Evidence for reverse flux through pyruvate kinase in skeletal muscle

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1Advanced Imaging Research Center and Departments of 2Internal Medicine and 3Radiology, University of Texas Southwestern Medical Center, Dallas; 4Department of Chemistry, University of Texas at Dallas, Richardson; and 5Veterans Affairs North Texas Health Care System, Dallas, Texas

Submitted 20 November 2008; accepted in final form 24 January 2009

Jin ES, Sherry AD, Malloy CR. Evidence for reverse flux through pyruvate kinase in skeletal muscle. Am J Physiol Endocrinol Metab 296: E748–E757, 2009. First published February 3, 2009; doi:10.1152/ajpendo.90935.2008.—Conversion of lactate to glucose was examined in myotubes, minced muscle tissue, and rats exposed to 2H2O or 13C-enriched substrates. Myotubes or minced skeletal muscle incubated with [U-13C3]lactate released small amounts of [1,2,3-13C3]- or [4,5,6-13C3]glucose. This labeling pattern is consistent with direct transfer from lactate to glucose without randomization in the tricarboxylic acid (TCA) cycle. After exposure of incubated muscle to 2H2O, [U-13C3]lactate, glucose, and glutamine, there was minimal release of synthesized glucose to the medium based on a low level of 2H enrichment in medium glucose but 50- to 100-fold greater 13H enrichment in glucosyl units from glycogen. The 13C enrichment pattern in glycogen from incubated skeletal muscle was consistent only with direct transfer of lactate to glucose without exchange in TCA cycle intermediates. 13C nuclear magnetic resonance (NMR) spectra of glutamate from the same tissue showed flux from lactate through pyruvate dehydrogenase but not flux through pyruvate carboxylase into the TCA cycle. Carbon from an alternative substrate for glucose production that requires metabolism through the TCA cycle, propionate, did not enter glycogen, suggesting that TCA cycle intermediates do not exchange with phosphoenolpyruvate. In vivo, the 13C labeling patterns in hepatic glycogen and plasma glucose after administration of [U-13C3]lactate did not differ significantly. However, skeletal muscle glycogen was substantially enriched in [1,2,3-13C3]- and [4,5,6-13C3]glucose units that could only occur through skeletal muscle glycogen synthesis rather than glyconeogenesis. Lactate serves as a substrate for glycogen synthesis in vivo without exchange into symmetric intermediates of the TCA cycle.

pyruvate kinase; phosphoenolpyruvate carboxykinase; glycogen synthesis; nuclear magnetic resonance; skeletal muscle

GLYCOGEN IN SKELETAL MUSCLE serves multiple well-accepted roles in systemic glucose homeostasis. Postprandial hyperglycemia normally results in insulin-stimulated transport of glucose into skeletal muscle followed by direct glucose phosphorylation and incorporation into muscle glycogen. This process, termed glycogenesis, clears glucose from the systemic circulation and is impaired among patients with type 2 diabetes (3). Skeletal muscle glycogen also serves as an energy source for muscle during anaerobic glycolysis, and lactate produced from glycogen during a fast or prolonged exercise is a major substrate for hepatic glycogen synthesis. Less clear is the capacity of skeletal muscle for de novo synthesis of glycogen from 3-carbon precursors, or glyconeogenesis. Only one pathway is generally accepted for conversion of pyruvate to phosphoenolpyruvate (PEP) in mammalian tissues: carboxylation of pyruvate to oxaloacetate followed by decarboxylation to PEP via phosphoenolpyruvate carboxykinase (PEPCK). The alternative pathway, direct conversion of pyruvate to PEP via pyruvate kinase (ATP + pyruvate → ADP + PEP), is usually held to be insignificant (4, 16, 17).

Although this conclusion is standard teaching in textbooks (21), early studies (14) reported that the reaction is reversible based on the transfer of 32P from PEP to ATP in rat muscle extracts. Later, Hiatt et al. (9) found essentially equal 14C labeling in positions 1, 2, 5, and 6 in glucosyl units from glycogen synthesized by the liver exposed to [2-14C]pyruvate. This glucose-labeling pattern would be expected if pyruvate undergoes carboxylation to form a dicarboxylic acid in exchange with the symmetric intermediate, fumarate, before conversion to PEP. Diaphragm muscle, however, generated glycosyl units without any enrichment in glucose positions 1 or 6. These data demonstrated unequivocally that the [2-14C]pyruvate was not converted to the symmetrical tricarboxylic acid (TCA) cycle intermediate, fumarate, in the pathway to glucose production (9). Donovan and Pagliassotti (6), studying rabbit skeletal muscle, found that glycogen synthesis from lactate was identical when measured with [1,14C2]-, [2,14C2]-, or [U-14C]lactate. If intermediates of the TCA cycle were involved in glyconeogenesis, site-specific loss of tracer and therefore different rates of glycogen synthesis would be expected. Krimsky (13) and Dyson et al. (7) found that reverse flux through skeletal muscle pyruvate kinase is sufficient to support significant rates of glyconeogenesis. More recently, Dobson et al. (5) argued that technical limitations in early studies of pyruvate kinase coupled with the high sensitivity of the reaction to free [Mg2+] and pH interfered with initial studies of the thermodynamics of the reaction. They found that, under in vivo conditions, the mass action ratio was within a factor of three to six of the equilibrium value, and, based on this analysis, proposed that the reaction may be reversed under some conditions (5). In summary, thermodynamic studies suggest that reverse flux through pyruvate kinase may occur in vivo. Experiments examining the conversion of 14C-enriched lactate to glucose by striated muscle are consistent with two possible reaction pathways, reverse flux through the pyruvate kinase reaction or flux in the oxaloacetate/malate pool without exchange with fumarate. In this report, both pathways will be termed “direct transfer” because both pathways preserve the 14C labeling pattern of pyruvate in glucose.

