Published in final edited form as:

Magn Reson Med. 2015 August ; 74(2): 312–319. doi:10.1002/mrm.25419.

Hyperpolarized ^{13}C NMR detects rapid drug-induced changes in cardiac metabolism

Chalermchai Khemtong¹, Nicholas R. Carpenter¹, Lloyd L. Lumata¹, Matthew E. Merritt¹, Karlos X. Moreno¹, Zoltan Kovacs¹, Craig R. Malloy¹, and A. Dean Sherry^{1,2}

¹Advanced Imaging Research Center, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd. Dallas, TX 75390

²Department of Chemistry, University of Texas at Dallas, 800 West Campbell Rd., Richardson, TX

Abstract

Purpose—The diseased myocardium lacks metabolic flexibility and responds to stimuli differently compared to healthy hearts. Here, we report the use of hyperpolarized ^{13}C NMR spectroscopy to detect sudden changes in cardiac metabolism in isolated, perfused rat hearts in response to adrenergic stimulation.

Methods—Metabolism of hyperpolarized $[1-^{13}\text{C}]$ pyruvate was investigated in perfused rat hearts. The hearts were stimulated in situ by isoproterenol shortly after the administration of hyperpolarized $[1-^{13}\text{C}]$ pyruvate. The hyperpolarized ^{13}C NMR results were corroborated with ^1H NMR spectroscopy of tissue extracts.

Results—Addition of isoproterenol to hearts after equilibration of hyperpolarized $[1-^{13}\text{C}]$ pyruvate into the existing lactate pool resulted in a sudden, rapid increase in hyperpolarized $[1-^{13}\text{C}]$ lactate signal within seconds after exposure to drug. The hyperpolarized $\text{H}^{13}\text{CO}_3^-$ and hyperpolarized $[1-^{13}\text{C}]$ alanine signals were not affected by the isoproterenol-induced elevated cardiac workload. Separate experiments confirmed that the new hyperpolarized $[1-^{13}\text{C}]$ lactate signal that arises after stimulation by isoproterenol reflects a sudden increase in total tissue lactate derived from glycogen.

Conclusions—These results suggest that hyperpolarized pyruvate and ^{13}C MRS may be useful for detecting abnormal glycogen metabolism in intact tissues.

Keywords

imaging; metabolism; magnetic resonance spectroscopy; isoproterenol

Introduction

The mammalian heart primarily uses free fatty acids (FFA) for energy production. Glucose, lactate, ketone bodies and amino acids can also be metabolized but these alternative substrates account for only a small proportion of cardiac energy requirements. Myocardial substrate metabolism is highly flexible with rapid alterations in substrate utilization occurring within seconds, as needed, as hearts respond to changes in hormones, substrate availability, and external stimuli. Examples include insulin-induced activation of glycolysis and glucose oxidation [1], increased lactate metabolism via pyruvate due to elevated circulating lactate during exercise [2], and promotion of glucose utilization through inhibition of fatty acid oxidation by an antianginal agent, trimetazidine [3]. It has been known for some time that the diseased myocardium lacks metabolic flexibility and responds differently to external stimuli compared to the healthy heart [4]. Therefore, the development of diagnostic tools for diagnosis of cardiac abnormalities by detecting the changes in myocardial metabolism could be quite useful clinically.

Magnetic resonance imaging of hyperpolarized (HP) nuclei offers the potential to detect rapid changes in metabolism. The use of hyperpolarized ^{13}C -enriched substrates produced by dynamic nuclear polarization (DNP) improves NMR sensitivity by 10,000-fold or greater [5]. With such gains in signal, DNP now allows the possibility of imaging less sensitive nuclei such as ^{13}C , ^{15}N , ^6Li , ^{89}Y and others at sensitivities approaching that of water. This has opened the door to imaging a variety of biological processes using hyperpolarized reporter molecules [6–10]. $[1-^{13}\text{C}]$ -pyruvate (^{13}C -enriched at the carboxylate carbon only) has become the most widely studied metabolic substrate to date. In metabolically active tissues, HP pyruvate is rapidly metabolized and distinct NMR signals from HP bicarbonate, lactate, alanine, and glutamate have all been detected at a temporal resolution of a few seconds [9, 11–13]. This high temporal resolution allows for possible detection of rapid changes in metabolite pool sizes and/or enzyme kinetics by NMR. Our lab and others have demonstrated that HP $[1-^{13}\text{C}]$ pyruvate can differentiate between normal and hypertrophied heart tissue, pyruvate dehydrogenase (PDH) activity, and changes in metabolism in the failing heart [9, 11, 14, 15]. Metabolism of HP pyruvate has also been explored as potential diagnostic and prognostic marker of cancer in tumor-bearing animals. It has been shown that the Warburg effect can be characterized in tumors by spectroscopic imaging (MRSI) by quantifying the conversion of HP pyruvate to HP lactate [12, 16, 17]. Assessment of the tumor microenvironment including an estimate of tumor pH has also reported using HP bicarbonate [18]. These studies and others have shown that HP ^{13}C -enriched metabolic substrates and exogenous agents have considerable clinical potential for detecting metabolic abnormalities in heart tissue and in staging of malignant tumors.

Herein, we report the use of HP $[1-^{13}\text{C}]$ pyruvate and ^{13}C NMR spectroscopy to detect sudden changes in cardiac metabolism as a result of *in situ* β -agonist stimulation. We hypothesized that the high sensitivity and fast temporal resolution of HP substrates would allow for the real-time detection of drug-induced abrupt changes in substrate metabolism in the heart. In this study, the effect of β -agonist stimulation on myocardial metabolism of HP $[1-^{13}\text{C}]$ pyruvate was investigated in Langendorff-perfused rat hearts where rapid and precise delivery of HP pyruvate and the β -adrenergic agonist can be achieved. ^{13}C NMR

spectroscopy revealed a sudden increase in HP [1-¹³C]lactate signal within seconds after stimulation of cardiac function by isoproterenol, a β -agonist. This abrupt increase in HP [1-¹³C]lactate was subsequently traced to rapid production of lactate from tissue glycogen on a timescale of a few seconds. These results were confirmed by high resolution ¹³C NMR spectroscopy of tissue extracts from the hearts perfused with non-polarized ¹³C-enriched pyruvate as a tracer.

