraction of the enzyme; however, a large fraction of active PDH implies that acute mechanisms potentially have an increased role in regulating substrate selection. For example, high mitochondrial ratios of NADH/NAD^+ and acetyl CoA/CoA, as produced by fatty acid oxidation, are thought to limit pyruvate flux through PDH by both acute activation of PDK [5], and feedback inhibition [9,10].

Here, the *in vivo* result that malate infusion elevated $\text{H}^{13}\text{CO}_3^-$ production by 27% indicates that acute PDH inhibition decreased flux from the maximum level set by the activity of the PDH enzyme complex. Malate infusion decreased acetyl-CoA/CoA, which would be expected to relieve acute inhibition on PDH, yet increased NADH/NAD^+ , which would be expected to further inhibit PDH flux. In isolated mitochondria, direct feedback inhibition on PDH flux is more sensitive to shifts in the acetyl-CoA/CoA ratio than to small perturbations in the redox potential [9], whereas acute inactivation of PDH by PDK has been shown to occur due to small perturbations in redox state that overcome larger shifts in acetyl-CoA/CoA [9]. Therefore, our results also suggest that malate infusion specifically increased PDH flux by means of alleviating direct feedback inhibition on the enzyme complex, rather than altering its activity via PDK. In the fed state, plasma fatty acid levels are on the order of 0.2 mM [15]; feedback inhibition of pyruvate oxidation may play an even more prominent role in situations of elevated plasma fatty acids, such as during high fat feeding [7,12]. Alternately, acute activation of PDK may dominate PDH regulation in conditions characterized by elevated NADH/NAD^+ including ischemia [21,22] and pressure overload hypertrophy [40].

Limitations of the study

Undoubtedly the metabolic fate of malate infusion into a living animal is complex, and it was outside the scope of this non-invasive study to determine the full route of malate disposal, and to quantitatively measure the effects of malate on cytosolic and mitochondrial levels of the low-concentration metabolic intermediates acetyl-CoA, CoA, oxaloacetate, NADH and NAD^+ . To achieve our study aims we confirmed that intravenous malate was taken up into myocytes, and subsequently referenced the multitude of previous studies examining malate and pyruvate respiration in isolated mitochondria, to estimate the alterations in mitochondrial acetyl CoA/CoA and NADH/NAD^+ in our *in vivo* study.

A second limitation of this study was that our source of MR signal required infusion of supraphysiological pyruvate. Pyruvate is known to independently inhibit PDK [6], and thus to stimulate PDH flux [41]. An 80 μM pyruvate infusion into a ~300 g rat raises plasma pyruvate levels to 250 μM , 30 s after infusion [42], suggesting that the protocol used here would have resulted in physiological plasma pyruvate levels of approximately 125 μM [41]. It is possible that this pyruvate infusion could have increased the measured $k_{\text{pyr} \rightarrow \text{bic}}$ to some extent. However, as the pyruvate infusion protocol was identical among all experimental groups, and did not result in excessively high pyruvate levels, we do not believe our experimental protocol could have affected the differences in $k_{\text{pyr} \rightarrow \text{bic}}$ we observed in the presence and absence of malate.

Conclusion

In summary, this study has used the non-invasive technique of hyperpolarized MR to distinguish between the mechanisms that govern cardiac PDH flux in the fasted and fed states. In the fasted state, the characteristic upregulation of PDK protein dominated the regulation of flux through PDH. In the fed state, modulating the acetyl-CoA/CoA ratio caused a statistically significant increase in PDH flux *in vivo*. These observations suggested that pyruvate oxidation is directly limited by feedback inhibition, caused by a high acetyl-CoA/CoA ratio, in the fed state when PDH activity is high. The approach presented here for non-invasively assessing PDH regulation may be applicable in additional enzymatic systems. The potential to use hyperpolarized ^{13}C MR to ascertain why metabolic fluxes are altered may enhance the specificity of the technique, thus expanding it into a more robust diagnostic method to pinpoint a range of cardiac pathologies.

