Lactate Contributes to Glyceroneogenesis and Glycogen Synthesis in Skeletal Muscle by Reversal of Pyruvate Kinase*

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Background: Lactate can contribute to glycogen in skeletal muscle, but the pathway of this conversion is uncertain.
Results: Glyceroneogenesis and glycogen synthesis from lactate occur in muscle of rats with high blood glucose without prior mixing in the citric acid cycle.
Conclusion: Trioses are generated via reversal of pyruvate kinase.
Significance: Lactate contributes to glycerol and glycogen in skeletal muscle via reversal of pyruvate kinase.

Phosphoenolpyruvate (PEP) generated from pyruvate is required for de novo synthesis of glycogen and glycerol in skeletal muscle. One possible pathway involves synthesis of PEP from the citric acid cycle intermediates via PEP carboxykinase, whereas another could involve reversal of pyruvate kinase (PK). Earlier studies have reported that reverse flux through PK can contribute carbon precursors for glycogen synthesis in muscle, but the physiological importance of this pathway remains uncertain especially in the setting of high plasma glucose. In addition, although PEP is a common intermediate for both glycogenesis and glycogen synthesis, the importance of reverse PK in de novo glycogen synthesis has not been examined. Here we studied the contribution of reverse PK to synthesis of glycogen and the glycerol moiety of acylglycerols in skeletal muscle of animals with high plasma glucose. Rats received a single intraperitoneal bolus of glucose, glycerol, and lactate under a fed or fasting state. Only one of the three substrates was 13C-labeled in each experiment. After 3 h of normal awake activity, the animals were sacrificed, and the contribution from each substrate to glycogen and the glycerol moiety of acylglycerols was evaluated. The fraction of 13C labeling in glycogen and the glycerol moiety of acylglycerols exceeded the possible contribution from either plasma glucose or muscle oxaloacetate. The reverse PK served as a common route for both glycogenesis and glyceroneogenesis in the skeletal muscle of rats with high plasma glucose. The activity of pyruvate carboxylase was low in muscle, and no PEP carboxykinase activity was detected.

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since pyruvate is readily produced from lactate, and a high concentration of pyruvate could favor conversion of pyruvate to PEP via PK by mass action. It is unknown whether reversal of PK contributes significantly to glycogen synthesis or under more normal physiological conditions such as in the presence of high levels of circulating glucose. In this condition, the availability of excess glucose and subsequently glucose 6-phosphate could inhibit de novo glycogen synthesis from lactate through any pathway.

Glyceroneogenesis refers to the de novo synthesis of the glycerol moiety of acylglycerols from pyruvate or equivalent molecules other than glucose or free glycerol (11, 12). Because the conversion of pyruvate to a triose is required for both glyconeogenesis and glyceroneogenesis, carbon tracer labeling in the glycerol moiety of acylglycerols provides an alternative source of information about the origin of three-carbon units. Glyceroneogenesis in muscle has been reported to play a significant role in fatty acid esterification in mammals (13, 14). The details of the involved pathways, however, are not clear. Although cytosolic PEPCK was suggested as a reaction step for glyceroneogenesis (15, 16), we did not detect any PEPCK activity in muscle (8). The roles of some other enzymes in skeletal muscle such as pyruvate carboxylase and glycerol kinase are not well established (13, 17–21). For example, the activity of glycerol kinase in extrahepatic organs was generally considered not to be significant, but it was suggested to have an important role in fatty acid esterification in muscle (13, 20, 21).

In this study we tested the hypothesis that reversal of PK plays a role in de novo glycerol synthesis. Because the triose pool in exchange with glycerol also contributes as precursors for glycogen synthesis, the role of the reverse pathway in glyconeogenesis was assessed simultaneously in animals with high levels of circulating glucose. We also performed in situ hind limb perfusions to verify the results observed in muscle in vivo. The results demonstrated that reversal of PK contributes to both glyconeogenesis and glyceroneogenesis in the presence of high levels of glucose. PEPCK activity was not detected, and the activity of pyruvate carboxylase was negligible. Glycerol kinase was important for the supply of glycerol backbone of acylglycerols in skeletal muscle.