METHODS

Heart Perfusion

All procedures were approved by the IACUC at UTSW. Hearts were rapidly excised from male rats under general anesthesia (1.5% isoflurane in oxygen) and perfused at a constant pressure (100-mm Hg) with a modified Krebs-Henseleit (KH) solution oxygenated with a 95:5 mixture of O₂/CO₂. A balloon secured to a catheter was inserted into the left ventricle for monitoring of heart rate and developed pressure. The hearts were placed in an 18 mm NMR tube placed into the bore of a 14.1T magnet and shimmed on the ²³Na fid to a linewidth of ~17 Hz. The heart rate, cardiac flow rate, developed pressure, and oxygen partial pressure (pO₂) were recorded during this time. A concentrated sucrose solution (250 mM) was delivered directly to the bottom of the NMR tube (2 mL/min) to flush all other HP species outside the heart away from the NMR-sensitive volume.

Polarization Procedure and Delivery of HP [1-¹³C]Pyruvic acid

A solution of OX063 radical (15 mM) and ProHance® ([Gd] = 2 mM) in 4 μ L [1-¹³C]pyruvic acid was polarized in a HyperSense polarizer (Oxford Instruments, UK).[19] After ~90 min, the frozen sample was dissolved in superheated PBS (pH 7.4, 4 mL). The estimated liquid-state enhancement for [1-¹³C]pyruvic acid was >16,000-fold. This corresponds to ~15% liquid state ¹³C polarization. A 3 mL aliquot of the HP solution was quickly mixed with 20 mL of KH ([HP-pyruvate] = 2 mM) and was injected directly above the heart via a polyethylene catheter (0.99 mm inner diameter). The flow rate during the injection of HP-pyruvate was approximately 12 mL/min. The injection generally lasted ~90 s. The ¹³C NMR acquisition was initiated at the beginning of the injection of HP substrate (Fig. 1). Thirty seconds after the injection of HP-pyruvate, a solution of isoproterenol (1 μ M) in KH buffer was administered to the heart in the same manner as the HP-pyruvate. After the completion of all ¹³C NMR acquisitions, hearts were perfused for an additional 10 min with [3-¹³C]pyruvate before being freeze-clamped. Over the course of each experiment, the perfusate was periodically sampled and checked for pH and pO₂ using a GEM Premiere 3000 blood gas analyzer (Instrumentation Laboratory, Bedford, MA). Coronary flow (mL/min) was measured by collecting samples of recirculating perfusate for a fixed period of time, typically 30 s.

¹³C NMR Spectroscopy of Perfused Hearts

¹³C NMR spectra were acquired at 14.1 T (Varian INOVA) in an 18 mm broadband probe (Doty Scientific) using 20° pulses. Serial, single FIDs were acquired with proton decoupling (AT=1 s) with a delay time of 1 s between scans. These data were zero-filled before Fourier transformation and the relative peak areas were measured by integration using ACDLabs

SpecManager (ACD/Labs, Canada). Peak areas were normalized to the total peak areas of all metabolites and plotted as a function of time.

High Resolution ^{13}C and ^1H NMR Spectroscopy of Tissue Extracts

Two groups of perfusion studies were done outside the NMR spectrometer to (1) quantify myocardial lactate concentration by ^1H NMR, and (2) identify the sources of endogenous lactate. In the first experiment, eight hearts (2 groups, $n = 4$) were perfused under the same conditions as in the hyperpolarized ^{13}C studies. First, four hearts were perfused with the KH buffer containing 1 mM unlabeled pyruvate for 30 min. At $t = 0$ s, 2 mM $[3-^{13}\text{C}]$ pyruvate in KH was directly injected above the heart. ~66 s later, each heart was rapidly arrested and freeze-clamped. A second group of four hearts were perfused with 1 mM unlabeled pyruvate in KH for 30 min, at $t = 0$, 2 mM $[3-^{13}\text{C}]$ pyruvate injected into the perfusate as before. Approximately 30 s later, isoproterenol in KH (1 μM) was injected to stimulate cardiac function and each heart was freeze-clamped at ~66 s. In another experiment, the hearts were perfused with KH only without oxidizable substrates for 45 min to deplete tissue glycogen. Each heart was then perfused with 10 mM $[\text{U}-^{13}\text{C}]$ glucose and insulin (50 μU) in KH for 1 h to replenish glycogen. After the glycogen replenishment, one group of hearts was perfused with 1 mM unlabeled pyruvate for 30 min followed by 2 mM unlabeled pyruvate (time = 0 s) before being freeze-clamped at ~66 s. In a second group, hearts were perfused with 1 mM unlabeled pyruvate for 30 min to the steady-state. Then at $t = 0$ s, 2 mM unlabeled pyruvate was injected followed by 1 μM isoproterenol at ~30 s. Again, these hearts were freeze-clamped at ~66 s.

The frozen heart tissue was pulverized in a cold mortar and extracted with 5% perchloric acid. The extracts were then neutralized, freeze-dried, and re-constituted in D_2O containing 1 mM EDTA and 0.5 mM DSS for ^1H NMR and ^1H -decoupled ^{13}C NMR. All high resolution NMR spectra were acquired on a 600 MHz spectrometer (Agilent). The concentrations of lactate and alanine were determined from ^1H NMR spectra using Chenomx NMR Suite (Chenomx, Canada), using DSS as internal standard. ^1H chemical shifts were referenced to DSS (0 ppm). All NMR spectra were processed using ACD/NMR Processor.

