Metabolomics (2014) 10:986–994
DOI 10.1007/s11306-014-0630-5

ORIGINAL ARTICLE

Hyperpolarized ^{13}C lactate as a substrate for in vivo metabolic studies in skeletal muscle

Jessica A. M. Bastiaansen · Hikari A. I. Yoshihara · Yuhei Takado · Rolf Gruetter · Arnaud Comment

Received: 6 November 2013 / Accepted: 27 January 2014 / Published online: 6 February 2014

Abstract Resting skeletal muscle has a preference for the oxidation of lipids compared to carbohydrates and a shift towards carbohydrate oxidation is observed with increasing exercise. Lactate is not only an end product in skeletal muscle but also an important metabolic intermediate for mitochondrial oxidation. Recent advances in hyperpolarized MRS allow the measurement of substrate metabolism in vivo in real time. The aim of this study was to investigate the use of hyperpolarized ^{13}C lactate as a substrate for metabolic studies in skeletal muscle in vivo. Carbohydrate metabolism in healthy rat skeletal muscle at rest was studied in different nutritional states using hyperpolarized [1-^{13}C]lactate, a substrate that can be injected at physiological concentrations and leaves other oxidative processes undisturbed. ^{13}C label incorporation from lactate into bicarbonate in fed animals was observed within seconds but was absent after an overnight fast, representing inhibition of the metabolic flux through pyruvate dehydrogenase (PDH). A significant decrease in ^{13}C labeling of alanine was observed comparing the fed and fasted group, and was attributed to a change in cellular alanine concentration and not a decrease in enzymatic flux through alanine transaminase. We conclude that hyperpolarized [1-^{13}C]lactate can be used to study carbohydrate oxidation in resting skeletal muscle at physiological levels. The herein proposed method allows probing simultaneously both PDH activity and variations in alanine tissue concentration, which are associated with metabolic dysfunctions. A simple alteration of the nutritional state demonstrated that the observed pyruvate, alanine, and bicarbonate signals are indeed sensitive markers to probe metabolic changes in vivo.

Keywords Lactate · Metabolism · Skeletal muscle · Hyperpolarization · 13C MRS

Abbreviations

ALT	Alanine transaminase
LDH	Lactate dehydrogenase
PDH	Pyruvate dehydrogenase
TCA	Tricarboxylic acid cycle
MCT	Monocarboxylate transporter
CA	Carbonic anhydrase

Electronic supplementary material The online version of this article (doi:10.1007/s11306-014-0630-5) contains supplementary material, which is available to authorized users.

J. A. M. Bastiaansen (✉) · R. Gruetter
Laboratory for Functional and Metabolic Imaging, LIFMET-IPSB-SB, Ecole Polytechnique Fédérale de Lausanne (EPFL), Station 6, 1015 Lausanne, Switzerland
e-mail: jessica.bastiaansen@epfl.ch

H. A. I. Yoshihara · Y. Takado · A. Comment
Institute of Physics of Biological Systems, Ecole Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland

H. A. I. Yoshihara
Department of Cardiology, Lausanne University Hospital, 1011 Lausanne, Switzerland

Y. Takado
Laboratory of Biological Geochemistry, Ecole Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland

R. Gruetter
Department of Radiology, Geneva University Hospital and Faculty of Medicine, University of Geneva, 1211 Geneva 4, Switzerland

R. Gruetter
Department of Radiology, University of Lausanne, 1015 Lausanne, Switzerland

1 Introduction

To meet its energy demands resting skeletal muscle has a preference for the oxidation of lipids compared to that of carbohydrates but with increasing exercise intensity, a fuel shift is observed from lipid towards carbohydrate utilization and during intense exercise nearly 100 % of oxidative capacity is used for the oxidation of carbohydrates (Brooks and Mercier 1994; Brooks 1998). Lactate concentrations in skeletal muscle and plasma are known to increase during exercise, and skeletal muscle is a major producer of lactic acid in the body.

However, lactate is not only the product of anaerobic metabolism but also a source of pyruvate, and resting skeletal muscle is even viewed as the most likely primary consumer of lactic acid as a respiratory fuel (Gladden 2004; Roth 1991; Brooks 2000). Lactate enters the cell via monocarboxylate transporters and by free diffusion at higher concentrations (>10 mM) (Poole and Halestrap 1993; Juel 1991). Changes in cellular lactate concentrations are affected by the cytosolic redox potential, thus affecting production and utilization of pyruvate. In the cytosol it is transformed into pyruvate by lactate dehydrogenase (LDH) and pyruvate is converted to alanine via alanine transaminase (ALT) (Fig. 1). Pyruvate is shuttled into the mitochondria and transformed via pyruvate dehydrogenase (PDH) into acetylCoA, a key entry molecule for the tricarboxylic acid (TCA) cycle. The flux through PDH also results in the formation of CO_2 which equilibrates with bicarbonate. These aforementioned metabolic pathways can be studied using ^{13}C magnetic resonance spectroscopy (MRS), and lactate, being a simple carbohydrate, has previously been used as a substrate for carbohydrate metabolism in resting skeletal muscle and was shown to be readily oxidized (Bertocci et al. 1997; Bertocci and Lujan 1999).

Only the most abundant metabolites can be detected by conventional ^{13}C MRS in vivo. Recent developments in hyperpolarized ^{13}C MRS allow for the detection of less abundant metabolites by increasing the signal-to-noise ratio by several orders of magnitude (Ardenkjaer-Larsen et al. 2003). However, pyruvate, which has been the most widely used substrate for hyperpolarized ^{13}C studies of carbohydrate metabolism, has some disadvantages. Hyperpolarized pyruvate administration results in supraphysiological levels and pyruvate also influences the oxidation of other metabolic substrates. Excess pyruvate of ~10 mM inhibits the oxidation of palmitate as well as lactate (Yoshida et al. 2007). Conversely, ~10 mM excess lactate fails to inhibit the oxidation of palmitate and pyruvate (Yoshida et al. 2007) and does not influence the uptake of glucose, alanine output, or the glycogen store up to ~30 mM (Gladden et al. 1994).