The carbon tracer labeling patterns in glucose cannot distinguish the two possible direct transfer pathways, but these alternatives could be resolved by examining the 13C labeling...
pattern of oxaloacetate or malate. Reverse flux through the pyruvate kinase reaction bypasses the oxaloacetate/malate pool, whereas carboxylation of pyruvate to oxaloacetate/malate followed by decarboxylation to PEP without rearrangement due to exchange with fumarate will produce a unique labeling pattern in oxaloacetate. Because the concentration of oxaloacetate is low, the $^{13}$C labeling pattern in glutamate can be used to measure oxaloacetate labeling in positions 1, 2, and 3. In this study, $^2$H and $^{13}$C nuclear magnetic resonance (NMR) analysis of glucose, glucosyl units from glycogen, or glutamate was performed on cultured myoblast cells, isolated muscle tissue, and whole animals treated with $^2$H$_2$O and/or $^{13}$C-labeled tracers. The $^{13}$C labeling pattern in glucose released in the medium from myotubes or minced, incubated muscle demonstrated small excess labeling due to direct transfer. However, the $^{13}$C labeling pattern in glycogen from minced muscle was dramatically different from that seen in glucose in the medium and demonstrated glucose production exclusively by direct trans-

METHODS

Materials. [U-$^{13}$C$_3$]lactate (98%), [U-$^{13}$C$_2$]propionate (99%), $^3$H$_2$O (99.9%), and deuterated acetonitrile (99.8%) were obtained from Cambridge Isotopes (Andover, MA). Dulbecco’s modified Eagle’s medium (DMEM), FBS, Dulbecco’s PBS (D-PBS), Dowex 50WX8–200 (a cation-exchange column), Amberlite IRA-67 (an anion-exchange column), and other common chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Muscle cell culture. C$_2$C$_12$ (a mouse myogenic cell line) myoblasts were proliferated in growth media consisting of DMEM and 20% FBS in 5% CO$_2$ at 37°C. When myoblasts were grown to confluence, the media was changed to differentiation media consisting of DMEM and 2% horse serum. The differentiation media was changed every 2 days. After 5–7 days in differentiation media, myotubes with multiple nuclei were formed. These myotubes were washed with D-PBS and then further incubated for 5 h with either D-PBS containing 20 mM glucose, 5% $^3$H$_2$O, and 2% FBS (protocol 1); D-PBS containing 20 mM [U-$^{13}$C$_3$]lactate, 5% $^3$H$_2$O, 2% FBS, 5 mM glucose, and 2 mM glutamine (protocol 2); and D-PBS containing 20 mM [U-$^{13}$C$_2$]lactate, 5% $^3$H$_2$O, 2% FBS, 5 mM glucose, and 10 mM glutamine (protocol 3). After 5 h incubation, media from three petri dishes ($150 \times 25$ mm) (protocol 1) or six (protocols 2 and 3) were pooled and extracted with 7% perchloric acid by volume.

Skeletal muscle tissue incubation. The study was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. After a fasting period of 24 h, male Sprague-Dawley rats (350–450 g) were anesthetized with isoflurane. Muscle from hindlimbs was dissected out and chopped into thin (less than ~0.5-mm) pieces in saline. Muscle tissue (~10 g, wet) was placed in a petri dish ($150 \times 25$ mm, modified for gas tubing) with 60 ml of oxygenated Krebs-Henseleit bicarbonate buffer containing the following: 0.1% BSA, 10 mM glucose, 10 mM glutamine, 20 mM lactate (20% [U-$^{13}$C$_2$]lactate), and 5% $^3$H$_2$O (protocol 4) and 0.1% BSA, 10 mM glucose, 10 mM glutamine, 20 mM lactate, or 6 mM [U-$^{13}$C$_3$]propionate, and 5% $^3$H$_2$O (protocol 5). The mixture was incubated by shaking at 37°C, and the mixture was gassed continuously with 95% O$_2$–5% CO$_2$ for 5 h. After incubation, muscle tissues were treated with 30% KOH for glycogen isolation or treated with 8% perchloric acid for glutamate isolation. The medium was treated with perchloric acid for glucose isolation.

Whole animal studies. The study was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. Twenty-four-hour-fast rats (~430–500 g) received an intraperitoneal injection of sodium lactate (2 g/kg body wt) enriched with 10% [U-$^{13}$C$_3$]lactate under isoflurane anesthesia. The rats were placed back into their cage where they quickly awakened and were allowed free access to water. After 3 h of injection, blood was drawn from the inferior vena cava under pentobarbital sodium anesthesia, and the liver and muscle tissue from the hindlimbs were freeze-clamped and kept under −80°C for subsequent processing.