Materials and Methods

**Animal Studies**—The study was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center. Male Sprague-Dawley rats (300–350 g) were fed or fasted for 24 h with free access to water. Rats received one of the following three mixtures: (i) 6 mM glucose, 2 mM lactate, 0.2 mM pyruvate, 0.2 mM [U-13C6]glucose, 0.6 mM long chain fatty acids, and 4% BSA and (ii) 2 mM NH4Cl, 15 mM lactate, 1.5 mM pyruvate, 1.5 mM acetate, and 2 mM [U-13C6]propionate at a flow rate of 10 ml/min. Oxygenation of the perfusion fluid was maintained by flow over an oxygenating net in a humidified atmosphere of O2/CO2 (95:5, v/v). Non-circulating perfusion was continued for 60 min, and then the tissues from hind limbs were freeze-clamped with liquid nitrogen.

**Sample Processing for NMR Analysis**—Glycogen was extracted from muscle tissues, purified, and hydrolyzed as described previously (8). Blood was 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 was purified and converted to monoacetone glucose (MAG) as reported previously (22). MAG was dissolved in 90% deuterated acetonitrile, 10% water (160 μl) for 13C NMR acquisition.

Acylglycerols from muscle tissues (~7 g) were extracted with a mixture of CHCl3 and methanol (2:1), and the glycerol moiety of acylglycerols was hydrolyzed using a mixture of KOH and methanol (1:1, 1 N KOH, 90% methanol) as described previously (23). The extracted glycerol moiety was dissolved in 2H2O (160 μl) for 13C NMR acquisition.

Skeletal muscle tissues were treated with perchloric acid to extract water-soluble components and neutralized with KOH, centrifuged, and the supernatant was dried. The dried residue was dissolved in 2H2O (160 μl) for 13C NMR acquisition for the analysis of the citric acid cycle intermediates and exchanging pools. After 13C NMR acquisition, if necessary, the extracts were further purified using ion-exchange chromatography for aspartate and glutamate isolation.

**NMR Spectroscopy**—All NMR spectra were collected using a Varian INOVA 14.1 T spectrometer (Agilent, Santa Clara, CA) equipped with a 3-mm broadband probe with the observe coil tuned to 13C (150 MHz). 13C NMR spectra of MAG were collected using a 58° pulse (7.0 μs), 20,330 Hz sweep width, 60,992 data points, and a 1.5-s acquisition time with a 1.5-s delay at 25 °C. Proton decoupling was performed using a standard WALTZ-16 pulse sequence. Proton-decoupled 13C NMR spectra of extracts 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. All NMR spectra were analyzed using ACD/Labs NMR spectral analysis program (Advanced Chemistry Development, Inc., Toronto, Canada).

**Estimating 13C Enrichment in Glycogen and in the Glycerol Moiety of Acylglycerols**—The 13C enrichment in glycogen or in the glycerol moiety of acylglycerols was measured as described previously (23–25). Briefly the 13C enrichment in glycogen was calculated by the sum of the following isotopomers measured in glucosyl units: [1,2-13C2], [2,3-13C2], [1,2,3-13C3], [4,5-13C2], [5,6-13C2], [4,5,6-13C3], and [U-13C6]glucose. The enrichment of each isotopomer was measured using an internal reference of MAG and standard samples. The 13C enrichment in
the glycerol moiety of acylglycerols was estimated by the analysis of the glycerol moiety carbon 1 (C1) and C3 resonances at 63.5 ppm or the glycerol moiety C2 resonances at 73.0 ppm (Fig. 1). Given the low probability of forming either singly enriched [1-13C1]glycerol, [3-13C1]glycerol, or [2-13C1]glycerol moiety from any of exogenous labeled substrates, it was assumed that the singlet component corresponds to natural abundance 13C (1.1%).

Quantitative Assessment of the Relative Contributions of Glucose and Lactate Carbons to Glycogenolysis and Glyceroneogenesis—In animals given [U-13C3]lactate plus glucose and glycerol, excess 13C in glycogen or the glycerol moiety of acylglycerols in muscle was assumed to originate from lactate and blood glucose. Excess 13C in blood glucose was possible through glycogenolysis from [U-13C3]lactate in the liver. In the estimation of the relative contributions from glucose and lactate to glycogen or to the glycerol moiety, NMR analysis was performed on several samples; MAG derived from blood glucose, lactate from muscle, MAG derived from muscle glycogen, and the glycerol moiety of acylglycerols from muscle (Fig. 1).