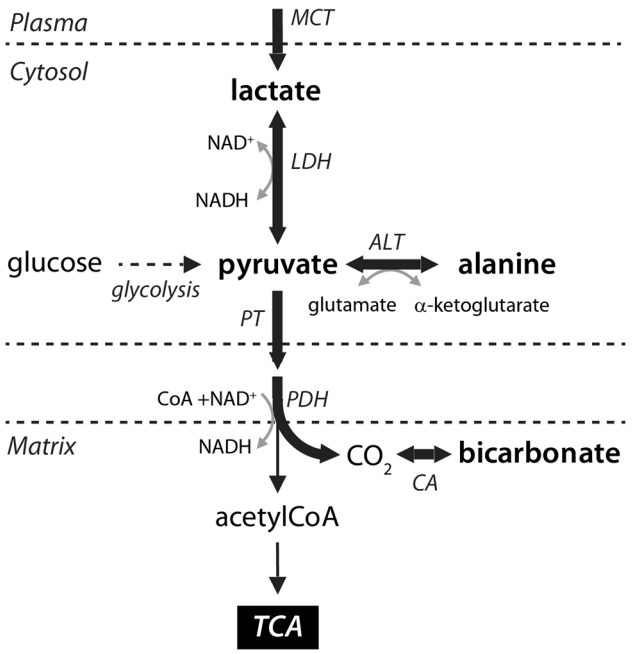

Fig. 1 Scheme of [1-^{13}C]lactate metabolism in skeletal muscle in vivo. The ^{13}C label propagation is indicated by the bold arrows and metabolites observed following [1-^{13}C]lactate oxidation are in bold typeface. Lactate enters the cell through MCT transporters and is converted to pyruvate by LDH. Pyruvate is transformed by ALT to alanine via a reversible transamination reaction. Pyruvate in turn can enter the mitochondria via PDH resulting in acetylCoA and bicarbonate. *ALT* Alanine transaminase; *LDH* lactate dehydrogenase; *PDH* pyruvate dehydrogenase; *TCA* tricarboxylic acid cycle; *MCT* monocarboxylate transporter; *CA* carbonic anhydrase; *PT* pyruvate transporter

Lactate does not influence the metabolism of other substrates, and endogenous lactate exceeds cytosolic pyruvate concentrations by an order of magnitude. Another benefit of using lactate as a hyperpolarized ^{13}C substrate is that the formation and detection of the pyruvate resonance can be used directly as a marker of the intracellular ^{13}C concentration. This can be used as a reference for scaling the signals of the downstream metabolites alanine and bicarbonate, which then become independent of the initial polarization level. The applicability of hyperpolarized [1-^{13}C]lactate as a metabolic substrate has recently been demonstrated (Chen et al. 2008) and was used to measure the activity of LDH in tumors (Kennedy et al. 2012) and changes in the flux through PDH in hearts subjected to dichloroacetate treatment (Mayer et al. 2012). The aim of this study was to explore the use of hyperpolarized [1-^{13}C]lactate as a suitable probe for investigating carbohydrate oxidation in skeletal muscle in vivo. To determine the sensitivity of hyperpolarized lactate metabolism to metabolic changes, two nutritional situations were studied: the fed and fasted states.

2 Materials and methods

2.1 Samples preparation and hyperpolarization protocol

Sodium [1-^{13}C]lactate and TEMPOL nitroxyl radicals were obtained from Sigma Aldrich (Buchs, Switzerland). Sodium [1-^{13}C]lactate was mixed with d_8-glycerol in a 2:1 weight ratio to a concentration of 4.0 M with a final radical concentration of 50 mM. The solutions were rapidly frozen in liquid nitrogen to form 2 ± 0.5 µL beads that were inserted into a sample cup which was further cooled to 1 ± 0.05 K in a 7 T custom-designed DNP polarizer (Comment et al. 2007), where the ^{13}C nuclei were dynamically polarized with microwave irradiation (197.25 MHz and 55 mW) for 90 min, with a buildup time of 60 min. Using an automated process, the sample was rapidly dissolved using 6.0 mL of pressurized, heated D_2O and transferred within 2 s following dissolution to a separator/infusion pump, containing 0.6 mL of PBS and heparin, located inside a 9.4 T horizontal bore magnet (Cheng et al. 2013; Comment et al. 2007). Subsequently, 1.5 mL of the hyperpolarized solution at physiological temperature and pH was automatically infused into the animal during 8 s. The ^{13}C polarization level at the time of the injection was 17 ± 2 %.

2.2 Animals

All animal studies conformed to institutional guidelines. Wild type male Sprague–Dawley rats (293 ± 19 g) were anesthetized with 1.5 % isoflurane in oxygen. A catheter was placed into the femoral vein for intravenous delivery of the hyperpolarized solution. A second catheter was placed in the artery to monitor the blood pressure, to measure glucose and lactate concentrations, pH, PCO_2, PO_2 and bicarbonate concentrations. Plasma samples were taken before the experiment, right after the hyperpolarized [1-^{13}C]lactate injection and at 5–10 min intervals thereafter. The respiration rate and temperature were monitored and maintained constant during the experiment. Two groups of animals were studied, fed animals (n = 5) and fasted animals (n = 5). The animals in the fasted group were fasted overnight by placing them in a clean cage without food the evening before the experiment, starting at around 5 pm. This resulted in a fasting period of about 16 h. All animal experiments were performed in the morning. Each animal was injected three times consecutively with [1-^{13}C]hyperpolarized lactate. The lactate injections did not significantly change the physiology of the animal in terms of pH, PCO_2, PO_2, bicarbonate and glucose levels (Supplemental Table 1). Lactate concentration changes are described in the results section.

2.3 In vivo magnetic resonance spectroscopy

Measurements were carried out on a 9.4 T/31 cm actively shielded animal scanner (Varian/Magnex). A custom-made radiofrequency (RF) hybrid probe consisting of a 10 mm diameter ^{13}C surface coil and two 10 mm diameter ^{1}H surface coils placed in quadrature was positioned over the hind leg of the rat for transmission and reception, localizing the signals from the skeletal muscle. Acquisition of gradient echo proton images confirmed the correct position of the coils to probe the gastrocnemius muscles. Shimming was performed to reduce the localized proton line width in a voxel of 6 × 10 × 10 mm to 20–30 Hz using FASTESTMAP (Gruetter and Tkac 2000). The NMR console was triggered to start acquisition at the beginning of the automated injection process, 6 s after dissolution of the hyperpolarized sample. Series of single pulse acquisitions were recorded using 30° adiabatic RF pulses (BIR-4) applied every 3 s with ^{1}H decoupling using WALTZ-16. Typically 40–60 FIDs were acquired with 4,129 complex data points over a 20,000 Hz bandwidth. The adiabatic pulse offset and power were calibrated to ensure a 30° excitation pulse for all observed metabolites in the entire tissue of interest. Following data acquisition, 200 µL liquid samples were extracted from the separator/infusion pump to precisely determine the infused [1-^{13}C]lactate concentration using a high-resolution 400 MHz MR spectrometer (Bruker, Fällanden, Switzerland).