Acknowledgements

M. Schroeder gratefully acknowledges the Wellcome Trust, the Newton Abraham Scholarship Foundation for her D.Phil. studentship, and NIH grant #1-F31-EB006692-01A1. This work was funded by research grants from the Medical Research Council [MRC Grant G0601490] and the British Heart Foundation [BHF Grant PG/07/070/23365] and through equipment support from GE-Healthcare.

Abbreviations

3-Gd	(1,3,5-tris-(N-(DO3A-acetamido)-N-methyl-4-amino-2-methylphenyl)-[1,3,5]triazinane-2,4,6-trione)
AMARES	Advanced Method for Accurate, Robust and Efficient Spectral Fitting
ANOVA	Analysis of Variance
ATMA	Automatic Tuning and Matching
ATP	Adenosine Triphosphate
CoA	Coenzyme A
DC	Direct Current
ECG	Electrocardiography
jMRUI	Java Magnetic Resonance User Interface

NADH/NAD⁺	Nicotinamide Adenine Dinucleotide
OMC	Oxoglutarate-Malate Carrier
PCA	Principal Component Analysis
PDH	Pyruvate Dehydrogenase
PDK	Pyruvate Dehydrogenase Kinase
PLS-DA	Partial Least Squares Discriminant Analysis
PPAR	Peroxisome Proliferator-Activated Receptor
TSP	sodium-3-(tri-methylsilyl)-2,2,3,3-tetradeuteriopropionate

References

1. Randle PJ. Fuel selection in animals. *Biochem Soc Trans.* 1986; 14:799–806. [PubMed: 3536635]
2. Reed LJ. Regulation of mammalian pyruvate dehydrogenase complex by a phosphorylation-dephosphorylation cycle. *Curr Top Cell Regul.* 1981; 18:95–106. [PubMed: 7273851]
3. Linn TC, Pettit FH, Reed LJ. Alpha-keto acid dehydrogenase complexes. X. Regulation of the activity of the pyruvate dehydrogenase complex from beef kidney mitochondria by phosphorylation and dephosphorylation. *Proc Natl Acad Sci U S A.* 1969; 62:234–241. [PubMed: 4306045]
4. Cooper RH, Randle PJ, Denton RM. Stimulation of phosphorylation and inactivation of pyruvate dehydrogenase by physiological inhibitors of the pyruvate dehydrogenase reaction. *Nature.* 1975; 257:808–809. [PubMed: 171583]
5. Kerbey AL, Randle PJ, Cooper RH, Whitehouse S, Pask HT, et al. Regulation of pyruvate dehydrogenase in rat heart. Mechanism of regulation of proportions of dephosphorylated and phosphorylated enzyme by oxidation of fatty acids and ketone bodies and of effects of diabetes: role of coenzyme A, acetyl-coenzyme A and reduced and oxidized nicotinamide-adenine dinucleotide. *Biochem J.* 1976; 154:327–348. [PubMed: 180974]
6. Pratt ML, Roche TE. Mechanism of pyruvate inhibition of kidney pyruvate dehydrogenase kinase and synergistic inhibition by pyruvate and ADP. *J Biol Chem.* 1979; 254:7191–7196. [PubMed: 457675]
7. Holness MJ, Smith ND, Bulmer K, Hopkins T, Gibbons GF, et al. Evaluation of the role of peroxisome-proliferator-activated receptor alpha in the regulation of cardiac pyruvate dehydrogenase kinase 4 protein expression in response to starvation, high-fat feeding and hyperthyroidism. *Biochem J.* 2002; 364:687–694. [PubMed: 12049632]
8. Sugden MC, Holness MJ. Recent advances in mechanisms regulating glucose oxidation at the level of the pyruvate dehydrogenase complex by PDKs. *Am J Physiol Endocrinol Metab.* 2003; 284:E855–862. [PubMed: 12676647]
9. Hansford RG, Cohen L. Relative importance of pyruvate dehydrogenase interconversion and feed-back inhibition in the effect of fatty acids on pyruvate oxidation by rat heart mitochondria. *Arch Biochem Biophys.* 1978; 191:65–81. [PubMed: 216317]
10. Hopkins TA, Sugden MC, Holness MJ, Kozak R, Dyck JR, et al. Control of cardiac pyruvate dehydrogenase activity in peroxisome proliferator-activated receptor-alpha transgenic mice. *Am J Physiol Heart Circ Physiol.* 2003; 285:H270–276. [PubMed: 12663261]
11. Lydell CP, Chan A, Wambolt RB, Sambandam N, Parsons H, et al. Pyruvate dehydrogenase and the regulation of glucose oxidation in hypertrophied rat hearts. *Cardiovasc Res.* 2002; 53:841–851. [PubMed: 11922894]
12. Orfali KA, Fryer LG, Holness MJ, Sugden MC. Long-term regulation of pyruvate dehydrogenase kinase by high-fat feeding. Experiments in vivo and in cultured cardiomyocytes. *FEBS Lett.* 1993; 336:501–505. [PubMed: 8282119]