The 13C NMR spectra of lactate C2, glucose C2, and glucose C5 are composed of nine resonances: a singlet, a doublet due to J12 (or J34) in the glucose C5), a doublet due to J23 (or J45 in the glucose C5), and a doublet of doublets or quartet due to 13C in C1, C2, and C3 (or C4, C5, and C6 in the glucose C5). The contributions of [1,2-13C2]- and [2,3-13C2]lactate or [1,2-13C2] and [2,3-13C2]glucose were summed and represented as D, doublet. The same procedure was applied for analysis of glucose C5. Because of degeneracy in 13C-13C coupling constants in glycerol, [1,2,3-13C3]glycerol appears as a 1:2:1 triplet (T). The following abbreviations were used to refer to the metabolites in the equations: GLC (glucose), LAC (lactate), GLY (glucosyl units of glycogen), and GCL (glycerol moiety of acylglycerols). The fractions of double-labeled (D;13C-13C) and triple-labeled (T or Q;13C-13C-13C) isotopeomers in each metabolite were obtained as follows.

\[
\begin{align*}
\text{GLC(D)} &= D(D=Q) \\
\text{GLC(Q)} &= Q(D=Q) \\
\text{LAC(D)} &= D(D=Q) \\
\text{LAC(Q)} &= Q(D=Q) \\
\text{GLY(D)} &= D(D=Q) \\
\text{GLY(Q)} &= Q(D=Q) \\
\text{GCL(D)} &= D(D=T) \\
\text{GCL(T)} &= T(D=T)
\end{align*}
\]

The doublets shown in Fig. 1 (D12 and D23 in glucosyl units of glycogen, D12 and D23 in lactate, and so on) can only arise after metabolism in the citric acid cycle because the administered 13C-labeled precursors were uniformly enriched.

As shown in Fig. 1, the 13C labeling pattern in glycogen differed from that in plasma glucose or muscle lactate, indicating that glycogen had contributions from both sources. The relative contributions from plasma glucose (x) and muscle lactate (y) to recently synthesized muscle glycogen or the glycerol moiety of acylglycerols can be calculated as follows.

Because there are two unknowns (x and y) and two equations for each case, the relative contribution from glucose (x) and that from lactate (y) can be obtained.

Detection of Pyruvate Dehydrogenase, Pyruvate Carboxylase, or PEPCK Activity Based on Aspartate and Glutamate Analysis—Rats given [U-13C6]glucose plus lactate and glycerol produced almost exclusively [U-13C3]pyruvate in skeletal muscle as evidenced by the lactate resonances (Fig. 2A). [U-13C3]Pyruvate may enter the citric acid cycle through pyruvate dehydrogenase (PDH) or pyruvate carboxylase (Fig. 2, B and C). [1,2-13C2]Acetyl-CoA produced through PDH condenses with oxaloacetate to produce [4,5-13C2]citrate and consequently [4,5-13C2]α-ketoglutarate, which is in exchange with glutamate. Thus, the detection of [4,5-13C2]glutamate demonstrates flux through PDH. Through forward turn of the citric acid cycle, α-ketoglutarate C1 is decarboxylated, becoming [1,2-13C2]- or [3,4-13C2]fumarate, since fumarate is a symmetric molecule. Fumarate is in exchange with oxaloacetate that is also in rapid exchange with aspartate. Because extensive exchange would be expected over the 3 h of this experiment, the labeling pattern of aspartate was assumed to reflect that of oxaloacetate. Thus [1,2-13C2]- or [3,4-13C2]aspartate is produced from the double-labeled fumarate. If [1,2-13C2]- and [3,4-13C2]oxaloacetate go through one complete turn of the citric acid cycle including condensing with [1,2-13C2]acetyl-CoA, [1,2,3-13C3]- and [2,3,4-13C3]oxaloacetate would be produced (Fig. 2B).

The entry of [U-13C3]pyruvate through pyruvate carboxylase produces [1,2,3-13C3]oxaloacetate first and then [2,3,4-13C3]-oxaloacetate after back-scrambling with fumarate. However, the observation of [1,2,3-13C3]- and [2,3,4-13C3]aspartate is not concrete evidence for pyruvate carboxylase activity, as they can also be produced through PDH followed by extensive turns of the citric acid cycle as noted above. When [1,2,3-13C3]- and [2,3,4-13C3]oxaloacetate go through a forward turn of the cycle after condensing with unlabeled acetyl-CoA, [2,3,4-13C3] and [1,2,3-13C3]glutamate were produced (Fig. 2C). Through the forward turn of the cycle, as illustrated in Fig. 2D, oxaloacetate C2-C4 became glutamate C1-C3 in a reverse order (OAA C2 → Glu C3; OAA C3 → Glu C2; OAA C4 → Glu C1). Thus, an equivalent labeling pattern is expected in aspartate C3 and glutamate C2. The equivalent pattern is also expected between aspartate C2 and C3 due to extensive back-scrambling of oxaloacetate with the symmetric fumarate and succinate pool, exactly as observed.