Sample processing for NMR analysis. A 5– to 10-g portion of skeletal muscle tissue and liver tissue was used for glycogen extraction and purification (18). Isolated glycogen was dissolved in ~5 ml of sodium acetate solution (10 mM, pH 4.8) and incubated with amyloglucosidase (50 mg glycogen/20 units) for 4 h at 50°C for hydrolysis into glucose. Blood from animals and media from myotube or tissue incubation were deproteinized by adding cold perchloric acid to a final concentration of 7% by volume. After neutralization with KOH and centrifugation, the supernatant was lyophilized. Glucose in the dried residue was purified by passing through two columns, a cation-exchange column and an anion-exchange column. Glucose was eluted from the columns with deionized water, lyophilized, and subsequently converted to monoaacetone glucose (MAG, Fig. 1). This was accomplished by suspending the dried glucose in 3.0 ml of acetone containing 120 μl of concentrated sulfuric acid. The mixture was stirred for 4 h at room temperature to yield diacetone glucose. After addition of 3 ml of water, the pH was increased to 2.0 by addition of Na$_2$CO$_3$ (1.5 M). The mixture was stirred for 24 h at room temperature to convert diacetone glucose into MAG. The pH was then further increased to ~8.0 by adding Na$_2$CO$_3$. Acetone was evaporated under a vacuum, and the sample was freeze-dried. MAG was extracted into 3 ml of hot ethyl acetate (5×) that was subsequently removed by vacuum evaporation. The resulting MAG was further purified by passage through a 3-ml DSC-18 cartridge using 5% acetonitrile as eluent. The efficient was freeze-dried and stored dry before NMR analysis. For isolation of glutamate, muscle tissue (~5–10 g) was treated with cold perchloric acid (8%), neutralized

![Fig. 1. Structure of monoaacetone glucose (MAG) derived from glucose. The six carbons labeled by 1–6 and seven aliphatic hydrogens attached to those carbons originated from glucose. Two methyl groups labeled by M1 and M2 are added during the deprotonization of glucose, and they are independent from metabolism. The resonances of methyl groups of $^2$H or $^{13}$C nuclear magnetic resonance (NMR) spectra are used as references for the estimation of isotope enrichment of glucose.](http://aipendao.physiology.org/)
with KOH, and freeze-dried. Glutamate was isolated by ion-exchange chromatography as described previously (11).

For ²H NMR measurements, MAG was dissolved in a mixture of 90% acetonitrile-10% water (160 µl). After each ²H NMR acquisition, the MAG samples were lyophilized and resuspended in 90% deuterated acetonitrile-10% water for ¹³C NMR measurements. Glutamate was dissolved in ²H₂O (160 µl) for ¹³C NMR measurement.

NMR spectroscopy. All NMR spectra were collected using a Varian ANOVA 14.1 T spectrometer (Varian Instruments, Palo Alto, CA) equipped with a 3-mm broadband probe with the observe coil tuned to ²H (92.1 MHz) or ¹³C (150 MHz). Proton-decoupled ²H NMR spectra were acquired using a 90° pulse (12.5 µs), 920-Hz sweep width, 1,836 data points, and a 1-s acquisition time with no further delay at 50°C. Typically 20,000–80,000 scans were averaged for MAG requiring ~5–24 h. Proton decoupling was performed using a standard WALTZ-16 pulse sequence. ¹³C NMR spectra of MAG were collected using 52° pulse (6.06 µs), 20,330-Hz sweep width, 60,992 data points, and a 1.5-s acquisition time with no further delay at 25°C (10). Typically, 10,000–40,000 scans were averaged requiring ~5–18 h.

Proton-decoupled ¹³C NMR spectra of glutamate were obtained using a 45° pulse (5.0 µs), 34,965-Hz sweep width, 104,986 data points, and a 1.5-s interpulse delay at 25°C. Spectra were averaged 7,000–30,000 scans requiring ~6–25 h.

The notations used in ¹³C NMR analysis such as C5D56 represents the following: “C5” for the carbon position of glucose and “D56” for doublet arising from spin-spin coupling of carbons 5 and 6. The Q in C5Q means quartet or doublet of doublets arising from coupling of carbon-5 with both carbons-4 and -6. C5S means the singlet resonance of carbon-5. Notations for other carbon resonances are used in a similar way in this study. All NMR spectra were analyzed using the curve-fitting routine supplied with NUTS PC-based NMR spectral analysis program (Acorn NMR, Freemont, CA).

Measurement of excess isotope enrichment. A ²H NMR spectrum of MAG derived from glucose without isotope enrichment (Fig. 2A) shows ²H resonances in the seven aliphatic hydrogens in the glucose skeleton and in the two methyl groups in the monoacetone adduct. The peak area of each ²H resonance was defined as \( A_{H1} \), \( A_{H6S} \) depending on hydrogen position in glucose. The total peak areas of two ²H

Fig. 2. ²H NMR spectra of MAG derived from natural abundance glucose (A), medium glucose from myotube culture (B, protocol 3), medium glucose from skeletal muscle tissue incubation (C, protocol 4), and glycogen from skeletal muscle tissue incubation (D, protocol 4). The scale of spectrum (D) is ~1/8 of the others. Protocol 3 is myotube culture using Dulbecco’s PBS (D-PBS) containing 20 mM [U-¹³C3]lactate, 5% ²H₂O, 2% FBS, 5 mM glucose, and 10 mM glutamine. Protocol 4 is muscle tissue incubation using 0.1% BSA, 10 mM glucose, 10 mM glutamine, 20 mM lactate (20% [U-¹³C3]lactate), and 5% ²H₂O.
natural abundance methyl resonances were defined as $A_{\text{methyl}}$. The ratio of each MAG resonance in the $^2$H NMR spectrum compared with $A_{\text{methyl}}$ is theoretically 1:6 since there are 6 hydrogens that contribute to the two methyl groups. The measured ratios for natural abundance glucose were slightly higher (~0.20), likely because of a longer spin-lattice relaxation time of deuterons in the methyl position (Table 1). With the use of this correction factor, the $^2$H enrichment above natural abundance (termed excess enrichment) could be determined by comparing the areas of each $^2$H resonance in MAG derived from a tissue sample with the natural abundance signals arising from the two methyl resonances, M1 and M2 (Fig. 1).