RESULTS

The effect of isoproterenol on cardiac metabolism as assayed by HP $[1-^{13}\text{C}]$ pyruvate

Hearts were first perfused with non-polarized $[3-^{13}\text{C}]$ pyruvate to achieve a steady-state ^{13}C isotopomer distribution in all TCA cycle intermediates [20] before being exposed to hyperpolarized pyruvate. Addition of 2 mM HP $[1-^{13}\text{C}]$ pyruvate ($t = 0$) resulted in rapid appearance of all downstream metabolites. The resonance intensities of HP $[1-^{13}\text{C}]$ lactate (183.1 ppm), HP $[1-^{13}\text{C}]$ alanine (176.5 ppm), HP $[^{13}\text{C}]$ carbon dioxide (125.4 ppm), and HP $[^{13}\text{C}]$ bicarbonate (160.9 ppm) all increased with time, reached an apex in peak intensity (see Fig. 2a and 2b), then gradually declined with time due to T_1 relaxation of each polarized species [9]. The resulting intensity *versus* time curves are a unique characteristic of HP metabolic studies.

To test whether sudden changes in cardiac function that parallel β -adrenergic stimulation might alter these HP signals, a second perfusion protocol was designed to allow for rapid stimulation of cardiac function by isoproterenol (1 μ M) while HP-pyruvate was being metabolized by the myocardium. As illustrated in Figure 1, isoproterenol was added to the perfusion medium ~30 s after addition of HP-[1- 13 C]pyruvate, a time chosen to correspond to a few seconds after the signals of HP lactate, alanine and bicarbonate had reached their apex and begun to decline (Fig. 2c). As expected, an elevated cardiac workload (as measured by HR and DP, Supporting Information Fig. S2) was achieved approximately 15 s after the addition of isoproterenol. Almost immediately thereafter, the signal intensity of HP-lactate which had begun to decay after reaching a first apex sharply increased in intensity and reached a second apex (Fig. 2c). The signals of HP-alanine and HP-H 13 CO $_3^-$ did not display this same pattern after addition of isoproterenol (Fig. 2c). This illustrates that stimulation of cardiac function did not significantly alter the total HP- 13 CO $_2$ /H 13 CO $_3^-$ signal or exchange of HP-pyruvate with HP-alanine. The appearance of the second apex in the HP-lactate curve was clearly linked to the stimulatory effects of isoproterenol since the same phenomenon was not observed in control hearts not stimulated by isoproterenol. Fig. 2d compares the intensity *versus* time curves for HP-lactate from two separate hearts, perfused under identical conditions except for the addition of isoproterenol. The appearance of the second apex in the HP-lactate curve suggests that an increase in cardiac workload stimulated either 1) production of more HP [1- 13 C]lactate directly from any remaining HP [1- 13 C]pyruvate or 2) production of more unenriched lactate from another source which then equilibrates rapidly with any remaining HP [1- 13 C]pyruvate. Further experiments were then designed to differentiate between these two possibilities.

Quantification of 13 C-enriched lactate by high resolution NMR spectroscopy

To investigate the origin of the second apex in the HP [1- 13 C]lactate kinetic curve, total tissue lactate and alanine and the 13 C fractional enrichment in these two metabolites were measured in extracts of hearts perfused on the bench (no NMR) using identical concentrations of non-hyperpolarized pyruvate. These hearts were freeze-clamped at the same time that would have corresponded to the second apex of the HP-lactate kinetic curve (66 s) had HP pyruvate been used. Group 1 hearts received unenriched pyruvate for a period of 30 min then given a bolus of 2 mM [3- 13 C]pyruvate for ~66 s before freeze-clamping. Group 2 hearts received identical amounts of [3- 13 C]pyruvate except 1 μ M isoproterenol was added 30 s after the bolus of 2 mM [3- 13 C]pyruvate. Typical 1 H NMR spectra of tissue extracts of hearts in groups 1 and 2 are compared in Figure 3a. In both spectra, three doublets (due to J_{HH} spin-spin coupling) were observed for the methyl proton resonances of lactate (1.22, 1.32, 1.43 ppm) and the methyl proton resonances of alanine (1.37, 1.47, 1.58 ppm). The outer doublets (reflecting one-bond J_{CH} spin-spin coupling) in each metabolite resonance could be assigned to [3- 13 C]lactate and [3- 13 C]alanine, both derived from [3- 13 C]pyruvate, while the center doublet in each grouping reflects unlabeled lactate and alanine derived from endogenous sources. The concentrations of lactate and alanine (13 C enriched and natural abundance) were calculated from peak intensities of the three resonances, respectively, using 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard. Table 1 summarizes the average concentrations of 13 C-enriched lactate and alanine

(that portion derived from [3-¹³C]pyruvate) and the total tissue lactate and alanine concentrations.

