Metabolism of Glycerol, Glucose, and Lactate in the Citric Acid Cycle Prior to Incorporation into Hepatic Acylglycerols*

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Background: The contribution of glyceroneogenesis to hepatic acylglycerol synthesis is controversial.

Results: Exogenous glucose and glycerol contribute to the glycerol backbone of acylglycerols through both direct and indirect pathways.

Conclusion: The citric acid cycle plays a major role in acylglycerol synthesis.

Significance: A method is presented that measures the direct and indirect contributions to the glycerol backbone by 13C NMR.

During hepatic lipogenesis, the glycerol backbone of acylglycerols originates from one of three sources: glucose, glycerol, or substrates passing through the citric acid cycle via glyceroneogenesis. The relative contribution of each substrate source to glycerol in rat liver acylglycerols was determined using 13C-enriched substrates and NMR. Animals received a fixed mixture of glucose, glycerol, and lactate; one group received [U-13C6]glucose, another received [U-13C3]glycerol, and the third received [U-13C3]lactate. After 3 h, the livers were harvested to extract fats, and the glycerol moiety from hydrolyzed acylglycerols was analyzed by 13C NMR. In either fed or fasted animals, glucose and glycerol provided the majority of the glycerol backbone carbons, whereas the contribution of lactate was small. In fed animals, glucose contributed >50% of the total newly synthesized glycerol backbone, and 35% of this contribution occurred after glucose had passed through the citric acid cycle. By comparison, the glycerol contribution was ~40%, and of this, 17% of the exogenous glycerol passed first through the cycle. In fasted animals, exogenous glycerol became the major contributor to acylglycerols. The contribution from exogenous lactate did increase in fasted animals, but its overall contribution remained small. The contributions of glucose and glycerol that had passed through the citric acid cycle first increased in fasted animals from 35 to 71% for glucose and from 17 to 24% for glycerol. Thus, a substantial fraction from both substrate sources passed through the cycle prior to incorporation into the glycerol moiety of acylglycerols in the liver.

It is well established that the glycerol moiety of triglycerides and other acylglycerols in adipose tissue can be derived directly from glucose (1, 2). The role of the citric acid cycle in conversion of pyruvate or equivalent molecules to glycerol for production of acylglycerols was demonstrated in studies of adipose tissue more than 40 years ago (3, 4); this process is termed glyceroneogenesis. In the past decade, attention has turned to the sources of the glycerol moiety of acylglycerols in liver (5, 6). Unlike adipose tissue, liver has the capacity to phosphorylate free glycerol via glycerol kinase to yield glycerol 3-phosphate (G3P),2 which then becomes esterified with fatty acids. Thus, it is now generally accepted that the carbons in the glycerol backbone of acylglycerols synthesized in the liver are derived from three potential sources: glucose via glycolysis to the level of triose phosphates, glycerol via glycerol kinase, or glyceroneogenesis from pyruvate and intermediates of the citric acid cycle (see Fig. 1A).

Recently, studies with labeled water (2H2O or 3H2O) have been adapted for assessment of the relative contributions of the various sources to the glycerol backbone. In the presence of labeled water, the number of hydrogen atoms (2H or 3H) incorporated into glycerol will differ depending on the source of the glycerol moiety (5, 6). Several studies using the water tracer method found that glyceroneogenesis contributed significantly to the glycerol moiety in liver triglycerides (7–9). Glyceroneogenesis was defined as the synthesis of G3P from precursors other than glycerol or glucose, including pyruvate, lactate, alanine, and intermediates of the citric acid cycle (9). According to this definition, conversion of glucose to pyruvate followed by carboxylation to oxaloacetate and subsequent decarboxylation to phosphoenolpyruvate and metabolism back to the glycerol moiety would not be considered glyceroneogenesis. This pathway, illustrated in Fig. 1B, would be considered an indirect pathway from conversion of glucose carbons to the glycerol backbone after passing through the citric acid cycle. The total contribution of glucose to hepatic triglyceride-glycerol, defined as the sum of direct and indirect pathways, has been reported to be modest, ~11–28% of total triglyceride-glycerol depending upon the nutritional state. In contrast to the total contribution from glucose, the contribution from glyceroneogenesis was reported to be much larger (~60%) and to be independent of nutritional state (9). Glyceroneogenesis was also reported to be

2 The abbreviation used is: G3P, glycerol 3-phosphate.
increased in hepatic lipogenesis in the setting of type 2 diabetes as determined using the labeled water tracer method (10).