Similar considerations apply to analysis of the $^{13}$C spectra. Because each $^{13}$C is present at natural abundance levels of 1.1%, the probability of having two $^{13}$Cs as nearest neighbors in the same molecule is 0.000121 or 0.0121%. A $^{13}$C NMR spectrum of MAG containing only natural abundance levels of $^{13}$C is shown in Fig. 3a (only the carbon-2 and carbon-5 resonances are shown for clarity). The small doublets surrounding the more intense singlets in each resonance arise from 1.1% of the $^{13}$C atoms in the molecule. The natural abundance $^{13}$C signal from the methyl groups of MAG was also observed. The area of the $^{13}$C singlet of each carbon was defined as $A_{\text{C1S}} - A_{\text{COS}}$, the areas of doublets as $A_{\text{C12D}} - A_{\text{C5D56}}$, and the summed peak areas of two $^{13}$C natural abundance methyl resonances were defined as $A_{\text{methyl}}$. The ratio of each doublet area compared with $A_{\text{methyl}}$ in the $^{13}$C NMR spectrum of natural abundance MAG should be 0.000121/(2 x 0.011) = 0.0055, exactly as observed within experimental error (Table 2). Any enrichment above this level was considered excess.

The 1.1% enrichment in each neighboring carbon (D12 and D23 in the carbon-2 resonance and D45 and D56 in the carbon-5 resonance) arises from a tissue sample with the natural abundance signals arising from the two methyl resonances, M1 and M2 (Fig. 1). The natural abundance $^{13}$C signal from the methyl groups of MAG was also observed. The area of the $^{13}$C singlet of each carbon was defined as $A_{\text{C1S}} - A_{\text{COS}}$, the areas of doublets as $A_{\text{C12D}} - A_{\text{C5D56}}$, and the summed peak areas of two $^{13}$C natural abundance methyl resonances were defined as $A_{\text{methyl}}$. The ratio of each doublet area compared with $A_{\text{methyl}}$ in the $^{13}$C NMR spectrum of natural abundance MAG should be 0.000121/(2 x 0.011) = 0.0055, exactly as observed within experimental error (Table 2). Any enrichment above this level was considered excess.

Relative contributions of glyconeogenesis vs. direct glycolysis in intact animals. [U-13C3]lactate, provided to a fasted animal, serves as a substrate for hepatic glycolysis and glyconeogenesis as well as for muscle glucose and glycogen synthesis. The isotopic enrichment of [U-13C3]lactate in the muscle glycogen was measured by 2H NMR analysis of MAG.

Table 1. $^2$H NMR analysis of MAG

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<tr>
<td>Hydrogen-1</td>
<td>0.21 ± 0.01</td>
<td>0.23 ± 0.04</td>
<td>0.36 ± 0.05 (0.011)</td>
<td>0.43 ± 0.06 (0.016)</td>
<td>0.53 ± 0.18 (0.023)</td>
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<td>Hydrogen-2</td>
<td>0.21 ± 0.01</td>
<td>0.26 ± 0.07</td>
<td>0.37 ± 0.09 (0.011)</td>
<td>0.59 ± 0.13 (0.027)</td>
<td>0.75 ± 0.23 (0.041)</td>
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<td>Hydrogen-3</td>
<td>0.20 ± 0.01</td>
<td>0.18 ± 0.02</td>
<td>0.19 ± 0.05</td>
<td>0.21 ± 0.02</td>
<td>0.21 ± 0.04</td>
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<tr>
<td>Hydrogen-4</td>
<td>0.18 ± 0.02</td>
<td>0.18 ± 0.03</td>
<td>0.21 ± 0.06</td>
<td>0.23 ± 0.06</td>
<td>0.25 ± 0.06 (0.006)</td>
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<tr>
<td>Hydrogen-5</td>
<td>0.21 ± 0.03</td>
<td>0.21 ± 0.03</td>
<td>0.21 ± 0.04</td>
<td>0.23 ± 0.07</td>
<td>0.30 ± 0.06 (0.006)</td>
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<td>Hydrogen-6a</td>
<td>0.20 ± 0.02</td>
<td>0.20 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>0.23 ± 0.04</td>
<td>0.26 ± 0.07</td>
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<tr>
<td>Hydrogen-6b</td>
<td>0.18 ± 0.03</td>
<td>0.18 ± 0.02</td>
<td>0.18 ± 0.05</td>
<td>0.21 ± 0.05</td>
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Values are means ± SE. NMR, nuclear magnetic resonance; MAG, monocarboxylate. MAG was derived from natural abundance (NA) glucose (n = 6), from medium glucose of myotubes (protocols 1-3, n = 6 for each), and from medium glucose (n = 10) and skeletal muscle glycogen (n = 4) in tissue incubation.