The 13C labeling pattern in aspartate is important because, if flux through PEPCK is active, then the labeling pattern of oxaloacetate (as determined aspartate resonances) must be transmitted to the glucosyl units of glycogen and the glycerol moiety of acylglycerols.

Glucose and Lactate Assay—Plasma lactate was assayed enzymatically by detecting NADH released through lactate
FIGURE 1. Excess $[1,2,3-^{13}C_3]$ labeling in muscle glycerol and glycogen compared with plasma glucose. All the $^{13}C$ NMR spectra (of glucose from plasma, the glucosyl units of glycogen from muscle, the glycerol moiety of acylglycerols from muscle, and lactate from muscle) are from a fasted rat given $[U-^{13}C]lactate$ plus glucose and glycerol. The doublets ($^{13}C-^{13}C$) were dominant in $C_2$ and $C_5$ regions of plasma glucose, whereas the quartet ($^{13}C-^{13}C-^{13}C-^{13}C$) was dominant in $C_2$ of muscle lactate. The labeling pattern in glycogen was intermediate between plasma glucose and muscle lactate, and the quartets were the major signals in $C_2$ and $C_5$ regions of the glucosyl units of glycogen. In the glyceral moeity $C_2$ region of acylglycerols, the signal from triplet ($^{13}C-^{13}C-^{13}C$) was 2-fold that of the doublet. Insets: $D_{12}$, doublet from coupling of $C_1$ with $C_2$; $D_{23}$, doublet from coupling of $C_2$ with $C_3$; $D_{45}$, doublet from coupling of $C_4$ with $C_5$; $D_{56}$, doublet from coupling of $C_5$ with $C_6$; $T$, triplet from coupling of adjacent carbon; $Q$, doublet of doublets, or quartet, arising from coupling of $C_2$ with both $C_1$ and $C_3$ or from coupling of $C_5$ with both $C_4$ and $C_6$; $S$, singlet; open circle = $^{12}C$; filled circle = $^{13}C$. 

**blood**

**muscle**

**glycerol moiety C2**

**glycerol moiety (acylglycerol)**

**glucose**

**glucosyl unit C2**

**glucosyl unit C5**

**glycogen**

**PEP → PK → Pyruvate / lactate**

**lactate C2**

**lactate C5**

$D_{12}$, $D_{23}$, $D_{45}$, $D_{56}$, $Q$, $T$, $S$.
FIGURE 2. $^{13}$C labeling in glutamate and oxaloacetate after the entry of $[U^{-13}C_6]$pyruvate to the citric acid cycle. Rats receiving a mixture of $[U^{-13}C_6]$glucose, glycerol, and lactate showed dominant $[U^{-13}C_3]$lactate in skeletal muscle among $^{13}$C-labeled lactate isotopomers after 3 h of the mixture administration (A). $[U^{-13}C_6]$Pyruvate entry to the citric acid cycle through PDH led to $[4,5^{-13}C_2]$-ketoglutarate ($\alpha$-kg) and consequently $[4,5^{-13}C_2]$glutamate. After decarboxylation, the $\alpha$-ketoglutarate was converted to $[1,2^{-13}C_2]$- and $[3,4^{-13}C_2]$fumarate (fum; a symmetric molecule), which was in exchange with oxaloacetate (OAA) and then aspartate (Asp) producing $[1,2^{-13}C_2]$- and $[3,4^{-13}C_2]$aspartate. The condensation of $[1,2^{-13}C_2]$- and $[3,4^{-13}C_2]$oxaloacetate with $[1,2^{-13}C_2]$acetyl-CoA and subsequent one turn through the citric acid cycle may produce $[1,2,3^{-13}C_3]$- and $[2,3,4^{-13}C_3]$aspartate (B). In contrast, $[U^{-13}C_6]$pyruvate entry through pyruvate carboxylase (PC) produced $[1,2,3^{-13}C_3]$oxaloacetate first and then $[2,3,4^{-13}C_3]$oxaloacetate after back-scrambling with fumarate, which became $[2,3^{-13}C_2]$- and $[1,2,3^{-13}C_3]$glutamate, respectively, through the forward turn of the citric acid cycle (C). Because oxaloacetate C2-C4 are equivalent with $\alpha$-ketoglutarate C1-C3 in a reverse order, the same $^{13}$C labeling pattern is expected between aspartate C3 and glutamate C2 (D). In panel D the resonances from glutamate and aspartate were from the skeletal muscle of a fed rat received $[U^{-13}C_6]$glucose, glycerol, and lactate. Signals from glutamate were stronger than those from aspartate; they are not on the same scale. Open circle = $^{12}$C; filled circle = $^{13}$C; gray circle = $^{12}$C or$^{13}$C.
conversion to pyruvate via lactate dehydrogenase. The level of glucose was estimated using glucose oxidase method (YSI 2300 Glucose Analyzer; GMI, Inc.).