These observations are not consistent with the conventional concept that glycolysis to the level of trioses is the major source for G3P needed for fatty acid esterification. The conclusion that glyceroneogenesis provided most of the glycerol backbone in hepatic acylglycerol production was based on a technique using 14C-labeled glucose in combination with tritiated water (3H2O) to quantify the contribution of glucose to the glycerol moiety via the citric acid cycle. This indirect contribution of [U-14C6]glucose via lactate was estimated based on the appearance of triglyceride-[2,3-14C2]glycerol. However, this labeling pattern is not the only isotopomer produced by [U-14C6]glucose via lactate/pyruvate; triglyceride-[1,2-14C2]glycerol plus triglyceride-[U-14C6]glycerol may also be generated during passage through the citric acid cycle. Additional triglyceride-[1,2-14C2]glycerol formation may be possible because [2,3-14C2]G3P generated from the citric acid cycle is in the equilibrium of glycerol, a symmetric molecule, producing [1,2-14C2]G3P. This approach is important because it recognizes that labeled water tracers cannot distinguish glyceroneogenesis from glucose metabolism to the glycerol backbone via the citric acid cycle. However, this method may underestimate the contribution of glucose to the glycerol backbone through the indirect pathway because not all possible glycerol isotopomers are considered.

In addition to glucose, glycerol via glycerol kinase has been believed to be a significant source of the glycerol backbone of acylglycerols in the liver. Nonetheless, the contribution of free glycerol to the glycerol backbone is often not measured or is reported as a minor contribution by use of the water tracer method (8, 9). Like glucose, free glycerol could conceivably contribute to the glycerol backbone via cycling through the citric acid cycle. Free glycerol is in equilibrium with triose phosphates and can be metabolized to pyruvate, oxaloacetate, phosphoenolpyruvate, and gluconeogenesis. However, the contribution of free glycerol to the glycerol backbone after metabolism in the citric acid cycle (see Fig. 1C) has not been considered previously.

Because fatty liver, defined as overproduction and storage of hepatic triglycerides, is a major and growing clinical problem (11), it is important to understand the contribution of each nutritional source of carbon to the glycerol backbone. Furthermore, it is important to develop a simple method using stable isotopes to quantify these pathways because studies with 3H and 14C are not acceptable for patients. Deuterated water (2H2O) can be given to humans (8, 10), but as noted, glyceroneogenesis may be overestimated, and the glucose contribution may be underestimated using this technique. Here, we explored an alternative approach to examine the sources of glycerol in hepatic acylglycerols of whole animals using one of three 13C-enriched substrates: [U-13C6]glucose, [U-13C3]glycerol, or [U-13C3]lactate. 13C NMR analysis of glycerol hydrolyzed from liver fats enabled us to measure the independent contribution of each substrate to the glycerol backbone and also distinguishes between the direct versus indirect pathway contributions of glucose or glycerol to the glycerol moiety in the livers of whole animals. This study demonstrates that glucose and glycerol are indeed the main sources of the glycerol backbone but that a significant portion of this contribution occurs after metabolism of glucose or glycerol to the level of pyruvate, followed by carboxylation to oxaloacetate and subsequent synthesis to the glycerol backbone. Because the indirect pathway is detected as glyceroneogenesis by labeled water methods, the results from these earlier studies should be interpreted considering the possibility of cycling involved in glucose and glycerol contributions.

EXPERIMENTAL PROCEDURES

Materials—[U-13C6]Glycerol (99%), [U-13C3]lactate (98%), and [U-13C3]glucose (99%) were obtained from Cambridge Isotopes (Andover, MA). Dowex 50WX8–200 (a cation-exchange resin) and other common chemicals were purchased from Sigma-Aldrich.

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 studied in two different nutritional states. One group had free access to food and water. The other group was fasted for 24 h with free access to water. All animals received an intraperitoneal injection of a mixture of glucose (2 g/kg of body weight), glycerol (0.5 g/kg of body weight), and lactate (0.5 g/kg of body weight) under isoflurane anesthesia. Only one substrate was enriched in 13C in any given experiment, but all three substrates were present in each experiment. After the injection, rats were placed back into their cage, where they quickly awakened and were allowed free access to water. After 3 h, blood, liver, and skeletal muscle tissues were harvested under sodium pentobarbital anesthesia, and they were further processed for NMR analysis.

Sample Processing for NMR Analysis—Liver tissue (7–8 g) ground to a powder under liquid nitrogen was transferred into a beaker containing CHCl3/methanol (2:1, 40 ml). The mixture was stirred for 1 h and filtered using a Whatman filter paper. Deionized water (5 ml) was added, and the mixture was swirled manually for 1 min. The swirled mixture was allowed to settle at room temperature for organic-aqueous layer separation and further centrifuged at a low rpm for clear separation. The upper aqueous layer was aspirated, and the remaining organic layer was dried under a vacuum using a liquid nitrogen trap. The dried residue was dissolved in 4 ml of 1N KOH and centrifuged, and the supernatant was dried. The perchloric acid to extract water-soluble components, neutralized with KOH, and centrifuged, and the supernatant was dried. The dried residue was dissolved in 2H2O (160 μl) for 13C NMR acquisition.