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RESULTS

Glucose released by myotubes. Myotubes were exposed to 5% $^2$H2O and unlabeled glucose (protocol 1). $^2$H enrichment in medium glucose at the hydrogen-2 position tended to be higher than natural abundance $^2$H, but the difference did not reach statistical significance (P = 0.08, Table 1). There was no excess enrichment in hydrogen-6 (Table 1). In a second series of experiments, myotubes were exposed to conditions designed to stimulate glucose production: 20 mM [U-13C3]lactate, 5% $^2$H2O, 5 mM glucose, and 2 mM glutamine (protocol 2). In these experiments, excess $^2$H enrichment was observed at both

Statistical analysis. The data are expressed as means ± SD. Comparisons between groups were performed using one-way ANOVA. Differences in mean values were considered statistically significant at a probability level of <5% (P < 0.05).

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<tr>
<td>Hydrogen-4</td>
<td>0.18 ± 0.02</td>
<td>0.18 ± 0.03</td>
<td>0.21 ± 0.06</td>
<td>0.23 ± 0.06</td>
<td>0.25 ± 0.06 (0.006)</td>
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<tr>
<td>Hydrogen-5</td>
<td>0.21 ± 0.03</td>
<td>0.21 ± 0.03</td>
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glycogen (D) is much higher compared with the rest (see Table 2). Carbon-5; D56, doublet from coupling of carbon-5 with carbon-6; S, singlet. With both carbons-4 and -6; D45, doublet from coupling of carbon-4 with coupling of carbon-2 with both carbons-1 and -3, or from coupling of carbon-5 with both carbons-4 and -6; D45, doublet from coupling of carbon-4 with carbon-5, D56, doublet from coupling of carbon-5 with carbon-6, S, singlet. hydrogen-1 and hydrogen-2 positions of medium glucose, about two times natural abundance (Table 1), but again there was no excess enrichment in position 6. When the glutamine concentration was increased from 2 to 10 mM (protocol 3), the excess enrichments at hydrogen-1 and hydrogen-2 positions were even higher (Table 1 and Fig. 2B), but 2H labeling in position 6 was not significantly different from natural abundance. The presence of excess 2H label in position 2 is generally accepted as evidence of proton exchange at the level of glucose 6-phosphate isomerase, and excess 2H label in position 1 is due to equilibration with mannose-6-phosphate (2). Together, the low level of 2H enrichment in each carbon compared with the concentration of 2H in the medium suggests a low rate of gluconeogenesis.

In the 13C NMR analysis, the dominant resonances from glucose in the medium were singlets resulting from natural abundance 13C and the doublets due to adjacent natural abundance 13C nuclei (doublets due to J1,2, J2,3, J4,5, and J5,6). However, in some instances, glucose from the medium also showed a quartet due to [4,5,6-13C3]glucose at levels greater than natural abundance in protocols 2 and 3 (Fig. 3B and Table 2). The appearance of CSQQ demonstrates that it originated from [U-13C3]lactate because natural abundance [4,5,6-13C3]glucose cannot be detected by 13C NMR analysis in this study (inner two components of the CSQ quartet overlap with the singlet in Fig. 3B). Although resonances due to J4,5 and J5,6 were detected, these signals were not significantly greater than natural abundance. Interestingly, there was no evidence of the existence of excess [1,2,3-13C3]glucose, which would be expected under the equilibrium of the triose phosphate isomerase reaction. The 13C spectra demonstrated a small amount of glucose synthesis in myotubes by direct transfer.

Analysis of incubated, minced skeletal muscle. 2H enrichment in glucose from the medium of incubated skeletal muscle was essentially indistinguishable from the glucose produced by myotubes (Table 1). In contrast to glucose isolated from the medium, muscle glycogen from incubated, minced muscle showed very intense 2H enrichment in glucose units (Table 1 and Fig. 2, C and D). The excess enrichment in the hydrogen-2 position of glycogen was 40-fold higher than medium glucose. Because the excess enrichments labeled M1 and M2 are the natural abundance 2H signals from MAG, these signals provide an internal standard for comparison of Fig. 2, C and D. The difference between the labeling of glucose isolated from the medium and glycogen demonstrates that the overwhelming majority of glucose 6-phosphate produced in muscle was directed toward muscle glycogen rather than released in the medium. Experiments performed with [U-13C3]propionate in the incubation medium in addition to unlabeled lactate (protocol 5) did not affect the 2H enrichment pattern in muscle glycogen and medium glucose (spectra not shown).

A very small amount of excess [1,2,3-13C3]glucose and [4,5,6-13C3]glucose was observed in the medium (Table 2 and Fig. 3C) and was qualitatively similar to results from myotubes. However, glycogen derived from [1,2,3-13C3]glucose and [4,5,6-13C3]glucose isotopomers amounted to 1.13 and 1.39%, respectively, perhaps 1,000-fold greater than that found in medium glucose. This dramatic difference is easily appreciated by comparing Fig. 3, C and D. Because only 20% of the total lactate provided in the incubation medium was [U-13C3]lactate, excess enrichment in [1,2,3-13C3]glucose or [4,5,6-13C3]glucose of 1% corresponds to 5% of the tissue glycogen. The marked difference in labeling patterns of medium glucose vs. skeletal muscle glycogen again confirmed that glucose 6-phosphate synthesized from lactate was stored.
almost exclusively as glycogen rather than being released into the medium.