2.4 Metabolite extraction and NMR spectroscopy

Wild type male Sprague–Dawley rats (n = 6) were anesthetized with 1.5 % isoflurane in oxygen. They were divided in two groups, either fed or fasted overnight. Metabolite extraction and ^{1}H spectroscopy was performed as described in (Duarte et al. 2007). In brief, skeletal muscle tissue was excised, placed immediately in liquid nitrogen and stored at −80 °C until extraction. Water-soluble metabolites were extracted with 7 % (v/v) perchloric acid (PCA) and dried with a sample concentrator (SpeedVac DNA 120, Thermo Fisher Scientific, Wohlen, Switzerland). The dried extracts were dissolved in D_2O (99.9 % D, Sigma-Aldrich) and 1.2 mmol sodium fumarate (Sigma-Aldrich) was added as internal standard for quantification by ^{1}H NMR spectroscopy. ^{1}H NMR spectra were acquired on a 14.1 T DRX-600 spectrometer equipped with a 5-mm cryoprobe (Bruker BioSpin SA, Fällanden, Switzerland) to determine alanine concentrations in the tissue samples. Peak areas were quantified by curve fitting in jMRUI.

2.5 Data analysis

The resonance peaks of the infused substrates and their corresponding metabolic products were assigned according to their chemical shifts. ^{13}C NMR time courses were summed for the time period during which the hyperpolarized [1-^{13}C]lactate resonance had a signal-to-noise ratio higher than 5 (typically 16 spectra satisfied that criterion). On average this corresponds to the time interval from 9 to 57 s after the injection, in both animal groups. The resulting summed spectra were fitted to Lorentzian line shapes using Bayesian analysis (Bretthorst 1990). These summed integrals were used to determine the ratios of one substrate relative to the other. Metabolite time courses were obtained by applying a 10 Hz line broadening on the raw data, followed by fitting in jMRUI (Naressi et al. 2001). Statistical differences between the ratios of observed metabolites were calculated using a two-tailed *t* test for unpaired data with equal variance. Error bars in figures and in the manuscript indicate ± standard error of the mean (SEM) unless otherwise indicated.

3 Results

Carbohydrate metabolism was studied in healthy resting skeletal muscle in vivo in fed and fasted animals using hyperpolarized [1-^{13}C]lactate. Overnight fasting resulted in a 30 % decrease in plasma glucose and a 41 % decrease in plasma lactate compared with animals which were fed ad libitum (Table 1). Lactate metabolism was detected in the skeletal muscle of healthy rats in vivo ($n = 10$) and the following metabolite resonances were observed: [1-^{13}C]pyruvate (171.2 ppm), [1-^{13}C]alanine (177.0 ppm) and ^{13}C bicarbonate (161.3 ppm). The spectral time courses of fed animals injected with hyperpolarized [1-^{13}C]lactate showed (Fig. 2a) that within seconds, lactate was transformed into pyruvate in the cytosol. The ^{13}C MR signal of the [1-^{13}C]lactate resonance increased due to perfusion of the substrate into the tissue, and decayed with time as a consequence of a combination of the loss of spin polarization related to the longitudinal relaxation time, the loss of longitudinal magnetization due to repeated RF excitations and the metabolic conversion of lactate to its downstream metabolites. The ^{13}C MR signal of the other metabolites increased due to metabolic conversion, and decreased due to repeated RF excitations and relaxation processes. Although in principle these parameters need to be taken into account to obtain quantitative measures of kinetic rate constants by means of mathematical modeling, it was shown that kinetic rates obtained from the mathematical modeling of metabolite time courses are directly proportional to calculated metabolite ratios (Bastiaansen et al. 2013).

Table 1 Plasma glucose and lactate concentrations in fed and overnight fasted animals with ± standard deviation

Plasma concentration	Fed	Fasted
Lactate (mM)	2.1 ± 0.2	1.3 ± 0.1
Glucose (mM)	8.2 ± 1.0	5.7 ± 0.5

The observation of bicarbonate in summed spectra (Fig. 2b) indicates that part of the labeled pyruvate was transported into the mitochondrion for subsequent oxidation in the TCA cycle. The time evolution of the signal integrals of lactate, alanine, pyruvate and bicarbonate (Fig. 3) show that alanine attained higher signal intensities compared to pyruvate but the maximum was reached at a later time point compared with pyruvate in all experiments. On average, alanine reached its maximum at 28 ± 4 s and pyruvate at 22 ± 4 s in the fed state (±standard deviation). In the fasted state, the maximum for alanine was reached at 28 ± 3 s and for pyruvate at 22 ± 2 s. There were no significant differences in the time to maximum for both metabolites in both metabolic states. The linewidths of detected ^{13}C resonances were on average 22 Hz. The injected lactate concentration was between 100 and 250 mM. After a rapid elevation to 8.2 ± 1.5 mM following the delivery of the hyperpolarized solution, the arterial lactate concentration returned to its baseline level within 20 min (Supplemental Figure 1). The resonance of ^{13}C bicarbonate was absent in animals which were fasted overnight ($n = 5$). In the fed group ($n = 5$), bicarbonate was only detected in nine out of fifteen experiments because of the low SNR in experiments with lower lactate dose. In both groups, the same relationship was observed between the SNR of the lactate signal and the injected dose.