Statistical Analysis—The data are expressed as the means ± S.E. Comparisons between groups were performed using a t test. Differences in mean values were considered statistically significant at a probability level of <5% (p < 0.05).

Results

Three hours after the administration of glucose, glycerol, and lactate, plasma glucose was found to be 9.6 ± 0.3 mM (n = 7) in fasted rats and 8.8 ± 0.2 mM (n = 10) in fed rats, whereas plasma lactate was measured at 2.6 ± 0.4 mM in fasted rats and 3.5 ± 0.3 mM in fed rats.

Contributions of Exogenous Substrates to Glycogen in Skeletal Muscle—

^13^C enrichment in glycogen was 1.67 ± 0.23% in fed rats and 7.25 ± 1.29% in fasted rats given [U-^13^C_6]glucose plus glycerol and lactate. This enrichment level was 6–9-fold greater in comparison to that measured in animals given the same substrate combination but with the label originating in either [U-^13^C_3]glycerol or [U-^13^C_3]lactate (Fig. 3A). Fig. 1 shows typical ^13^C NMR spectra of MAG derived from plasma glucose and muscle glycogen and muscle lactate of a fasted rat given [U-^13^C_3]lactate plus glucose and glycerol. It is easy to appreciate that the doublets (^13^C_2-^13^C_3) are dominant in C2 and C5 regions of plasma glucose, whereas the quartet (^13^C_2-^13^C_4-^13^C_5) is dominant in C2 of muscle lactate. The labeling pattern in glycogen is intermediate between plasma glucose and muscle lactate as it was derived from both. The excess ^13^C in glycogen from rats given [U-^13^C_3]lactate plus glucose and glycerol was mainly from ^13^C-labeled lactate, 56% in fed rats and 62% in fasted animals. The contribution from ^13^C-labeled plasma glucose (derived from hepatic gluconeogenesis) was 44% in fed animals and 38% in fasted animals (Fig. 3B). Although not typical, one rat showed an identical labeling pattern between muscle lactate and glycogen that was different from plasma glucose (Fig. 4).

Conservation of the [1,2,3-^13^C_3] backbone in glycogen from lactate indicates either PEP was formed directly from [1,2,3-^13^C_3]pyruvate via reversal of PK or carboxylation of [1,2,3-^13^C_3]pyruvate was followed without scrambling in the citric acid cycle by immediate decarboxylation to form [1,2,3-^13^C_3]PEP. The latter pathway, examined further below, seems unlikely. If PEP from pyruvate is formed via oxaloacetate through pyruvate carboxylase and PEPCK, then the ^13^C labeling pattern in [1,2,3-^13^C_3]lactate would not be expected in gly-
Evidence for glyconeogenesis from \([U-^{13}C_3]lactate\) through reversal of PK. \(^{13}C\) NMR spectra of plasma glucose, muscle glycogen, and muscle lactate are from a fed rat given \([U-^{13}C_3]lactate\) plus glucose and glycerol. Quartets \((1^{13}C-1^{13}C-1^{13}C)\) are dominant in muscle lactate and glycogen resonances, whereas doublets \((1^{13}C-1^{13}C)\) are prominent in plasma glucose. This demonstrates that \([1,2,3-^{13}C_3]lactate\) was preserved in glycogen, which was possible via PK reversal. An alternative pathway through oxaloacetate \((pyruvate \rightarrow oxaloacetate \rightarrow PEP)\) cannot keep the intact \(^{13}C\) distribution in 3-units due to extensive \(^{13}C\) scrambling in the citric acid cycle intermediates including oxaloacetate. DHAP, dihydroxyacetone phosphate; GA3P, α-glyceraldehyde 3-phosphate.
cogen due to extensive $^{13}$C scrambling in the citric acid cycle intermediates including oxaloacetate.