If glycogen was being synthesized via the direct transfer pathway $[1,2,3,\text{13C}_3]\text{lactate} \rightarrow [1,2,3,\text{13C}_3]\text{pyruvate} \rightarrow [1,2,3,\text{13C}_3]\text{oxaloacetate} \rightarrow [1,2,3,\text{13C}_3]\text{PEP} \rightarrow [1,2,3,\text{13C}_3]\text{glucose}$, then the $\text{13C}$ labeling patterns in glucose would show a quartet but no signal due to doubly labeled glucose ($[1,3,4,\text{13C}_2]\text{glucose}$, $[2,4,5,\text{13C}_2]\text{glucose}$, $[4,5,6,\text{13C}_2]\text{glucose}$, or $[5,6,7,\text{13C}_2]\text{glucose}$). Because $[1,2,3,\text{13C}_3]\text{oxaloacetate}$ also serves as a substrate for citrate synthase to produce citrate, subsequently isocitrate, $\alpha$-ketoglutarate, and glutamate, $[1,2,3,\text{13C}_3]\text{oxaloacetate}$ would generate $[2,3,\text{13C}_2]\text{glutamate}$. However, $[2,3,\text{13C}_2]\text{glutamate}$ was not detected (Figs. 4 and 5). Instead, $\text{13C}$ NMR analysis of muscle glutamate showed $\text{C}_4\text{D}_4\text{S}$ plus relatively

Table 2. $\text{13C}$ NMR analysis of MAG derived from NA glucose, from medium glucose of myotubes, and from medium glucose and skeletal muscle glycogen in tissue incubation

<table>
<thead>
<tr>
<th>Glucose (NA)</th>
<th>Myotubes</th>
<th>Minced Skeletal Muscle, Protocol 4</th>
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<tr>
<td></td>
<td>Protocol 2</td>
<td>Protocol 3</td>
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<tr>
<td>C2D12</td>
<td>$0.0049 \pm 0.0003$</td>
<td>$0.0052 \pm 0.0009$</td>
</tr>
<tr>
<td>C2D23</td>
<td>$0.0053 \pm 0.0005$</td>
<td>$0.0051 \pm 0.0003$</td>
</tr>
<tr>
<td>C2Q</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>C5D45</td>
<td>$0.0056 \pm 0.0004$</td>
<td>$0.0051 \pm 0.0007$</td>
</tr>
<tr>
<td>C5D56</td>
<td>$0.0057 \pm 0.0003$</td>
<td>$0.0051 \pm 0.0004$</td>
</tr>
<tr>
<td>C5Q</td>
<td>ND</td>
<td>$0.0057 \pm 0.0014$ (0.0122)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. $\text{13C}$ NMR analysis of MAG derived from NA glucose (n = 6), from medium glucose of myotubes (protocols 2–3, n = 6 for each), and from medium glucose (n = 10) and skeletal muscle glycogen (n = 6) in tissue incubation. Each value is the ratio of peak area of each resonance normalized by areas of methyl resonances of MAG on $\text{13C}$ NMR spectra ($A_{\text{each resonance}}/A_{\text{methyl}}$). Nos. in parentheses are excess $\text{13C}$ enrichment (%). The protocols are described in the text and are summarized in the legend to Table 1. ND, not detectable. *Significantly different from NA glucose, $P < 0.05$.

Fig. 4. Schematic diagram of metabolic pathways and $\text{13C}$ labeling patterns related to reverse flux through pyruvate kinase in skeletal muscle after $[U-\text{13C}_3]\text{lactate}$ administration. The direct conversion of $[U-\text{13C}_3]\text{lactate}$ to $[U-\text{13C}_3]\text{pyruvate}$ followed by reverse flux through pyruvate kinase results exclusively in $[1,2,3,\text{13C}_3]\text{glucose}$, $[2,3,\text{13C}_2]\text{glucose}$, $[4,5,\text{13C}_2]\text{glucose}$, or $[5,6,\text{13C}_2]\text{glucose}$. Because $[1,2,3,\text{13C}_3]\text{oxaloacetate}$ also serves as a substrate for citrate synthase to produce citrate, and subsequently isocitrate, $\alpha$-ketoglutarate, and glutamate, $[1,2,3,\text{13C}_3]\text{oxaloacetate}$ would generate $[2,3,\text{13C}_2]\text{glutamate}$. However, $[2,3,\text{13C}_2]\text{glutamate}$ was not detected (Figs. 4 and 5). Instead, $\text{13C}$ NMR analysis of muscle glutamate showed $\text{C}_4\text{D}_4\text{S}$ plus relatively

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small C2D12 and excess C3S (Figs. 4 and 5). This pattern can only arise from flux of lactate to pyruvate and entry into the TCA cycle via pyruvate dehydrogenase because 13C enrichment in the acetyl group of acetyl-CoA results in [4,5,13C2]glutamate and subsequently both [1,2,13C2]glutamate and [3,13C1]glutamate after one turn of the TCA cycle. There was no evidence of pyruvate carboxylase flux from the 13C enrichment pattern in glutamate.