Spectra were summed according to the criteria mentioned in the Methods section and there was no significant shift in the time interval used for summing between the fed and fasted state. The summed signals were expressed relative to [1-^{13}C]lactate, and also to the two other intracellular ^{13}C enriched metabolites, namely [1-^{13}C]pyruvate and [1-^{13}C]alanine. These ratios are independent of variations in the initial polarization level of [1-^{13}C]lactate. Comparing fed and fasted states, the bicarbonate signal was undetectable in the fasted group ($p < 0.0005$), whereas the ratio of bicarbonate to lactate in the fed group was 0.0029 ± 0.0005 (Fig. 4a). The ratio of bicarbonate relative to pyruvate was 0.10 ± 0.02 (Fig. 4c) and the ratio relative to alanine 0.04 ± 0.01. The ratio of pyruvate to lactate increased marginally from 0.030 ± 0.002 to 0.034 ± 0.002 ($p < 0.2$, Fig. 4a) and alanine decreased significantly from 0.072 ± 0.004 to 0.058 ± 0.004 when comparing fed with fasted animals ($p < 0.05$, Fig. 4a). The

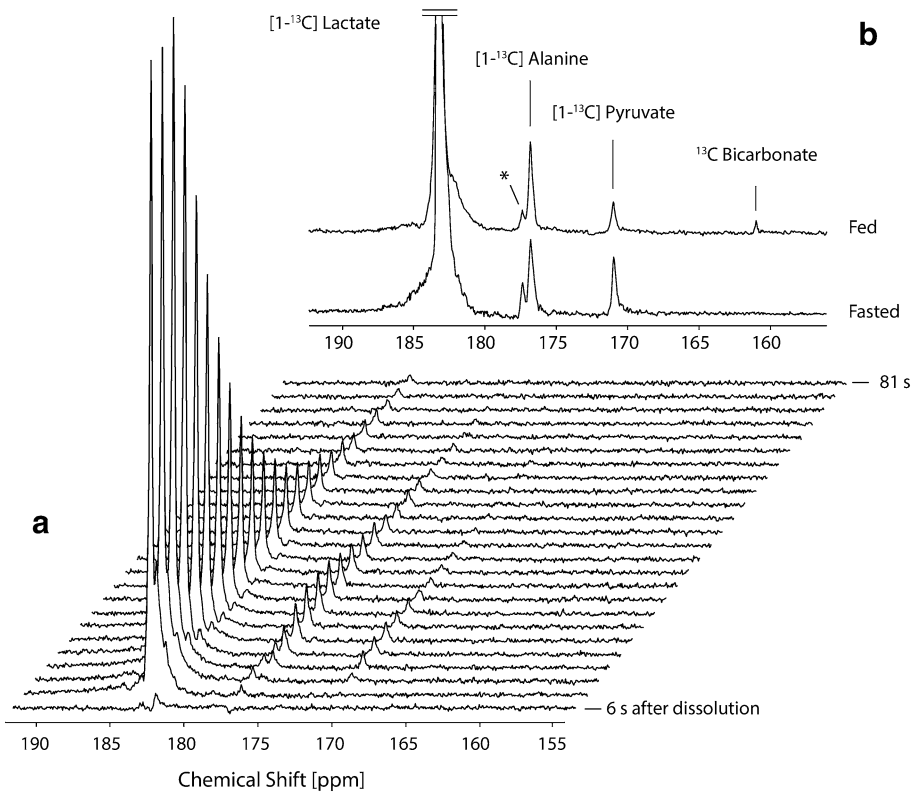

Fig. 2 Spectra acquired from the rat skeletal muscle using 30° adiabatic pulses applied every 3 s after the injection of hyperpolarized [1-^{13}C]lactate show the formation of [1-^{13}C]alanine, [1-^{13}C]pyruvate and ^{13}C bicarbonate (**a**). Spectra shown here were line broadened with 10 Hz. **b** Representative summed spectrum acquired from rat skeletal muscle in vivo after hyperpolarized [1-^{13}C]lactate injection in a fed (*top*) and fasted (*bottom*) animal. Fifteen spectra were summed and are displayed using 5 Hz line broadening. *Asterisks* The resonance on the shoulder of alanine observed at 177.5 ppm was attributed to an impurity in the [1-^{13}C]lactate solution (**b**)

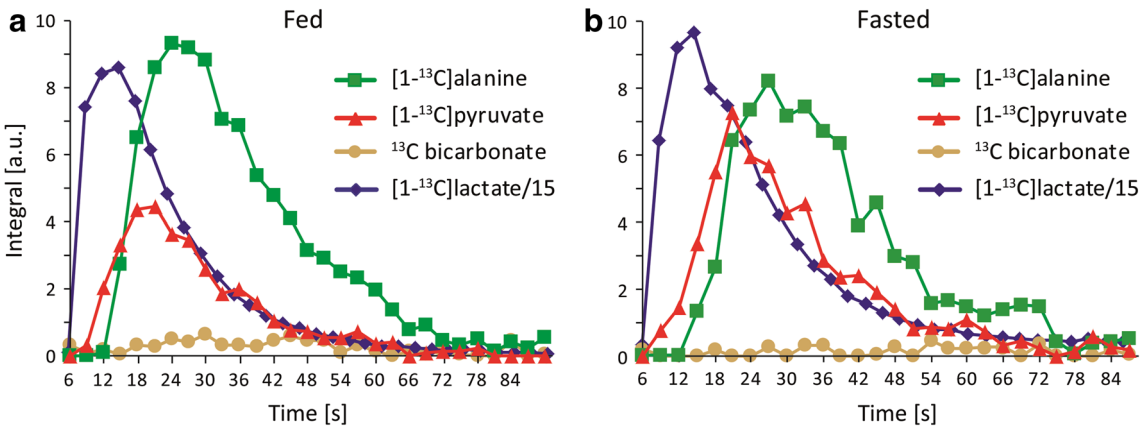

Fig. 3 Timecourses of integrated spectra showing the evolution of the injected hyperpolarized substrate [1-^{13}C]lactate and its downstream metabolites pyruvate, alanine and bicarbonate in a fed (**a**) and a fasted (**b**) animal. The hyperpolarized lactate integrals were fifteenfold downscaled. Spectral acquisition started 6 s after the dissolution process with a repetition time of 3 s and flip angle of 30°

ratio of pyruvate to alanine increased significantly ($p < 0.005$) by 42 % from 0.43 ± 0.03 to 0.61 ± 0.04 (Fig. 4b).