Representative ¹³C NMR spectra of tissue extracts showing glutamate C3 and C4 resonances of control hearts (group 1, spectra 3) and hearts exposed to isoproterenol (group 2, spectra 2) are shown in Figure 3b (for full spectra, see Supporting Information Fig. S4). These are compared with ¹³C spectra of an isoproterenol-stimulated heart exposed to [3-¹³C]pyruvate throughout (spectra 1) and a heart perfused with 2 mM non-labeled pyruvate for 30 min (spectra 4). A non-steady state isotopomer analysis of the glutamate multiplets in spectra ii and iii provided an estimate of [3-¹³C]pyruvate contribution to the acetyl-CoA entering the TCA cycle in each group [21]. The results showed that $24.9 \pm 3.6\%$ of the acetyl-CoA entering the TCA cycle came from the supplied [3-¹³C]pyruvate in hearts not stimulated by isoproterenol and $48.7 \pm 7.6\%$ ($p < 0.05$) in hearts exposed to isoproterenol (Table 1). This indicates that isoproterenol rapidly increases the fraction of acetyl-CoA derived from [3-¹³C]pyruvate to acetyl-CoA. The steady-state ¹³C NMR spectra of tissue extracts from the hearts that received 1 mM [3-¹³C]pyruvate for 10 min after the injection of 2 mM [3-¹³C]pyruvate and isoproterenol shows characteristic ¹³C multiplets of the metabolites (Supporting Information Fig. S5). A steady-state isotopomer analysis showed that the exposure of hearts to a bolus of isoproterenol followed by washout (increased HR for ~45s) did not alter the steady-state contribution of [3-¹³C]pyruvate to acetyl-CoA ($76 \pm 3\%$ w/o isoproterenol and $79 \pm 6\%$ with isoproterenol).

These ¹H and ¹³C NMR data illustrate two important points. First, in agreement with the HP experiments, total tissue alanine was no different between the two groups, consistent with no change in HP-alanine with increased cardiac activity stimulated by isoproterenol. Second, the most striking finding was that although total tissue lactate was about 2-fold higher in hearts exposed to isoproterenol (9.4 ± 3.8 versus 20.9 ± 5.9 $\mu\text{mol/g dw}$), there was no change in the amount of [3-¹³C]lactate in the lactate pool (2.0 ± 0.6 versus 2.2 ± 0.4 $\mu\text{mol/g dw}$). Results from separate experiments where [3-¹³C]pyruvate was used as exogenous pyruvate sources during both initial 30-min perfusion and ~60 s injection also showed a significant increase in the total lactate pool with no change in the [3-¹³C]lactate contribution (Supporting Information Fig. S6). This illustrates that the lactate generated in response to increased cardiac workload came from an endogenous source.

To identify the origin of this endogenous source, we first depleted stored glycogen and then replenished glycogen with ¹³C-enriched glucosyl units. These hearts were subsequently perfused under identical conditions as described in Fig. 3 with and without isoproterenol but without any ¹³C enrichment in pyruvate. The ¹H NMR spectra of tissue extracts from these hearts are shown in Fig 4. Here, a small amount of ¹³C enriched lactate and alanine was again be detected as “wings” symmetrically placed around the central non-enriched lactate and alanine resonances but, in this case, these satellite resonances appear as doublets of triplets reflecting long range spin-spin coupling in [U-¹³C₃]lactate or [U-¹³C₃]alanine. The spectra illustrate that in hearts perfused with pyruvate and not exposed to isoproterenol, a small amount of tissue lactate (31%) is derived from glycogen. Less glycogen contributed to tissue alanine (13%). In hearts stimulated by isoproterenol, the glycogen contribution to tissue lactate increased to 55% while the amount contributing to alanine also increased (to

23%) but this increase was less than that found for lactate. These observations demonstrate that significant glycogenolysis occurs upon stimulation of hearts with isoproterenol, producing both lactate and, to a lesser extent, alanine.

DISCUSSION

The metabolic state of the heart is a valuable diagnostic and prognostic marker of cardiovascular disease. Healthy hearts can adapt rapidly and effectively to physiological changes responding to fluctuating energy demands. Noninvasive tools capable of detecting rapid metabolic adaptations in real-time may potentially offer clinical benefits for early detection of cardiovascular diseases given that ailing hearts are generally lacking in metabolic flexibility. To date, altered cardiac metabolism as a result of pharmacological stimulations and induced cardiac events are typically studied as two separate groups, control *versus* stimulated hearts [22]. HP ^{13}C NMR has recently become a novel tool to investigate altered substrate metabolism in the heart. Our group first used HP-pyruvate and ^{13}C NMR to examine substrate selection in perfused rat hearts [9, 19] by monitoring generation of HP- CO_2 and HCO_3^- from pyruvate generated by flux into the mitochondria through pyruvate dehydrogenase (PDH). In that study, decreased flux of HP-[1- ^{13}C]pyruvate into acetyl-CoA was observed when the hearts were perfused with a mix of HP-pyruvate and free fatty acids. Atherton *et al* also reported increased cardiac metabolism of HP pyruvate through PDH *in vivo* after addition of exogenous dichloroacetate to rats [14]. Altered cardiac metabolism has also been investigated in disease models such as ischemic heart disease [23, 24], diabetes [15], and hypertrophy [14, 25]. Drug-induced metabolism changes have also been previously investigated [26, 27]. Despite the clear evidence of altered substrate utilization in the heart observed by HP ^{13}C NMR, real-time detection of those altered metabolic events has not, to our knowledge, been observed previously.

The goal of this study was to evaluate whether hyperpolarized pyruvate could detect rapid changes in substrate utilization in the myocardium upon stimulation of cardiac function. The β -adrenergic agonist, isoproterenol, was chosen because it is known to induce rapid, positive inotropic stimulation resulting in an elevated cardiac workload in Langendorff perfused hearts. Initial bench experiments verified that addition of 1 μM isoproterenol to a perfused heart rapidly increased both heart rate (HR) and myocardial oxygen consumption (MVO_2). In bench experiments, hearts perfused with 2 mM unlabeled pyruvate displayed an average HR and MVO_2 ($n = 4$) of 289 ± 10 beats per min (bpm) and 15.5 ± 0.6 $\mu\text{mol}/\text{min}$ dw, respectively. After addition of 1 μM isoproterenol, the average HR and MVO_2 increased to 422 ± 28 bpm and 47.9 ± 1.9 $\mu\text{mol}/\text{min}$ dw, respectively. In parallel, the coronary flow (CF) increased from 9.2 ± 0.4 mL/min to 20.9 ± 1.0 mL/min after stimulation. This isoproterenol-induced increase in cardiac output provided an excellent platform for assessing the feasibility of using HP ^{13}C NMR spectroscopy for the detection of rapid changes in cardiac metabolism. It is worth noting that the increased cardiac workload did not affect the performance and viability of the heart as confirmed by ^{31}P NMR (Fig. S7).