The possibility that oxaloacetate is an intermediary pool in glucose production also was tested by addition of [U-13C3]propionate to the incubation medium of minced skeletal muscle. There was no 13C excess enrichment in muscle glycogen, but labeling was observed in muscle glutamate (spectra not shown). Because there is only one pathway for propionate to enter the TCA cycle, via succinyl-CoA (Fig. 4), the absence of 13C enrichment in glycogen demonstrates that flux through PEPCK is negligible under these conditions.

13C NMR of liver glycogen, plasma glucose, and skeletal muscle glycogen from intact animals. Blood glucose, liver glycogen, and skeletal muscle glycogen were isolated from rats after an intraperitoneal injection of [1,2,3-13C3]lactate. The 13C enrichment pattern of skeletal muscle glycogen shown in Fig. 6C is quite different from that of liver glycogen or blood glucose. The C2Q and C5Q are dominant in muscle glycogen while C2D12 and C5D56 are dominant in liver glycogen and blood glucose. Thus [1,2,3-13C3] and [4,5,6-13C3]glucose isotopeomers are abundant in muscle glycogen while [1,2,13C2] and [5,6-13C2]glucose are abundant in liver glycogen and blood glucose. The area of the quartet relative to the doublet due to J1,2 (AC2Q/AC2D12) in carbon-2 resonance was 0.59, 0.53, and 4.23 in liver glycogen, blood glucose, and skeletal muscle glycogen, respectively. The area of the quartet in carbon-5 relative to the doublet due to J5,6 (AC5Q/AC5D56) was 0.85, 0.73, and 4.90, respectively (Fig. 7A). Based on the disproportionate amplitude of the quartet signal in muscle glycogen, it was estimated that 88% of [1,2,3-13C3]glucose in muscle glycogen originated from glyconeogenesis and the remainder from blood glucose. Similarly, 87% of [4,5,6-13C3]glucose units originated from glyconeogenesis based on carbon-5 analysis. According to the calculation based on these percentages and the enrichments of glucose isotopeomers (Table 3), ~70% of multiple-labeled glucose units in skeletal muscle glycogen was synthesized through a direct transfer pathway from [U-13C3]lactate in whole animals while the remaining 30% was from blood glucose phosphorylation (Fig. 7B).

DISCUSSION

This study demonstrated direct transfer of lactate to glucose in skeletal muscle both in vitro and in vivo. It is the first...
demonstration of this pathway in vivo. Surprisingly, under these conditions, ~70% of multiple-labeled glucosyl units of skeletal muscle glycogen in whole animals originated through reverse flux of pyruvate kinase while ~30% originated from phosphorylation of blood glucose. Nevertheless, the physiological relevance of these observations is not known. In particular, it is already well-established that direct transfer of blood glucose into muscle glycogen plays an essential role in systemic glucose homeostasis, and it seems very unlikely that reverse flux could play any meaningful role under postprandial conditions where the concentration of lactate is low. It is conceivable that reverse flux could be relevant in glycogen replenishment after intense exercise when the concentration of lactate is increased up to 10–12 mM (8, 22), which is closer to

Table 3. \(^{13}\)C NMR analysis of MAG derived from liver glycogen, blood glucose, and skeletal muscle glycogen of whole animals treated with \([U-^{13}\text{C}_3]\)lactate

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Liver Glycogen</th>
<th>Blood Glucose</th>
<th>Muscle Glycogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2D12</td>
<td>0.5735±0.1052(^c)(1.4040)</td>
<td>0.4279±0.0325 (1.0446)</td>
<td>0.0453±0.0116(^c)(0.0999)</td>
</tr>
<tr>
<td>C2D23</td>
<td>0.1197±0.0164(^d)(0.2611)</td>
<td>0.0843±0.0078 (0.1805)</td>
<td>0.0174±0.0024(^d)(0.0277)</td>
</tr>
<tr>
<td>C5Q</td>
<td>0.3343±0.0465(^d)(0.7932)</td>
<td>0.2237±0.0100 (0.5306)</td>
<td>0.1882±0.0438 (0.4466)</td>
</tr>
<tr>
<td>C5D45</td>
<td>0.1612±0.0110(^d)(0.3362)</td>
<td>0.1159±0.0123 (0.2384)</td>
<td>0.0208±0.0057(^d)(0.0329)</td>
</tr>
<tr>
<td>C5D56</td>
<td>0.4735±0.0758(^c)(0.9931)</td>
<td>0.3675±0.0182 (0.7679)</td>
<td>0.0408±0.0114(^c)(0.0745)</td>
</tr>
<tr>
<td>C5Q</td>
<td>0.4006±0.0611(^d)(0.8579)</td>
<td>0.2686±0.0341 (0.5751)</td>
<td>0.1967±0.0424(^c)(0.4212)</td>
</tr>
</tbody>
</table>

Values are means ± SE. \(^{13}\)C NMR analysis of MAG derived from liver glycogen \((n = 6)\), blood glucose \((n = 6)\), and skeletal muscle glycogen \((n = 6)\) of whole animals treated with \([U-^{13}\text{C}_3]\)lactate. Each value is the ratio of peak area of each resonance normalized by areas of methyl resonances of MAG on \(^{13}\)C NMR spectra \((A_{resonance}/A_{methyl})\). Nos. in parentheses are excess \(^{13}\)C enrichment (%). Significantly different from blood glucose \((^\ast P < 0.01 \text{ and } ^\ddagger P < 0.001)\).
the concentration of lactate used in the minced muscle studies, 20 mM (Fig. 3D). This hypothesis remains to be examined.