The influence of the injected lactate dose on the formation of bicarbonate, alanine and pyruvate was also investigated: The metabolite ratios were compared to the administered lactate dose and no relationship was observed. There was no influence of repeated lactate injections in the same animal on the ratio of alanine and bicarbonate relative to pyruvate in the fed and fasted situation (Supplemental Figure 2).

In a separate set of experiments, animals were either fed (n = 3) or fasted overnight (n = 3). ^{1}H NMR analysis of tissue extracts showed a 78 % decrease in alanine pool size after an overnight fast, from 2.32 ± 0.66 to 0.50 ± 0.27 µmol/g (Table 2).

4 Discussion

In this study, it was shown that hyperpolarized [1-^{13}C]lactate is rapidly taken up by resting skeletal muscle

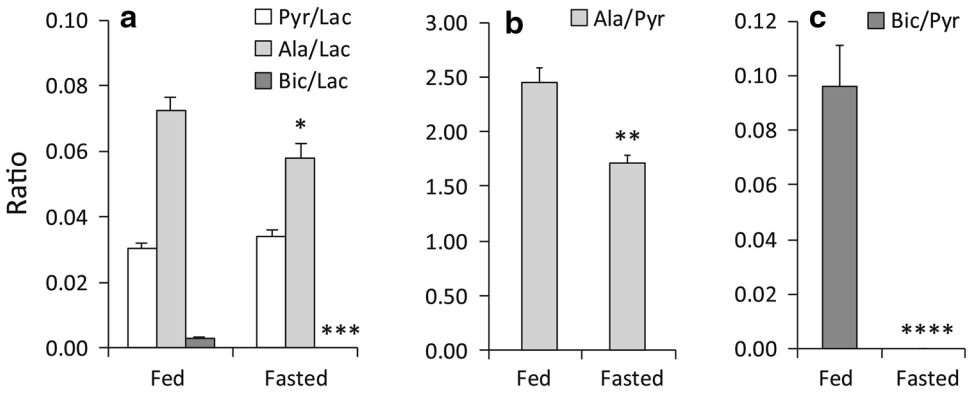

Fig. 4 Signal ratios of pyruvate (**a**) and bicarbonate (**b**, **c**) relative to lactate and alanine. Spectra were summed over a time interval between 9 and 57 s after the injection, corresponding to an SNR of [1-^{13}C]lactate >5. *Asterisks* denote statistically significant differences compared to the fed state between metabolite ratios where *$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$, ****$p < 0.00005$

Table 2 Alanine concentrations in skeletal muscle of fed and overnight fasted rats with ± standard deviation

Tissue concentration	Fed (n = 3)	Fasted (n = 3)
Alanine (μmol/g)	2.32 ± 0.66	0.50 ± 0.27

in vivo and quickly converted to pyruvate for subsequent oxidation in the mitochondrion. Lactate was chosen since it can be administered at physiological concentrations leaving other metabolic process undisturbed, unlike pyruvate (Yoshida et al. 2007; Gladden et al. 1994). In this particular study, no physiological changes were observed as a result of the hyperpolarized lactate injection in terms of heart rate, blood pressure, pH, PCO_2, PO_2, bicarbonate and glucose levels (Supplemental Table 1). The metabolic conversion rate of lactate was however sensitive to a simple alteration of nutritional state. Significant decreases in ^{13}C labeling of both bicarbonate and alanine were observed after an overnight fast. The absence of ^{13}C bicarbonate signal in the fasted group is a result of the inhibition of metabolic flux through PDH (Garland et al. 1964; Schroeder et al. 2008). Increases in the acetylCoA/CoA ratio or the NADH concentration further inhibit PDH. In general, alanine attained higher ^{13}C signal intensities compared to pyruvate, as was also observed in the abdomen and the rat heart (Mayer et al. 2012; Chen et al. 2008). This is attributed to the much larger pool size of alanine compared to pyruvate in both nutritional states (Berger et al. 1976; Jucker et al. 1997). It also suggests that the fractional enrichment of the cellular pyruvate pool was high enough to ensure efficient ^{13}C label transfer into the cellular alanine pool. Contribution to the signal from surrounding organs was expected to be negligible and has been previously discussed (Bastiaansen et al. 2013).

It has been shown that, after administration of hyperpolarized pyruvate or lactate, there is a rapid exchange of ^{13}C label between the pyruvate and lactate pools, and that observed changes in ^{13}C labeling of lactate and pyruvate is representative of the metabolite's pool size at the time of the measurements rather than originating from a net enzymatic flux through LDH (Day et al. 2007; Merritt et al. 2007; Kettunen et al. 2010). Since, like LDH, ALT is a reversible enzyme, fast isotope redistribution between the pyruvate and alanine pools implies that the variation in alanine to pyruvate ^{13}C signal ratio is essentially a consequence of changes in the endogenous relative concentrations of both metabolites. The significant reduction of this ratio observed in fasted fed animals could result from an increase in pyruvate signal, a decrease in alanine signal or a combination of both.

An increase in endogenous pyruvate concentration in the fasted state could be due to inhibition of pyruvate oxidation (Garland et al. 1964), or due to increased NAD^+ levels which shift the LDH-catalyzed reaction towards the formation of pyruvate (Houtkooper et al. 2010). However, as mentioned above, the slight increase in pyruvate to lactate ^{13}C signal ratio observed in our experiments was not statistically significant ($p < 0.2$). The largely significant decrease in alanine to pyruvate ^{13}C signal ratio observed in the fasted state is thus most likely mainly a consequence of a decrease of the alanine pool size.

In previous studies where hyperpolarized [1-^{13}C]pyruvate was used as a substrate, a decrease in the ^{13}C alanine signal was observed in a perfused liver model and in the liver in vivo after a fasting period and this reduction was ascribed to a cellular redox state change and a reduction in ALT activity respectively (Merritt et al. 2011; Hu et al. 2009). However, a direct comparison with results obtained using pyruvate as a hyperpolarized substrate is challenging since different metabolite pools are enriched and the reaction pathways and directions probed are different.

Moreover, the liver takes up alanine for gluconeogenic purposes while the muscle releases it.