In this study, HP ^{13}C NMR was used to monitor rapid alterations in metabolism that occur rather quickly after adrenergic stimulation. Each heart served as its own control because dynamic scans of all metabolites produced from HP-pyruvate (lactate, alanine, bicarbonate)

were collected before and after stimulation of cardiac activity using isoproterenol. The most interesting and surprising observation was a rapid increase in the hyperpolarized lactate signal after administration of isoproterenol which reached a second apex in its intensity versus time curve ~20 s after stimulation. Similar changes were not observed in the dynamic signals of either alanine or bicarbonate. The second apex in the lactate signal was traced to a sudden ~2-fold increase in tissue lactate generated from glycogen. These results are consistent with the “glycogen shunt” hypothesis put forth by Shulman and Rothman [28] who suggested that rapid breakdown of glycogen to lactate is absolutely required to buffer the short-term energy requirements of exercising muscle.

Given the increased energy demand driven by the positive inotropic response, we anticipated seeing increases in the magnitude of HP- $^{13}\text{CO}_2/\text{H}^{13}\text{CO}_3^-$ signal derived from HP-[1- ^{13}C]pyruvate. Unexpectedly, there was no increase in the intensity-time curve of HP- $\text{H}^{13}\text{CO}_3^-$ despite the ~2-fold increase flux of HP-pyruvate through PDH as verified by ^{13}C NMR isotopomer analysis of tissue extracts. In fact, if one carefully compares the shapes of the dynamic HP $\text{H}^{13}\text{CO}_3^-$ curves *versus* the dynamic HP-[1- ^{13}C]alanine signal, the HP bicarbonate signal appears to drop off more quickly (Fig 2c and Fig. S3) after stimulation by isoproterenol. We attribute this to the substantial increase in coronary flow [29, 30] that accompanies stimulation by isoproterenol which, in turn, washes away the tissue-produced HP $\text{H}^{13}\text{CO}_3^-$ quite rapidly from the sensitive volume of the NMR coil. In comparison, HP alanine does not leave the tissue so this polarized signal “relaxes” at a similar rate in both stimulated and unstimulated hearts. Given that carbonic anhydrase catalyzed conversion of HP- $^{13}\text{CO}_2$ to $\text{H}^{13}\text{CO}_3^-$ occurs largely in extracellular space of heart tissue [31], the increased coronary flow should rapidly remove both HP- $^{13}\text{CO}_2$ and HP- $\text{H}^{13}\text{CO}_3^-$ from the heart and this results in no apparent increase in HP- $\text{H}^{13}\text{CO}_3^-$ production. It is important to point out that the bathing medium surrounding the hearts in the NMR tube here was also continuously and rapidly “flushed” with sucrose throughout the ^{13}C experiment to purposely remove the intense extracellular HP-pyruvate signal as quickly as possible so that weaker HP metabolite signals could be more easily detected.

A scheme to illustrate our proposed mechanism for the appearance of the second “apex” in the dynamic HP lactate data is illustrated in Fig. 5. Hearts perfused with HP-pyruvate as the sole exogenous energy source mainly use pyruvate for oxidation as shown by high intensity of the HP- HCO_3^- signal and verified by ^{13}C isotopomer analysis. Upon addition of isoproterenol, the stimulated heart experiences a sudden increase in energy demand so quickly breaks down glycogen to lactate [28]. This results in generation of two pools of pyruvate and lactate. There is ample evidence in the literature showing that pyruvate produced from glycolysis does not fully equilibrate with exogenously supplied pyruvate, even under steady-state metabolic conditions [32–35]. Initially, before stimulation, there is a relatively small pool of endogenous lactate present in tissue that exchanges quickly with HP-pyruvate leading to the appearance of HP-lactate that reaches maximum intensity at ~15 s. Day *et al.* (34) have shown that exchange of HP-pyruvate into a pool of existing lactate can occur rapidly without net production of lactate by simple exchange of reducing equivalents in the active site of LDH. Thus, if a bigger lactate pool is already present in tissues when presented with HP-pyruvate, a larger signal of HP-lactate will result [36]. In this study,

stimulation of cardiac function at the mid-point of HP-pyruvate relaxation results in a rapid, nearly immediate increase in the size of the lactate pool, so any remaining HP-pyruvate once again exchanges rapidly into this newly formed pool of lactate to produce the second “apex”. Peuhkurinen *et al* [35], reported years ago that there are at least two different compartments of pyruvate in cardiac tissue, a glycolytic pool and a peripheral pool. The glycolytic pyruvate pool is thought to communicate more closely with glycolysis and tissue lactate while the “peripheral” pyruvate pool has a closer connection with extracellular and mitochondrial pyruvate. Later, we also used ^{13}C NMR isotopomer methods and found evidence for intracellular compartmentation of glycolytic and glycogenolytic enzymes in isolated rat hearts perfused with $[1-^{13}\text{C}]\text{glucose}$ [32]. The observation that one can detect changes in lactate derived from glycogen within seconds after stimulation of cardiac function by isoproterenol illustrates the power of using hyperpolarized substrates to detect rapid metabolic events. This feature offers new opportunities to assess the metabolic flexibility of individual hearts in a quick and relatively non-invasive imaging experiment. The ability to detect and assess the lost flexibility of the heart treated with pharmacological stimulants could prove greatly advantageous for the treatment and management of heart disease.