13. Seymour AM, Chatham JC. The effects of hypertrophy and diabetes on cardiac pyruvate dehydrogenase activity. *J Mol Cell Cardiol.* 1997; 29:2771–2778. [PubMed: 9344771]
14. Caruso M, Maitan MA, Bifulco G, Miele C, Vigliotta G, et al. Activation and mitochondrial translocation of protein kinase Cdelta are necessary for insulin stimulation of pyruvate dehydrogenase complex activity in muscle and liver cells. *J Biol Chem.* 2001; 276:45088–45097. [PubMed: 11577086]
15. Schroeder M, Cochlin L, Heather L, Clarke K, Radda G, Tyler D. In Vivo Assessment of Pyruvate Dehydrogenase Flux in the Heart Using Hyperpolarized Carbon-13 Magnetic Resonance. *Proceedings of the National Academy of Sciences USA.* 2008; 105:12051–12056.
16. Ardenkjaer-Larsen JH, Fridlund B, Gram A, Hansson G, Hansson L, et al. Increase in signal-to-noise ratio of > 10,000 times in liquid-state NMR. *Proc Natl Acad Sci U S A.* 2003; 100:10158–10163. [PubMed: 12930897]
17. Golman K, in't Zandt R, Thaning M. Real-Time Metabolic Imaging. *Proc Natl Acad Sci U S A.* 2006; 103:11270–11275. [PubMed: 16837573]
18. Tyler D, Schroeder M, Cochlin L, Clarke K, Radda G. The Application of Hyperpolarized Magnetic Resonance in the Study of Cardiac Metabolism. *Applied Magnetic Resonance.* 2008; 34:523–531.
19. Golman K, Petersson JS, Magnusson P, Johansson E, Akeson P, et al. Cardiac metabolism measured noninvasively by hyperpolarized ¹³C MRI. *Magn Reson Med.* 2008; 59:1005–1013. [PubMed: 18429038]
20. Merritt ME, Harrison C, Storey C, Jeffrey FM, Sherry AD, et al. Hyperpolarized ¹³C allows a direct measure of flux through a single enzyme-catalyzed step by NMR. *Proc Natl Acad Sci U S A.* 2007; 104:19773–19777. [PubMed: 18056642]
21. Merritt ME, Harrison C, Storey C, Sherry AD, Malloy CR. Inhibition of carbohydrate oxidation during the first minute of reperfusion after brief ischemia: NMR detection of hyperpolarized ¹³CO₂ and H¹³CO₃. *Magn Reson Med.* 2008; 60:1029–1036. [PubMed: 18956454]
22. Lopaschuk GD, Spafford MA, Davies NJ, Wall SR. Glucose and palmitate oxidation in isolated working rat hearts reperfused after a period of transient global ischemia. *Circ Res.* 1990; 66:546–553. [PubMed: 2297817]
23. Holness MJ, Sugden MC. Pyruvate dehydrogenase activities during the fed-to-starved transition and on re-feeding after acute or prolonged starvation. *Biochem J.* 1989; 258:529–533. [PubMed: 2705997]
24. Wu P, Sato J, Zhao Y, Jaskiewicz J, Popov KM, et al. Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart. *Biochem J.* 1998; 329(Pt 1):197–201. [PubMed: 9405294]
25. LaNoue KF, Williamson JR. Interrelationships between malate-aspartate shuttle and citric acid cycle in rat heart mitochondria. *Metabolism.* 1971; 20:119–140. [PubMed: 4322086]
26. Montgomery CM, Webb JL. Metabolic studies on heart mitochondria. I. The operation of the normal tricarboxylic acid cycle in the oxidation of pyruvate. *J Biol Chem.* 1956; 221:347–357. [PubMed: 13345824]
27. Naressi A, Couturier C, Castang I, de Beer R, Graveron-Demilly D. Java-based graphical user interface for MRUI, a software package for quantitation of in vivo/medical magnetic resonance spectroscopy signals. *Comput Biol Med.* 2001; 31:269–286. [PubMed: 11334636]
28. Zierhut ML, Yen YF, Chen AP, Bok R, Albers MJ, et al. Kinetic modeling of hyperpolarized ¹³C1-pyruvate metabolism in normal rats and TRAMP mice. *J Magn Reson.* 2010; 202:85–92. [PubMed: 19884027]
29. Atherton HJ, Schroeder MA, Dodd MS, Heather LC, Carter EE, et al. Validation of the in vivo assessment of pyruvate dehydrogenase activity using hyperpolarised (¹³C) MRS. *NMR Biomed.* 2010
30. Atherton HJ, Bailey NJ, Zhang W, Taylor J, Major H, et al. A combined ¹H-NMR spectroscopy- and mass spectrometry-based metabolomic study of the PPAR-alpha null mutant mouse defines profound systemic changes in metabolism linked to the metabolic syndrome. *Physiol Genomics.* 2006; 27:178–186. [PubMed: 16868074]