**Contributions of Exogenous Substrates to the Glycerol Moiety of Acylglycerols in Skeletal Muscle**—The enrichment in the glycerol moiety of acylglycerols was the same whether it was estimated based on C1 and C3 resonances or C2 resonances. In fed animals, $^{13}$C enrichment in the glycerol moiety was 0.32% in rats given $[U-^{13}$C$_6]$glucose/glycerol/lactate, 0.17% in rats given $[U-^{13}$C$_3]$glycerol/lactate, and 0.10% in rats given glucose/glycerol/[U-^{13}$C$_3$]lactate. Fasted animals showed similar results as fed animals (Fig. 5A). This indicates that over the duration of 3 h, only a small fraction of acylglycerols had turned over. In rats given [U-^{13}$C$_3$]lactate, the $^{13}$C-labeled glycerol moiety originated primarily from $^{13}$C-labeled lactate, 69% in fed animals and 77% in fasted animals. In contrast, the contribution from $^{13}$C-labeled glucose to the glycerol moiety was only 31% in fed animals and 23% in fasted animals (Fig. 5B).

Exogenous glycerol contributed substantially to the glycerol moiety, ~50% of the amount contributed by glucose. This unexpected observation led us to test whether glycerol undergoes phosphorylation in muscle by glycerol kinase. We performed *in situ* hind limb perfusions with [U-^{13}$C$_3$]glycerol (0.2 mM), and the tissues from the hind limbs were harvested. The glycerol moiety of acylglycerols from the hind limbs had 0.14% [U-^{13}$C$_3$]glycerol, and lactate in effluent from the perfusion had 0.34% [U-^{13}$C$_3$]lactate, confirming glycerol phosphorylation by glycerol kinase in muscle (Fig. 6).

**Lack of PEPCK Activity Based on Aspartate Analysis**—In principle, extensive transfer of the [1,2,3-^{13}$C$_3$]moiety from lactate to both glycerol and glycogen could be understood as flux from [1,2,3-^{13}$C$_3$]pyruvate $\rightarrow$ [1,2,3-^{13}$C$_3$]oxaloacetate $\rightarrow$ [1,2,3-^{13}$C$_3$]PEP. However, the $^{13}$C NMR spectrum of muscle aspartate was dominated by doublets due to [1,2-^{13}$C$_2$]- and [3,4-^{13}$C$_2$]-aspartate (Fig. 2D and 7). This suggests that the oxaloacetate pool did not contribute to PEP. If PEPCK were functioning, mostly double-labeled oxaloacetate would be transferred to PEP and consequently to both glycogen and the glycerol moiety of acylglycerols. In such a case, doublets should dominate in glycogen or the glycerol moiety as double-labeled isotopomers were also dominant in plasma glucose. However, as noted above, the $^{13}$C signal in glycogen or the glycerol moiety was dominated by the triple-labeled backbone (i.e. [U-^{13}$C$_3$]glycerol moiety, [1,2,3-^{13}$C$_3$]- and [4,5,6-^{13}$C$_3$]glucosyl units), which can be explained only by reversal of PK.

We also tested whether PEPCK could be detected when $^{13}$C enrichment in oxaloacetate occurs via a pathway that cannot involve PK. When the *in situ* hind limb was supplied with 2 mM [U-^{13}$C$_3$]propionate, $^{13}$C-labeled glutamate and aspartate were
observed indicating entry of \([U-^{13}C_3]\)propionate into the citric acid cycle through succinyl-CoA. However, no excess \(^{13}\)C was observed in glycogen (spectra not shown).