In summary, we have demonstrated the feasibility of using HP- $[1-^{13}\text{C}]\text{pyruvate}$ and NMR spectroscopy to detect rapid changes in cardiac metabolism. The results illustrated here suggest that HP ^{13}C NMR may prove useful for detecting rapid changes in substrate availability to the heart in response to various cardiac stimulants. The advantages of hyperpolarized substrates such as $[1-^{13}\text{C}]\text{pyruvate}$ for such studies is that the test is rapid (2 min) and could potentially applied multiple times in the same heart to evaluate the metabolic consequences of various cardiac agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank the National Institutes of Health [NIH 5R37-HL034557 to ADS and NIH 8P41-EB015908 to CRM] for financial support. Charles Storey and Angela Milde are acknowledged for their excellent technical support in the perfusion experiments.

REFERENCES

1. Stanley WC, Recchia FA, Lopaschuk GD. Myocardial substrate metabolism in the normal and failing heart. *Physiol Rev.* 2005; 85(3):1093–1129. [PubMed: 15987803]
2. Gertz EW, Wisneski JA, Stanley WC, Neese RA. Myocardial substrate utilization during exercise in humans. Dual carbon-labeled carbohydrate isotope experiments. *J Clin Invest.* 1988; 82(6):2017–2025. [PubMed: 3198763]
3. Kantor PF, Lucien A, Kozak R, Lopaschuk GD. The antianginal drug trimetazidine shifts cardiac energy metabolism from fatty acid oxidation to glucose oxidation by inhibiting mitochondrial long-chain 3-ketoacyl coenzyme A thiolase. *Circ Res.* 2000; 86(5):580–588. [PubMed: 10720420]
4. Taegtmeyer H, Golfman L, Sharma S, Razeghi P, van Arsdall M. Linking gene expression to function: metabolic flexibility in the normal and diseased heart. *Ann N Y Acad Sci.* 2004; 1015:202–213. [PubMed: 15201161]

5. Ardenkjaer-Larsen JH, Fridlund B, Gram A, Hansson G, Hansson L, Lerche MH, Servin R, Thaning M, Golman K. Increase in signal-to-noise ratio of >10,000 times in liquid-state NMR. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100(18):10158–10163. [PubMed: 12930897]
6. Gabellieri C, Reynolds S, Lavie A, Payne GS, Leach MO, Eykyn TR. Therapeutic target metabolism observed using hyperpolarized ^{15}N choline. *Journal of the American Chemical Society*. 2008; 130(14):4598–4599. [PubMed: 18345678]
7. Kovacs Z, Jindal AK, Merritt ME, Suh EH, Malloy CR, Sherry AD. Hyperpolarized (89)Y Complexes as pH Sensitive NMR Probes. *Journal of the American Chemical Society*. 2010; 132(6): 1784–1785. [PubMed: 20102196]
8. Kovacs Z, Lumata L, Jindal AK, Merritt ME, Malloy CR, Sherry AD. DNP by Thermal Mixing under Optimized Conditions Yields >60 000-fold Enhancement of (89)Y NMR Signal. *Journal of the American Chemical Society*. 2011; 133(22):8673–8680. [PubMed: 21539398]
9. Merritt ME, Harrison C, Storey C, Jeffrey FM, Sherry AD, Malloy CR. Hyperpolarized ^{13}C allows a direct measure of flux through a single enzyme-catalyzed step by NMR. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; 104(50):19773–19777. [PubMed: 18056642]
10. van Heeswijk RB, Uffmann K, Comment A, Kurdzesau F, Perazzolo C, Cudalbu C, Jannin S, Konter JA, Hautle P, van den Brandt B, et al. Hyperpolarized lithium-6 as a sensor of nanomolar contrast agents. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 2009; 61(6):1489–1493.
11. Golman K, Petersson JS, Magnusson P, Johansson E, Akeson P, Chai CM, Hansson G, Mansson S. Cardiac metabolism measured noninvasively by hyperpolarized ^{13}C MRI. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 2008; 59(5):1005–1013.
12. Golman K, Zandt RI, Lerche M, Pehrson R, Ardenkjaer-Larsen JH. Metabolic imaging by hyperpolarized ^{13}C magnetic resonance imaging for in vivo tumor diagnosis. *Cancer Res*. 2006; 66(22):10855–10860. [PubMed: 17108122]
13. Schroeder MA, Atherton HJ, Ball DR, Cole MA, Heather LC, Griffin JL, Clarke K, Radda GK, Tyler DJ. Real-time assessment of Krebs cycle metabolism using hyperpolarized ^{13}C magnetic resonance spectroscopy. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2009; 23(8):2529–2538. [PubMed: 19329759]
14. Atherton HJ, Dodd MS, Heather LC, Schroeder MA, Griffin JL, Radda GK, Clarke K, Tyler DJ. Role of pyruvate dehydrogenase inhibition in the development of hypertrophy in the hyperthyroid rat heart: a combined magnetic resonance imaging and hyperpolarized magnetic resonance spectroscopy study. *Circulation*. 2011; 123(22):2552–2561. [PubMed: 21606392]
15. Schroeder MA, Lau AZ, Chen AP, Gu Y, Nagendran J, Barry J, Hu X, Dyck JR, Tyler DJ, Clarke K, et al. Hyperpolarized ^{13}C magnetic resonance reveals early- and late-onset changes to in vivo pyruvate metabolism in the failing heart. *European journal of heart failure*. 2012; 15:130–140. [PubMed: 23258802]
16. Albers MJ, Bok R, Chen AP, Cunningham CH, Zierhut ML, Zhang VY, Kohler SJ, Tropp J, Hurd RE, Yen YF, et al. Hyperpolarized ^{13}C lactate, pyruvate, and alanine: noninvasive biomarkers for prostate cancer detection and grading. *Cancer Res*. 2008; 68(20):8607–8615. [PubMed: 18922937]
17. Lupo JM, Chen AP, Zierhut ML, Bok RA, Cunningham CH, Kurhanewicz J, Vigneron DB, Nelson SJ. Analysis of hyperpolarized dynamic ^{13}C lactate imaging in a transgenic mouse model of prostate cancer. *Magn Reson Imaging*. 2010; 28(2):153–162. [PubMed: 19695815]
18. Gallagher FA, Kettunen MI, Day SE, Lerche M, Brindle KM. ^{13}C MR spectroscopy measurements of glutaminase activity in human hepatocellular carcinoma cells using hyperpolarized ^{13}C -labeled glutamine. *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*. 2008; 60(2):253–257.
19. Moreno KX, Sabelhaus SM, Merritt ME, Sherry AD, Malloy CR. Competition of pyruvate with physiological substrates for oxidation by the heart: implications for studies with hyperpolarized $[1-^{13}\text{C}]$ pyruvate. *Am J Physiol Heart Circ Physiol*. 2010; 298(5):H1556–H1564. [PubMed: 20207817]