31. Gulston MK, Rubtsov DV, Atherton HJ, Clarke K, Davies KE, et al. A combined metabolomic and proteomic investigation of the effects of a failure to express dystrophin in the mouse heart. *J Proteome Res.* 2008; 7:2069–2077. [PubMed: 18386883]
32. Pearson DJ, Tubbs PK. Carnitine and derivatives in rat tissues. *Biochem J.* 1967; 105:953–963. [PubMed: 16742571]
33. Hardin CD, Lazzarino G, Tavazzi B, Di Pierro D, Roberts TM, et al. Myocardial metabolism of exogenous FDP is consistent with transport by a dicarboxylate transporter. *Am J Physiol Heart Circ Physiol.* 2001; 281:H2654–2660. [PubMed: 11709435]
34. Safer B. The Metabolic Significance of the Malate-Aspartate Cycle in Heart. *Circ Res.* 1975; 37:527–533. [PubMed: 172258]
35. LaNoue K, Nicklas WJ, Williamson JR. Control of citric acid cycle activity in rat heart mitochondria. *J Biol Chem.* 1970; 245:102–111. [PubMed: 4312474]
36. Lysiak W, Toth PP, Suelter CH, Bieber LL. Quantitation of the Efflux of Acylcarnitines from Rat-Heart, Brain, and Liver-Mitochondria. *Journal of Biological Chemistry.* 1986; 261:3698–3703.
37. Vincent G, Comte B, Poirier M, Rosiers CD. Citrate release by perfused rat hearts: a window on mitochondrial cataplerosis. *Am J Physiol Endocrinol Metab.* 2000; 278:E846–856. [PubMed: 10780941]
38. Huang B, Wu P, Popov KM, Harris RA. Starvation and diabetes reduce the amount of pyruvate dehydrogenase phosphatase in rat heart and kidney. *Diabetes.* 2003; 52:1371–1376. [PubMed: 12765946]
39. Wieland O, Siess E, Schulze-Wethmar FH, von Funcke HG, Winton B. Active and inactive forms of pyruvate dehydrogenase in rat heart and kidney: effect of diabetes, fasting, and refeeding on pyruvate dehydrogenase interconversion. *Arch Biochem Biophys.* 1971; 143:593–601. [PubMed: 4326833]
40. Lewandowski ED, O'Donnell JM, Scholz TD, Sorokina N, Buttrick PM. Recruitment of NADH shuttling in pressure-overloaded and hypertrophic rat hearts. *Am J Physiol Cell Physiol.* 2007; 292:C1880–1886. [PubMed: 17229809]
41. Bhattacharya P, Ross BD, Bunger R. Cardiovascular applications of hyperpolarized contrast media and metabolic tracers. *Exp Biol Med (Maywood).* 2009; 234:1395–1416. [PubMed: 19934362]
42. Atherton HJ, Schroeder MA, Dodd MS, Heather LC, Carter EE, et al. Validation of the in vivo assessment of pyruvate dehydrogenase activity using hyperpolarised (13)C MRS. *NMR Biomed.*

Figure 1.