There have been reports of decreased skeletal muscle tissue concentrations of alanine after a 24 h fast (Christop et al. 1971) and alanine concentrations were also diminished in overnight fasted animals based on ^{1}H NMR measurements of tissue extracts in a separate set of experiments (Table 2). Therefore, a decrease in ^{13}C alanine labeling can be most likely ascribed to a change in cellular alanine concentration. The endogenous alanine concentration is reduced in fasted muscles because of an enhanced alanine efflux (Macdonald et al. 1976; Felig 1973).

It is known that during fasting conditions and other situations of negative nitrogen balance, muscle is the major source of carbon and nitrogen for the synthesis of glucose and urea in the liver (Ruderman and Berger 1974; Mallette et al. 1969). In such conditions, amino acid catabolism increases (Odessey et al. 1974) and alanine is the principal amino acid released by skeletal muscle. Moreover, it was shown that the alanine release reflects the rate of de novo synthesis (Karl et al. 1976), and changes in alanine synthesis and release are related to pyruvate formation from amino acids (Garber et al. 1976), which increases during fasting. Although the formation and efflux of alanine is stimulated during fasting, the decreased hyperpolarized ^{13}C label transfer to alanine observed in this study is clearly not only dependent on the enzymatic flux through ALT but coupled with changes in fractional enrichments, pool sizes and formation of intermediate substrates. Since these factors are affected in several diseases, such as in diabetes where the release of lactate and alanine is increased (Karl et al. 1976; Berger et al. 1976), ketotic hypoglycemia where gluconeogenesis is impaired due to insufficient release of alanine (Pagliara et al. 1972), or maple syrup urine disease where decarboxylation of amino acids is impaired and alanine levels are three- to tenfold lower (Dancis et al. 1960), the alanine-to-pyruvate ratio could be a sensitive biological marker for the detection of skeletal muscle metabolic disorders.

The time evolution of the lactate resonance shows a buildup followed by decay and this is different from hyperpolarized acetate injections in skeletal muscle using an identical injection protocol (Bastiaansen et al. 2013). This difference could be attributed to a different uptake of both substrates. Acetate enters the cell via concentration gradient facilitated diffusion while lactate mainly enters via monocarboxylate transporters and also by free diffusion at concentrations >10 mM (Poole and Halestrap 1993; Juel 1991). Since the concentrations used in this study were ~8 mM, lactate was assumed to enter the cell largely via MCT transport.

The hyperpolarized lactate injections resulted in a fivefold elevation of endogenous lactate (~8 mM) compared to the resting state. Tissue concentrations of lactate can increase up to 10–50 mM in a state of exercise and our injected values are thus within physiological range (Gobatto et al. 2001; Watt et al. 1988). Lactate was cleared from the blood within minutes (Supplemental Figure 1). The ability to uptake and process lactate at these concentrations is also reflected by the high K_M values for MCT across the cellular membrane which were found to be between 13 and 40 mM (Juel and Halestrap 1999). MCT1 and MCT4 are the predominant isoforms expressed in skeletal muscle. No dose dependencies were observed in the metabolite-to-lactate ratios, which is indicative of lactate transport being proportional to blood lactate concentration and in agreement with the higher K_M value compared to the lactate concentrations.

Previous studies in the literature show that after a 24 and a 48 h fast, plasma lactate concentrations decrease but the concentration of lactate in rat skeletal muscle tissue remains unchanged (Goodman et al. 1974, 1990). In a study using overnight fasted rats, a 30 min infusion of lactate increased plasma lactate levels fourfold but did not increase the plasma concentrations of free fatty acids and alanine (Vettor et al. 1997). Infusing lactate up to 30 mM did not affect glucose uptake, pyruvate uptake/output, and alanine output in canine skeletal muscle (Gladden et al. 1994). Therefore, we conclude that the observed changes in ^{13}C label propagation are a likely consequence of an actual shift in lactate metabolism in skeletal muscle upon fasting.

5 Conclusions

The significant changes observed in ^{13}C labeling of bicarbonate and alanine after an overnight fast were attributed to decreased PDH flux and a decrease in cellular alanine concentration. We conclude that lactate can be administered at physiological concentrations without disturbing other oxidative processes and is readily oxidized in resting skeletal muscle. Therefore hyperpolarized [1-^{13}C]lactate is well suited for studying carbohydrate metabolism in skeletal muscle in vivo. The ratio of alanine to pyruvate is not only dependent on the enzymatic flux through ALT, but also on changes in fractional enrichments and pool size of intermediates. The fact that alanine concentration changes can be monitored in vivo using an endogenous substrate at nearly physiological concentrations will offer the opportunity to probe skeletal muscle metabolic disorders and to test the efficiency of drugs targeting specific muscular diseases.

Acknowledgments We would like to thank Tian Cheng and Andrea Capozzi for their suggestions and improvements on the lactate recipe

and Jacquelina Romero, Laure Bardouillet and Mario Lepore for veterinary support.

Conflict of interest The authors declare no conflicts of interest.

Animal studies All institutional and national guidelines for the care and use of laboratory animals were followed.

Funding This work was supported by the Swiss National Science Foundation (Grant PP00P2_133562 and 31003AB_131087), the Centre d'Imagerie BioMédicale (CIBM) of the UNIL, UNIGE, HUG, CHUV, EPFL, and the Leenaards and Jeantet Foundations.

References

Ardenkjaer-Larsen, J. H., Fridlund, B., Gram, A., Hansson, G., Hansson, L., Lerche, M. H., et al. (2003). 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, 100*(18), 10158–10163.

Bastiaansen, J. A. M., Cheng, T., Mishkovsky, M., Duarte, J. M. N., Comment, A., & Gruetter, R. (2013). In vivo enzymatic activity of acetylCoA synthetase in skeletal muscle revealed by 13C turnover from hyperpolarized [1-13C]acetate to [1-13C]acetylcarnitine. *Biochimica et Biophysica Acta, 1830*(8), 4171–4178. doi:10.1016/j.bbagen.2013.03.023.

Berger, M., Hagg, S. A., Goodman, M. N., & Ruderman, N. B. (1976). Glucose-metabolism in perfused skeletal-muscle—Effects of starvation, diabetes, fatty-acids, acetoacetate, insulin and exercise on glucose-uptake and disposition. *Biochemical Journal, 158*(2), 191–202.