20. Malloy CR, Sherry AD, Jeffrey FM. Analysis of tricarboxylic acid cycle of the heart using ^{13}C isotope isomers. *Am J Physiol.* 1990; 259(3 Pt 2):H987–H995. [PubMed: 1975735]
21. Malloy CR, Thompson JR, Jeffrey FM, Sherry AD. Contribution of exogenous substrates to acetyl coenzyme A: measurement by ^{13}C NMR under non-steady-state conditions. *Biochemistry.* 1990; 29(29):6756–6761. [PubMed: 1975750]
22. Lionetti V, Stanley WC, Recchia FA. Modulating fatty acid oxidation in heart failure. *Cardiovasc Res.* 2011; 90(2):202–209. [PubMed: 21289012]
23. Gallagher FA, Kettunen MI, Day SE, Hu DE, Ardenkjaer-Larsen JH, Zandt R, Jensen PR, Karlsson M, Golman K, Lerche MH, et al. Magnetic resonance imaging of pH in vivo using hyperpolarized ^{13}C -labelled bicarbonate. *Nature.* 2008; 453(7197):940–943. [PubMed: 18509335]
24. 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 $^{13}\text{CO}_2$ and H^{13}CO_3 . *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine.* 2008; 60(5):1029–1036.
25. Dodd MS, Ball DR, Schroeder MA, Le Page LM, Atherton HJ, Heather LC, Seymour AM, Ashrafian H, Watkins H, Clarke K, et al. In vivo alterations in cardiac metabolism and function in the spontaneously hypertensive rat heart. *Cardiovasc Res.* 2012; 95(1):69–76. [PubMed: 22593200]
26. Josan S, Park JM, Hurd R, Yen YF, Pfefferbaum A, Spielman D, Mayer D. In vivo investigation of cardiac metabolism in the rat using MRS of hyperpolarized $[1-^{13}\text{C}]$ and $[2-^{13}\text{C}]$ pyruvate. *NMR in biomedicine.* 2013; 26(12):1680–1687. [PubMed: 23904148]
27. Schroeder MA, Atherton HJ, Dodd MS, Lee P, Cochlin LE, Radda GK, Clarke K, Tyler DJ. The cycling of acetyl-coenzyme A through acetylcarnitine buffers cardiac substrate supply: a hyperpolarized ^{13}C magnetic resonance study. *Circulation Cardiovascular imaging.* 2012; 5(2): 201–209. [PubMed: 22238215]
28. Shulman RG, Rothman DL. The "glycogen shunt" in exercising muscle: A role for glycogen in muscle energetics and fatigue. *Proceedings of the National Academy of Sciences of the United States of America.* 2001; 98(2):457–461. [PubMed: 11209049]
29. Bardenheuer H, Schrader J. Relationship between Myocardial Oxygen-Consumption, Coronary Flow, and Adenosine Release in an Improved Isolated Working Heart Preparation of Guinea-Pigs. *Circulation Research.* 1983; 52(3):263–271. [PubMed: 6825219]
30. Headrick JP, Willis RJ. Adenosine formation and energy metabolism: a ^{31}P -NMR study in isolated rat heart. *Am J Physiol.* 1990; 258(3 Pt 2):H617–H624. [PubMed: 2316676]
31. Vandenberg JJ, Carter ND, Bethell HW, Nogradi A, Ridderstrale Y, Metcalfe JC, Grace AA. Carbonic anhydrase and cardiac pH regulation. *Am J Physiol.* 1996; 271(6 Pt 1):C1838–C1846. [PubMed: 8997183]
32. Anousis N, Carvalho RA, Zhao P, Malloy CR, Sherry AD. Compartmentation of glycolysis and glycogenolysis in the perfused rat heart. *NMR in biomedicine.* 2004; 17(2):51–59. [PubMed: 15052552]
33. Bunger R. Compartmented pyruvate in perfused working heart. *Am J Physiol.* 1985; 249(3 Pt 2):H439–H449. [PubMed: 2994495]
34. Mowbray J, Ottaway JH. The flux of pyruvate in perfused rat heart. *European journal of biochemistry / FEBS.* 1973; 36(2):362–368. [PubMed: 4354335]
35. Peuhkurinen KJ, Hiltunen JK, Hassinen IE. Metabolic compartmentation of pyruvate in the isolated perfused rat heart. *The Biochemical journal.* 1983; 210(1):193–198. [PubMed: 6405740]
36. Day SE, Kettunen MI, Gallagher FA, Hu DE, Lerche M, Wolber J, Golman K, Ardenkjaer-Larsen JH, Brindle KM. Detecting tumor response to treatment using hyperpolarized ^{13}C magnetic resonance imaging and spectroscopy. *Nature medicine.* 2007; 13(11):1382–1387.