(A) An example time course of spectra acquired over 30 s. The arrival of $[1-^{13}\text{C}]$ pyruvate can be seen at ~ 171 ppm followed by its subsequent decay back to thermal equilibrium levels. As highlighted by the single example spectrum ($t = 15$ s) in (B), the appearance of the metabolic products, $[1-^{13}\text{C}]$ lactate, $[1-^{13}\text{C}]$ alanine and $\text{H}^{13}\text{CO}_3^-$ can be seen shortly afterwards. (C) An example of the temporal variations in the fitted peak areas of each metabolite over the 60 s time course. The pyruvate area has been scaled by a factor of 10 to improve visualization.

Figure 2.

The observed $k_{\text{pyr} \rightarrow \text{bic}}$ for fed and fasted rats, infused with pyruvate alone and pyruvate plus malate. * $p < 0.05$ in comparison with the fed group infused with pyruvate alone. Data are expressed $\pm$ SEM.

(B) In Vitro Metabolic Changes Induced by Malate Co-Infusion

Figure 3.

(A) Assigned high-resolution 500 MHz ^1H NMR spectrum of an extract of cardiac tissue. The data generated was analyzed using multivariate statistics to determine metabolic differences between rats infused with pyruvate alone and pyruvate plus malate. *Peak 1*: valine/leucine/isoleucine, *peak 2*: β -hydroxybutyrate, *peak 3*: lactate, *peak 4*: alanine, *peak 5*: acetate, *peak 6*: glutamate, *peak 7*: glutamate and glutamine, *peak 8*: glutamine, *peak 9*: succinate, *peak 10*: malate, *peak 11*: citrate, *peak 12*: aspartate, *peak 13*: creatine, *peak 14*: choline, *peak 15*: phosphocholine/glycerophosphocholine, *peak 16*: taurine, *peak 17*:

glycine, *peak 18*: glucose, *peak 19*: glycerol backbone. (B) The percentage change to several key metabolites studied by multivariate data analysis as potentially driving the classification between the fed pyruvate and pyruvate plus malate groups. Data values were Pareto scaled and normalized to 100% in the pyruvate group, such that the pyruvate plus malate group reflects the percentage change to each 0.04 ppm region of the NMR spectrum. *Differed significantly between groups at the 95% confidence limit and were identified as significantly driving the difference between groups.

(a) Malate may be taken up into the myocyte by a dicarboxylate transporter (DCT), thus driving the production of excess oxaloacetate (OAA) and aspartate (Asp) and increasing the cytosolic NADH/NAD⁺ ratio. (b) Excess cytosolic malate will be taken up into the mitochondria via increased flux through the oxoglutarate-malate carrier (OMC). (c) Increased mitochondrial malate uptake will increase the mitochondrial OAA pool, increasing mitochondrial NADH/NAD⁺ and flux of OAA and acetyl-CoA through citrate synthase.

TCT: tricarboxylate transporter; AGC: aspartate-glutamate carrier; MPC: mitochondrial pyruvate carrier.

Table 1

A summary of the regulation of the enzymes pyruvate dehydrogenase (PDH), pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP).

Enzyme	Activated By	Inhibited By
Pyruvate dehydrogenase (PDH)	<ul style="list-style-type: none"> • PDP-dephosphorylation • Pyruvate 	<ul style="list-style-type: none"> • PDK-phosphorylation • End-product inhibition: $\uparrow$NADH/NAD⁺ $\uparrow$Acetyl-CoA/CoA
Pyruvate dehydrogenase kinase (PDK)	<ul style="list-style-type: none"> • $\uparrow$NADH/NAD⁺ • $\uparrow$Acetyl-CoA/CoA 	<ul style="list-style-type: none"> • Pyruvate
Pyruvate dehydrogenase phosphatase (PDP)	<ul style="list-style-type: none"> • Insulin • Ca²⁺ • Mg²⁺ 	