Blood, liver, and skeletal muscle tissues were treated with perchloric acid to extract water-soluble components, neutralized with KOH, and 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.
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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 $^{13}$C (150 MHz). $^{13}$C NMR spectra were collected using a 60° pulse, a 36,765-Hz sweep width, 110,294 data points, and a 1.5-s acquisition time with 1.5-s interpulse delay at 25 °C. Proton decoupling was performed using a standard WALTZ-16 pulse sequence. Spectra were averaged over ~3000–7000 scans requiring ~3–6 h. All NMR spectra were analyzed using the ACD/Labs PC-based NMR spectral analysis program (Advanced Chemistry Development, Inc., Toronto, Canada).

Statistical Analysis—Data are expressed as means ± S.E. Comparisons between groups were performed using Student’s t test. A p value of <0.05 was considered significant.

RESULTS

Contributions of Exogenous Glucose and Glycerol to the Glycerol Moiety of Acylglycerols via the Citric Acid Cycle—When a liver exposed to [U-$^{13}$C$_6$]glucose or [U-$^{13}$C$_3$]glycerol is producing acylglycerols, the appearance of an intact three-carbon [U-$^{13}$C$_3$]glycerol backbone in the acylglycerol pool would reflect “direct” formation of G3P from one of these precursors. In contrast, if [U-$^{13}$C$_6$]glucose or [U-$^{13}$C$_3$]glycerol is first metabolized to pyruvate, oxaloacetate, and the citric acid cycle before forming G3P (Fig. 1, A and C), doubly enriched ([1,2-$^{13}$C$_2$]glycerol and [2,3-$^{13}$C$_2$]glycerol) and uniformly enriched ([U-$^{13}$C$_3$]glycerol) isotopomers would then appear in the acylglycerol pool (Fig. 2). Hence, the appearance of [U-$^{13}$C$_3$]glycerol in the acylglycerol pool does not necessarily reflect the direct pathway from [U-$^{13}$C$_6$]glucose or [U-$^{13}$C$_3$]glycerol. To estimate the contribution of carbon coming solely from the citric acid cycle, [U-$^{13}$C$_3$]lactate was included as a third tracer. In this case, any contribution from [U-$^{13}$C$_3$]lactate to the glycerol backbone must reflect conversion to [U-$^{13}$C$_3$]pyruvate, entry into the citric acid cycle, and exit from the cycle through phosphoenolpyruvate carboxykinase to phosphoenolpyruvate and consequently G3P. [U-$^{13}$C$_3$]Pyruvate can enter the citric acid cycle via the pyruvate carboxylase pathway or the pyruvate dehydrogenase pathway (Fig. 2). [U-$^{13}$C$_3$]Pyruvate carboxylation to oxaloacetate produces [1,2,3-$^{13}$C$_3$]oxaloacetate, which fully equilibrates with a symmetric fumarate pool, producing [2,3,4-$^{13}$C$_3$]oxaloacetate. The conversion of [1,2,3-$^{13}$C$_3$]oxaloacetate and [2,3,4-$^{13}$C$_3$]oxaloacetate to phosphoenolpyruvate would result in [1,2-$^{13}$C$_2$]glycerol, [2,3-$^{13}$C$_2$]glycerol, and [U-$^{13}$C$_3$]glycerol in the acylglycerol pool (Fig. 2A). When the oxaloacetate isotopomers after equilibrium with fumarate undergo “forward” metabolism in the citric acid cycle, doubly labeled oxaloacetate ([1,2-$^{13}$C$_2$]oxaloacetate and [3,4-$^{13}$C$_2$]oxaloacetate) isotopomers are produced after one complete turn of the cycle. The doubly enriched oxaloacetate isotopomers would produce [1,2-$^{13}$C$_2$]glycerol, [2,3-$^{13}$C$_2$]glycerol, [1-$^{13}$C$_1$]glycerol, and [3-$^{13}$C$_1$]glycerol in the acylglycerol pool (Fig. 2A). Alternatively, entry of [U-$^{13}$C$_3$]pyruvate into the citric acid cycle through the pyruvate dehydrogenase pathway produces [4,5-$^{13}$C$_2$]citrate after condensation with unlabeled oxaloacetate (Fig. 2B). The forward turn of the citrate isotopomer through the cycle produces the same doubly labeled oxaloacetate isotopomers ([1,2-$^{13}$C$_2$]oxaloacetate and [3,4-$^{13}$C$_2$]oxaloacetate), resulting in [1,2-$^{13}$C$_2$]glycerol, [2,3-$^{13}$C$_2$]glycerol, [1-$^{13}$C$_1$]glycerol, and [3-$^{13}$C$_1$]glycerol in the acylglycerol pool.