Bertocci, L. A., Jones, J. G., Malloy, C. R., Victor, R. G., & Thomas, G. D. (1997). Oxidation of lactate and acetate in rat skeletal muscle: Analysis by C-13-nuclear magnetic resonance spectroscopy. *Journal of Applied Physiology, 83*(1), 32–39.

Bertocci, L. A., & Lujan, B. F. (1999). Incorporation and utilization of [3-C-13]lactate and [1,2-C-13]acetate by rat skeletal muscle. *Journal of Applied Physiology, 86*(6), 2077–2089.

Bretthorst, G. L. (1990). Bayesian analysis. I: Parameter estimation using quadrature NMR models. *Journal of Magnetic Resonance, 88*, 533–551.

Brooks, G. A. (1998). Mammalian fuel utilization during sustained exercise. *Comparative Biochemistry and Physiology B-Biochemistry and Molecular Biology, 120*(1), 89–107. doi:10.1016/S0305-0491(98)00025-X.

Brooks, G. A. (2000). Intra- and extra-cellular lactate shuttles. *Medicine and Science in Sports and Exercise, 32*(4), 790–799. doi:10.1097/00005768-200004000-00011.

Brooks, G. A., & Mercier, J. (1994). Balance of carbohydrate and lipid utilization during exercise: The crossover concept. *Journal of Applied Physiology, 76*(6), 2253–2261.

Chen, A. P., Kurhanewicz, J., Bok, R., Xua, D., Joun, D., Zhang, V., et al. (2008). Feasibility of using hyperpolarized [1-C-13]lactate as a substrate for in vivo metabolic C-13 MRSI studies. *Magnetic Resonance Imaging, 26*(6), 721–726. doi:10.1016/j.mri.2008.01.002.

Cheng, T., Mishkovsky, M., Bastiaansen, J. A. M., Ouari, O., Hautle, P., Tordo, P., et al. (2013). Method to minimize and monitor in situ the polarization losses in hyperpolarized biomolecules prior to in vivo MR experiments. *NMR in Biomedicine,*. doi:10.1002/nbm.2993.

Christop, J., Winand, J., & Hebbelin, M. (1971). Amino acid levels in plasma, liver, muscle, and kidney during and after exercise in fasted and fed rats. *American Journal of Physiology, 221*(2), 453–457.

Comment, A., van den Brandt, B., Uffmann, K., Kurdzesau, F., Jannin, S., Konter, J. A., et al. (2007). Design and performance of a DNP prepolarizer coupled to a rodent MRI scanner. *Concepts in Magnetic Resonance Part B: Magnetic Resonance Engineering, 31B*(4), 255–269.

Dancis, J., Hutzler, J., & Levitz, M. (1960). Metabolism of the white blood cells in maple-syrup-urine disease. *Biochimica et Biophysica Acta, 43*(2), 342–343. doi:10.1016/0006-3002(60)90448-0.

Day, S. E., Kettunen, M. I., Gallagher, F. A., Hu, D. E., Lerche, M., Wolber, J., et al. (2007). Detecting tumor response to treatment using hyperpolarized C-13 magnetic resonance imaging and spectroscopy. *Nature Medicine, 13*(11), 1382–1387. doi:10.1038/Nm1650.

Duarte, J. M., Cunha, R. A., & Carvalho, R. A. (2007). Different metabolism of glutamatergic and GABAergic compartments in superfused hippocampal slices characterized by nuclear magnetic resonance spectroscopy. *Neuroscience, 144*(4), 1305–1313. doi:10.1016/j.neuroscience.2006.11.027.

Felig, P. (1973). The glucose–alanine cycle. *Metabolism-Clinical and Experimental, 22*(2), 179–207. doi:10.1016/0026-0495(73)90269-2.

Garber, A. J., Karl, I. E., & Kipnis, D. M. (1976). Alanine and glutamine synthesis and release from skeletal-muscle. 2. precursor role of amino-acids in alanine and glutamine synthesis. *Journal of Biological Chemistry, 251*(3), 836–843.

Garland, P. B., Randle, P. J., & Newsholme, E. A. (1964). Regulation of glucose uptake by muscle. 9. Effects of fatty acids + ketone bodies + of alloxan-diabetes + starvation on pyruvate metabolism + on lactate/pyruvate + l-glycerol 3-phosphate/dihydroxyacetone phosphate concentration ratios in rat heart + rat diaphragm muscles. *Biochemical Journal, 93*(3), 665–678.

Gladden, L. B. (2004). Lactate metabolism: A new paradigm for the third millennium. *The Journal of Physiology, 558*(1), 5–30. doi:10.1113/jphysiol.2003.058701.

Gladden, L. B., Crawford, R. E., & Webster, M. J. (1994). Effect of lactate concentration and metabolic-rate on net lactate uptake by canine skeletal-muscle. *American Journal of Physiology, 266*(4), R1095–R1101.

Gobatto, C. A., de Mello, M. A. R., Sibuya, C. Y., de Azevedo, J. R. M., dos Santos, L. A., & Kokubun, E. (2001). Maximal lactate steady state in rats submitted to swimming exercise. *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology, 130*(1), 21–27. doi:10.1016/S1095-6433(01)00362-2.

Goodman, M. N., Berger, M., & Ruderman, N. B. (1974). Glucose-metabolism in rat skeletal-muscle at rest effect of starvation, diabetes, ketone-bodies and free fatty-acids. *Diabetes, 23*(11), 881–888.

Goodman, M. N., Dietrich, R., & Luu, P. (1990). Formation of gluconeogenic precursors in rat skeletal muscle during fasted-refed transition. *American Journal of Physiology, 259*(4 Pt 1), E513–E516.

Gruetter, R., & Tkac, I. (2000). Field mapping without reference scan using asymmetric echo-planar techniques. *Magnetic Resonance in Medicine, 43*(2), 319–323.

Houtkooper, R. H., Canto, C., Wanders, R. J., & Auwerx, J. (2010). The secret life of NAD(+): An old metabolite controlling new metabolic signaling pathways. *Endocrine Reviews, 31*(2), 194–223. doi:10.1210/Er.2009-0026.

Hu, S., Chen, A. P., Zierhut, M. L., Bok, R., Yen, Y. F., Schroeder, M. A., et al. (2009). In vivo carbon-13 dynamic MRS and MRSI of normal and fasted rat liver with hyperpolarized C-13-pyruvate. *Molecular Imaging and Biology, 11*(6), 399–407. doi:10.1007/s11307-009-0218-z.