**Low Pyruvate Carboxylase Activity Based on Aspartate and Glutamate Analysis**—\(^{13}\)C NMR spectra of skeletal muscle extracts from fed and fasted animals that had received \([U-^{13}C_6]\)glucose plus glycerol and lactate showed dominant signals characteristic of \([4,5-^{13}C_2]\)glutamate, \([1,2-^{13}C_2]\)aspartate, and \([3,4-^{13}C_2]\)aspartate. The presence of strong \([4,5-^{13}C_2]\)-glutamate reflects entry of pyruvate derived from \([U-^{13}C_6]\)glucose into the citric acid cycle via PDH (Fig. 7). Similarly, the strong signals from \([1,2-^{13}C_2]\)- and \([3,4-^{13}C_2]\)aspartate reflect decarboxylation of \([U-^{13}C_3]\)pyruvate through PDH followed by forward turn through the citric acid cycle. The signals from \([1,2,3-^{13}C_3]\)- and \([2,3,4-^{13}C_3]\)aspartate are much weaker compared with double-labeled isotopomers. If pyruvate carboxylase were significant, triple-labeled isotopomers should dominate oxaloacetate and aspartate pools.

The spectra of muscle tissue extracts isolated from fed animals showed an equivalent labeling pattern in aspartate C3 and glutamate C2 (Figs. 2D and 7A). In contrast, the NMR spectra of these same metabolites isolated from fasted animals showed a slightly higher fraction of \([2,3-^{13}C_2]\)glutamate compared with \([2,3,4-^{13}C_3]\)aspartate (Fig. 7B). Thus the ratio of D23/D12 in glutamate C2 was higher than the corresponding ratio of D23/D12 or D23/D34 in aspartate from fasted animals (Fig. 7C). This could reflect entry of \([U-^{13}C_3]\)pyruvate via pyruvate carboxylase followed by forward flux through the citric acid cycle without exchange with aspartate or fumarate; \([1,2,3-^{13}C_3]\)oxaloacetate → \([2,3,6-^{13}C_3]\)citrate → C6 decarboxylation → \([2,3-^{13}C_2]\)α-ketoglutarate or \([2,3-^{13}C_3]\)glutamate. Thus, a very low pyruvate carboxylase activity was detected in fasted animals, but the activity was not detected in fed animals.

**Discussion**

After the administration of \(^{13}\)C-enriched lactate, newly synthesized carbon skeletons of the glycerol moiety in acylglycerols or the glucosyl units of glycogen in skeletal muscle can originate from plasma glucose (labeled via hepatic gluconeogenesis), oxaloacetate (labeled via lactate metabolism in the citric acid cycle), or directly from pyruvate via reverse flux through PK. In rats presented with \([1,2,3-^{13}C_3]\)lactate, the \(^{13}\)C distribution in glycogen and glycerol as revealed by \(^{13}\)C NMR provided direct evidence for a substantial contribution from the \([1,2,3-^{13}C_3]\)-labeled precursor. However, the \([1,2,3-^{13}C_3]\) labeling in plasma glucose and muscle oxaloacetate (determined from aspartate) was not sufficient to account for the \([1,2,3-^{13}C_3]\) backbones observed in the glycerol moiety of acylglycerols and in glycogen. These observations are most consistent with metabolism of lactate to pyruvate and reverse flux of \([U-^{13}C_3]\)pyruvate through PK. Glucose, glycerol, and lactate all

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**FIGURE 6. Evidence for glycerol kinase activity in skeletal muscle.** *In situ* hind limb perfusion was performed using perfusate containing 0.2 mm \([U-^{13}C_3]\)glycerol. The glycerol moiety of acylglycerols from muscle had 0.14% \([U-^{13}C_3]\)glycerol, and lactate released from hind limbs had 0.34% \([U-^{13}C_3]\)lactate. The excess enrichments in these metabolites were not possible without \([U-^{13}C_3]\)phosphorylation via glycerol kinase (GK) in skeletal muscle. DHAP, dihydroxyacetone phosphate; GA3P, α-glyceraldehyde 3-phosphate; G3P, glyceral 3-phosphate.
contributed to glycogen and the glyceral production in muscle, and reverse flux of pyruvate to PEP was found to be the common route for both glyconeogenesis and glyceroneogenesis. Nevertheless, the major source of glycogen and the glycerol moiety in muscle originated from plasma glucose. Glycerol kinase was active in muscle and substantially important in fatty acid esterification by providing the backbone of acylglycerols. A low level of pyruvate carboxylase activity was detected in fasted but not fed rats, but we found no evidence for production of PEP from oxaloacetate via PEPCK.
Glyceroneogenesis and Glyconeogenesis in Muscle