The strongest evidence of the direct transfer comes from exposure of minced muscle tissues to 20% [U-13C3]lactate. If [1,2,3-13C3]oxaloacetate was produced, then exchange with fumarate must produce equal concentrations of [1,2,3,4,5-13C5]oxaloacetate and [2,3,4,5-13C4]oxaloacetate. The former must produce [1,2,3,4,5-13C5]- or [4,5,6-13C3]glucose, and the latter must produce [1,2-13C2]glucose or [5,6-13C2]glucose. These doubly labeled glucose isotopomers are easily distinguished from triply labeled glucose isotopomers in a NMR spectrum, and their absence in Fig. 3 distinguishes the direct transfer from lactate to glycogen in vivo. Although the absence of these isotopomers could be attributed to dilution by reverse exchange with fumarate, the absence of any labeled glucose isotopomers from the TCA cycle in this study strongly argues against reverse exchange. These data from the incubated skeletal muscle are fully consistent with the radiotracer studies by Hiatt et al. (9) in isolated rat diaphragm and by Donovan and Pagliassotti (6) in perfused rabbit skeletal muscle.

The current study extends earlier work by showing that the 13C enrichment patterns in skeletal muscle glycogen in vivo is also due predominantly to direct transfer from lactate to glucose. The in vivo experiment is somewhat more difficult to interpret than incubated skeletal muscle because hepatic gluconeogenesis from lactate will produce plasma glucose with complex labeling patterns due to recycling of 13C in the liver. However, analysis of 13C isotopomers in plasma glucose allowed for correction of the effects of hepatic gluconeogenesis followed by glycogen synthesis in skeletal muscle. The amount of [1,2,3,4,5-13C5]glucose, or [4,5,6-13C3]glucose, in skeletal muscle glycogen was far greater than could be contributed by plasma glucose and therefore must be due to in situ skeletal muscle gluconeogenesis.

Although these experiments confirmed a direct transfer pathway for glucose production by skeletal muscle, the 13C labeling pattern in glucose could in principle be consistent with flux into oxaloacetate followed by conversion to PEP without exchange with fumarate. This seems unlikely for a number of reasons. First, TCA cycle enzymes in skeletal muscle mitochondria are active, and “reverse flux” into fumarate from malate and oxaloacetate is well-studied and complete or nearly so in the liver (12, 15, 20). Second, the possibility that oxaloacetate was involved in gluconeogenesis was also tested by adding [U-13C3]propionate. Entry of [U-13C3]propionate in the TCA cycle via propionyl-CoA, methylmalonyl-CoA, and succinyl-CoA must produce a mixture of 13C isotopomers in PEP. If glycogen is derived from oxaloacetate through PEPC kinase, one would expect many more isotopomers to appear in glycogen. Because 13C enrichment was not observed in glycogen under these conditions, this eliminates the possibility of flux coming through PEPC. Third, 13C NMR analysis of muscle glutamate during exposure to [U-13C3]lactate confirmed flux through pyruvate dehydrogenase, but negligible flux through pyruvate carboxylase. Finally, the absence of [1,2,3,4,5-13C5]glucose, or [5,6-13C2]glucose, in the incubated skeletal muscle exposed to [U-13C3]lactate demonstrates the absence of reverse exchange with fumarate. The other doublet in position 2 or 5, due to [2,3,4-13C2]- or [4,5,6-13C3]glucose, could arise from “forward” flux through the cycle. The absence of these isotopomers could be attributed to dilution of TCA cycle intermediates by very high flux through anaplerotic pathways, which is typically at least 10 times TCA cycle flux. However, very high anaplerotic flux with unlabeled substrates is not consistent with the observed 13C spectrum of glutamate.

These results also bear on the functional significance of the newly discovered glucose-6-phosphatase β in muscle (19). The presence of this phosphatase would seem to allow glucose release in the circulation from skeletal muscle glycogen. However, in this study, the 2H and 13C labeling patterns in medium glucose were quite different from glycogen, and the whole animal data showed no evidence of glucose release from muscle. Based on both the 2H and 13C NMR data, glucose 6-phosphate formed from lactate is directed almost exclusively into glycogen. Although 2H labeling of glucose from 2H2O is a rich source of information, little work has been done using 2H NMR analysis of skeletal muscle metabolism.

In summary, the 13C distribution in glucose produced by gluconeogenesis in skeletal muscle from [U-13C3]lactate was not consistent with flux through a “well-mixed” TCA cycle. If lactate is converted to glucose in muscle via a TCA cycle intermediate, then other rather implausible conditions must be invoked. The alternative interpretation is significant reverse flux through the pyruvate kinase reaction. Despite early thermodynamic studies indicating that the reaction is essentially irreversible under cellular conditions (4, 16, 17), both tracer and subsequent thermodynamic studies in isolated systems (5–7, 9, 14) reported significant reverse flux. The current study demonstrated this pathway in vivo. Because skeletal muscle glycogen plays numerous important roles in carbohydrate homeostasis, it will be important to properly quantify all pathways feeding glycogen production and the physiological relevance of each.

ACKNOWLEDGMENTS

We thank Charles Storey and Angela Milde for excellent work in animal experiments and Zheng Yan for technical support.

GRANTS

This study was supported by National Institutes of Health Grants RR-02584, DK-16194, HL-34557, and DK-78933.

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