Jucker, B. M., Rennings, A. J. M., Cline, G. W., & Shulman, G. I. (1997). C-13 and P-31 NMR studies on the effects of increased plasma free fatty acids on intramuscular glucose metabolism in the awake rat. *Journal of Biological Chemistry, 272*(16), 10464–10473.

Juel, C. (1991). Muscle lactate transport studied in sarcolemmal giant vesicles. *Biochimica et Biophysica Acta, 1065*(1), 15–20. doi:10.1016/0005-2736(91)90004-R.

Juel, C., & Halestrap, A. P. (1999). Lactate transport in skeletal muscle—Role and regulation of the monocarboxylate transporter. *The Journal of Physiology, 517*(3), 633–642. doi:10.1111/j.1469-7793.1999.0633s.x.

Karl, I. E., Garber, A. J., & Kipnis, D. M. (1976). Alanine and glutamine synthesis and release from skeletal-muscle. 3. Dietary and hormonal-regulation. *Journal of Biological Chemistry, 251*(3), 844–850.

Kennedy, B. W. C., Kettunen, M. I., Hu, D. E., & Brindle, K. M. (2012). Probing lactate dehydrogenase activity in tumors by measuring hydrogen/deuterium exchange in hyperpolarized L-[1-C-13, U-H-2]lactate. *Journal of the American Chemical Society, 134*(10), 4969–4977. doi:10.1021/Ja300222e.

Kettunen, M. I., Hu, D. E., Witney, T. H., McLaughlin, R., Gallagher, F. A., Bohndiek, S. E., et al. (2010). Magnetization transfer measurements of exchange between hyperpolarized [1-C-13]pyruvate and [1-C-13]lactate in a murine lymphoma. *Magnetic Resonance in Medicine, 63*(4), 872–880. doi:10.1002/Mrm.22276.

Macdonald, M., Neufeldt, N., Park, B. N., Berger, M., & Ruderman, N. (1976). Alanine metabolism and gluconeogenesis in rat. *American Journal of Physiology, 231*(2), 619–626.

Mallette, L. E., Exton, J. H., & Park, C. R. (1969). Control of gluconeogenesis from amino acids in perfused rat liver. *Journal of Biological Chemistry, 244*(20), 5713–5723.

Mayer, D., Yen, Y. F., Josan, S., Park, J. M., Pfefferbaum, A., Hurd, R. E., et al. (2012). Application of hyperpolarized [1-13C]lactate for the in vivo investigation of cardiac metabolism. *NMR in Biomedicine, 25*(10), 1119–1124. doi:10.1002/Nbm.2778.

Merritt, M. E., Harrison, C., Sherry, A. D., Malloy, C. R., & Burgess, S. C. (2011). Flux through hepatic pyruvate carboxylase and phosphoenolpyruvate carboxykinase detected by hyperpolarized C-13 magnetic resonance. *Proceedings of the National Academy of Sciences of the United States of America, 108*(47), 19084–19089. doi:10.1073/pnas.1111247108.

Merritt, M. E., Harrison, C., Storey, C., Jeffrey, F. M., Sherry, A. D., & Malloy, C. R. (2007). Hyperpolarized 13C 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, 104*(50), 19773–19777. doi:10.1073/pnas.0706235104.

Naressi, A., Couturier, C., Devos, J. M., Janssen, M., Mangeat, C., de Beer, R., et al. (2001). Java-based graphical user interface for the MRUI quantitation package. *Magnetic Resonance Materials in Physics, Biology and Medicine, 12*(2–3), 141–152.

Odessey, R., Khairall, E. A., & Goldberg, A. L. (1974). Origin and possible significance of alanine production by skeletal-muscle. *Journal of Biological Chemistry, 249*(23), 7623–7629.

Pagliara, A. S., Kipnis, D. M., Karl, I. E., Devivo, D. C., & Feigin, R. D. (1972). Hypoalaninemia—A concomitant of ketotic hypoglycemia. *Journal of Clinical Investigation, 51*(6), 1440–1449. doi:10.1172/Jci106940.

Poole, R. C., & Halestrap, A. P. (1993). Transport of lactate and other monocarboxylates across mammalian plasma-membranes. *American Journal of Physiology, 264*(4), C761–C782.

Roth, D. A. (1991). The sarcolemmal lactate transporter: Transmembrane determinants of lactate flux. *Medicine and Science in Sports and Exercise, 23*(8), 925–934.

Ruderman, N. B., & Berger, M. (1974). Formation of glutamine and alanine in skeletal-muscle. *Journal of Biological Chemistry, 249*(17), 5500–5506.

Schroeder, M. A., Cochlin, L. E., Heather, L. C., Clarke, K., Radda, G. K., Tyler, D. J., et al. (2008). In vivo assessment of pyruvate dehydrogenase flux in the heart using hyperpolarized carbon-13 magnetic resonance. *Proceedings of the National Academy of Sciences of the United States of America, 105*(33), 12051–12056. doi:10.1073/pnas.0805953105.

Vettor, R., Lombardi, A. M., Fabris, R., Pagano, C., Cusin, I., RohnerJeanrenaud, F., et al. (1997). Lactate infusion in anesthetized rats produces insulin resistance in heart and skeletal muscles. *Metabolism-Clinical and Experimental, 46*(6), 684–690. doi:10.1016/S0026-0495(97)90014-7.

Watt, P. W., Maclennan, P. A., Hundal, H. S., Kuret, C. M., & Rennie, M. J. (1988). L(+)-lactate transport in perfused rat skeletal-muscle: Kinetic characteristics and sensitivity to ph and transport inhibitors. *Biochimica et Biophysica Acta, 944*(2), 213–222. doi:10.1016/0005-2736(88)90434-8.

Yoshida, Y., Holloway, G. P., Ljubicic, V., Hatta, H., Spriet, L. L., Hood, D. A., et al. (2007). Negligible direct lactate oxidation in subsarcolemmal and intermyofibrillar mitochondria obtained from red and white rat skeletal muscle. *The Journal of Physiology, 582*(3), 1317–1335. doi:10.1113/jphysiol.2007.135095.