Although the reaction is thermodynamically unfavorable under standard conditions, the reversal of PK has been demonstrated based on in vitro studies (4–7). We demonstrated it as a part of glyconeogenesis in the skeletal muscle of animals given a high dose (2 g/kg) of lactate (8). The current study tested the hypothesis that a significant fraction of trioses feeding both glycerol and glycogen were derived via reversal of PK in the presence of high plasma glucose. These results are more physiologically relevant because a smaller dose (0.5 g/kg) of lactate was used in the current study compared with the prior study. In animals given [U-13C3]lactate plus glucose and glyceral, 13C labeling in glycogen or the glycerol moiety of acylglycerols originated mainly from lactate through reversal of PK. 13C-Labeled glucose contribution was minor in these animals.

The concentration of oxaloacetate was too low to be detected by NMR, but aspartate was monitored. Oxaloacetate was found to be in rapid exchange with both aspartate and fumarate as evidenced by aspartate resonances. The labeling pattern of aspartate was consistent with minimal pyruvate carboxylation but active flux through PDH. Previously, we found no evidence for pyruvate carboxylase activity in muscle tissue incubated with [U-13C5]lactate based on an absence of the anticipated 13C-labeling in carbons 1–3 of glutamate produced through the pathway. In the current study the 13C isotopomers detected in carbons 1–3 of glutamate were fully accounted for by the entry of [U-13C5]pyruvate into the citric acid cycle via PDH followed by the forward flux. The labeling pattern in aspartate verified the conclusion. The equivalent labeling pattern between aspartate C2-C4 and glutamate C1-C3 was observed in fed animals. However, the slightly increased fraction of [2,3-13C2]glutamate compared with corresponding [2,3-13C2]-aspartate in fasted animals could be explained by a small pyruvate carboxylase activity assuming no oxaloacetate exchange with aspartate. In this case, pyruvate carboxylase was functioning, but it is considered a very minor based on small increment in the signal from [2,3-13C2]glutamate (Fig. 7, B and C).

Previous reports of pyruvate carboxylase activity in muscle are inconsistent. It was reported as anaplerosis into the citric acid cycle in a vitro setting, and it was stimulated by exercise (19, 26). Another study reported that the activities of pyruvate carboxylase and PEPCK of rat skeletal muscle were only residual based on enzyme assays (27). In the current study an analysis of aspartate isotopomers was consistent with the lack of PEPCK activity in muscle of whole animals. The absence was confirmed using in situ hind limb perfusion with [U-13C2]-propionate. In this setting, no excess 13C was observed in glycerogen even in the presence of 13C labeling in glutamate and aspartate (spectra not shown). If PEPCK were active, excess 13C should be observed in the glycerogen through glyconeogenesis from the citric acid cycle intermediates, which requires PEPCK activity.

Surprisingly, glucose was not the single dominant contributor to the glycerol moiety of acylglycerols. The contribution of free glycerol was approximately half of the contribution of glucose to acylglycerols in both fed and fasted animals. This is substantial as the dose of glycerol (0.5 g/kg) was only 1/4 of glucose (2 g/kg). The function of glycerol kinase was confirmed by in situ hind limb perfusion in the current study. Conventionally, the role of glycerol kinase in muscle triacylglycerol synthesis has been questioned as (i) expression of glycerol kinase in muscle was much lower compared with liver tissue, and (ii) glycosylation was considered to be the source of the glycerol backbone of triacylglycerols. In some cases it was suggested to be less important in fatty acid esterification compared with glyconeogenesis (13), but the current study demonstrated that the contribution through glycerol kinase exceeded glyconeogenesis. This observation is consistent with a few previous reports emphasizing the importance of glycerol kinase in the synthesis of triacylglycerols in muscle (20, 28).

In summary, the current study tested the hypothesis that direct conversion of pyruvate to PEP via PK plays a role in synthesis of both glycerol and glycogen in muscle and that this is a common route for both metabolic products derived from lactate. In the course of this study we also found that glycerol kinase is active in skeletal muscle and that both pyruvate carboxylate and PEPCK are minimally active. Studies of carbohydrate biosynthesis in skeletal muscle metabolism in vivo are difficult because, unlike the liver, muscle does not readily export these products systemically. Nevertheless, detailed understanding of intermediary metabolism is important because muscle plays a pivotal role in energy expenditure, systemic glucose homeostasis, and peripheral insulin resistance.

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