Figure 1.

A timeline for ^{13}C NMR acquisition of hyperpolarized data, substrate concentrations and drug additions.

Figure 2.

Plots of normalized ^{13}C resonance intensities versus time after addition of HP- $[1-^{13}\text{C}]$ pyruvate. (a) the time-dependent signal of HP $[1-^{13}\text{C}]$ pyruvate; (b) time-dependent signals of HP-lactate, alanine, and bicarbonate; (c) time-dependent signals of HP-lactate, alanine, and bicarbonate with additional of isoproterenol at 30 s after addition of HP $[1-^{13}\text{C}]$ pyruvate; (d) HP-lactate curves from the isoproterenol-treated heart compared with another heart perfused under the same conditions without isoproterenol.

Figure 3.

(a) Representative ^1H NMR spectra of tissue extracts from 1) a heart perfused with 1 mM unlabeled pyruvate for ~30 min followed by a bolus of 2 mM $[3\text{-}^{13}\text{C}]$ pyruvate (bottom) and 2) a heart perfused with 1 mM unlabeled pyruvate for ~30 min followed by a bolus of 2 mM $[3\text{-}^{13}\text{C}]$ pyruvate and followed by 1 μM isoproterenol (top). Both hearts were freeze-clamped at ~66 s after bolus injection of 2 mM $[3\text{-}^{13}\text{C}]$ pyruvate, a time that corresponds to the second apex in the HP-lactate curves. A singlet peak at 1.24 ppm is tentatively assigned to the methyl protons of β -hydroxyisovalerate. (b) Glutamate C3 and C4 resonances in from ^1H -

decoupled ^{13}C NMR spectra of extracts of hearts perfused with 1) 1 mM $[3-^{13}\text{C}]$ pyruvate for ~30 min followed by a bolus of 2 mM $[3-^{13}\text{C}]$ pyruvate, then 1 μM isoproterenol, 2) 1 mM unlabeled pyruvate for ~30 min followed by a bolus of 2 mM $[3-^{13}\text{C}]$ pyruvate, then 1 μM isoproterenol, 3) 1 mM non-labeled pyruvate for ~30 min followed by a bolus of 2 mM $[3-^{13}\text{C}]$ pyruvate, and 4) 2 mM unlabeled pyruvate for ~30 min. Hearts 1) – 3) were freeze-clamped at ~66 s after the injection of 2 mM $[3-^{13}\text{C}]$ pyruvate. C3 resonances shown in 2) – 4) were magnified to shown the multiplets. Full spectra of 2) – 4) are shown in Fig. S4. Abbreviations: S, singlet; T, triplet; D, doublet.

Figure 4.

^1H NMR spectra of tissue extracts. Hearts were depleted of glycogen then perfused with $[\text{U-}^{13}\text{C}]$ glucose plus insulin to replenish the glycogen pools with ^{13}C enriched glucosyl units. These hearts were subsequently perfused with pyruvate alone without stimulation (*bottom*) or with brief exposure to $1\ \mu\text{M}$ isoproterenol (*top*).

Figure 5.

A schematic showing the proposed metabolic events occurring in hearts perfused with HP- $[1-^{13}\text{C}]$ pyruvate. Exogenous HP-pyruvate enters the myocytes, exchanges quickly into the existing small lactate pool, and enters the mitochondria for oxidation in the TCA cycle. After stimulation of cardiac function by isoproterenol and rapid breakdown of glycogen, the lactate pool very quickly doubles in size (as designated by the larger oval structure) and any remaining HP-pyruvate quickly exchanges into the larger lactate pool to produce the second apex in the dynamic HP-lactate signal (see Fig. 2). The figure is drawn to emphasize that the pyruvate derived from glycogen does not mix with the exogenous HP-pyruvate. This may not be quantitatively correct but is shown here to simplify the discussion.

Table 1

Average tissue lactate and alanine concentrations (mean $\pm$ SEM; n = 4) calculated from ^1H NMR spectroscopy of the hearts perfused with 1 mM unlabeled pyruvate for ~30 min followed by a bolus of 2 mM $[3-^{13}\text{C}]$ pyruvate, with or without isoproterenol stimulation (1 μM). The hearts were freeze-clamped at ~66 s after injection of 2 mM $[3-^{13}\text{C}]$ pyruvate. The contribution of $[3-^{13}\text{C}]$ pyruvate to acetyl-CoA was estimated from ^{13}C NMR of tissue extracts using a non-steady state isotopomer analysis.¹⁶

	No Iso	1 μM Iso
[Lactate], $\mu\text{mol/g.dw}$		
^{13}C -Lac	2.0 ± 0.6	2.2 ± 0.4
^{12}C -Lac	7.3 ± 3.2	18.2 ± 5.6
Total Lac	9.4 ± 3.8	20.9 ± 5.9
^{13}C -Lac contribution to total Lac	$25.9 \pm 3.5\%$	12.0 ± 1.6
[Alanine], $\mu\text{mol/g.dw}$		
^{13}C -Ala	3.9 ± 0.8	2.9 ± 0.4
^{12}C -Ala	8.6 ± 2.3	6.9 ± 1.0
Total Ala	10.0 ± 1.8	8.3 ± 1.2
^{13}C -Ala contribution to total Ala	$39.2 \pm 3.7\%$	$34.5 \pm 1.0\%$
^{13}C -Pyr contribution to Ac-CoA	$24.9 \pm 3.6\% ^*$	$48.7 \pm 7.6\% ^*$

*
p-value < 0.05