The fate of [U-$^{13}$C$_3$]pyruvate through pyruvate carboxylase versus pyruvate dehydrogenase (i.e. acetyl-CoA) was confirmed by inspecting the labeling patterns of the citric acid cycle intermediates and exchanging pools. Fig. 3 shows the $^{13}$C NMR spectra of liver extracts from three groups of rats given a mixture of glucose, glycerol, and lactate (only one enriched in $^{13}$C). The resonances of succinate, glutamate, and glutamine are shown. Succinate is an intermediate of the citric acid cycle, whereas glutamate and glutamine are in exchange with $\alpha$-ketoglutarate, another intermediate of the citric acid cycle. Each metabolite contained a higher enrichment of $^{13}$C above natural abundance levels (as indicated by the singlet component in each resonance). This verifies that all three labeled substrates, [U-$^{13}$C$_6$]glucose, [U-$^{13}$C$_3$]glycerol, and [U-$^{13}$C$_3$]lactate, entered the citric acid cycle prior to formation of the glycerol backbone of acylglycerols. The appearance of triply labeled succinate ([1,2,3-$^{13}$C$_3$]succinate and [2,3,4-$^{13}$C$_3$]succinate), [2,3-$^{13}$C$_2$]glutamate,
or [2,3-13C2]glutamine in all spectra provides direct evidence for entry of [U-13C3]pyruvate largely through pyruvate carboxylase (PC) results in [1,2,3-13C3]oxaloacetate (OAA), which equilibrates in the symmetric fumarate (Fum) pool, producing both [1,2,3,4,5-13C5]oxaloacetate and [2,3,4,5-13C5]oxaloacetate. These two isotopomers can be converted to the [U-13C3]glycerol, [1,2-13C2]glycerol, and [2,3-13C2]glycerol moieties of acylglycerols in liver through the glyceroenic process. When the oxaloacetate isotopomers pass through citrate synthase, doubly labeled oxaloacetate isotopomers (i.e. [1,2,-13C2]oxaloacetate and [3,4-13C2]oxaloacetate) are formed and subsequently produce [1,2-13C2]glycerol, [2,3-13C2]glycerol, [1,1-13C1]glycerol, and [3,3-13C1]glycerol moieties. Metabolism of labeled pyruvate through the alternative pathway is shown in B. The entry of [U-13C3]pyruvate through pyruvate dehydrogenase (PDH) produces [4,5-13C2]citrate after condensation with oxaloacetate, resulting in [1,2,3-13C3]oxaloacetate and [3,4-13C2]oxaloacetate and consequently [1,2-13C2]glycerol, [2,3-13C2]glycerol, [1,1-13C1]glycerol, and [3,3-13C1]glycerol moieties. The appearance of [1,2,3-13C3]sucinate and [2,3,4-13C4]succinate (quartets in the C2 and C3 resonance in Fig. 3) and [2,3,4,5-13C5]glutamate (or glutamine; doublets in the C3 resonance in Fig. 3) are evidence of [U-13C3]pyruvate entry through the pyruvate carboxylase pathway. In contrast, the appearance of [4,5-13C2]glutamate (doublet in the C4 resonance in Fig. 3) is evidence of [U-13C3]pyruvate entry through the pyruvate dehydrogenase pathway. The metabolites underlined (i.e. the glycerol moiety of acylglycerols, succinate (Suc), glutamate and glutamine) were detected using 13C NMR. ○, 12C; ●, 13C. PEP, phosphoenolpyruvate; CAC, citric acid cycle; α-KG, α-ketoglutarate.
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As an example, the C2 resonance of glycerol isolated from the liver acylglycerols of a fed rat given [U-13C₆]glucose/glycerol/lactate showed five resonance components: a singlet (S), a doublet (D), and a triplet (T) (Fig. 5A). The singlet was assumed to

(independent of which substrate is enriched with 13C), then, the ratio can be used to evaluate the total carbon contribution to the glycerol moiety of acylglycerols coming from the citric acid cycle (the “indirect” pathway) in all other experiments.

FIGURE 3. 13C NMR spectra of tissue extracts from the livers of fed (left) and fasted (right) rats. The animals in A were given a mixture of [U-13C₆]glucose/glycerol/lactate, the animals in B were given glucose/[U-13C₃]glycerol/lactate, and the animals in C were given glucose/glycerol/[U-13C₃]lactate. Succinate (Suc) is an intermediate of the citric acid cycle, whereas glutamate and glutamine are in exchange with α-ketoglutarate, another intermediate of the cycle. The extensive enrichments in the intermediates of the citric acid cycle and exchange pools demonstrated the metabolism of [U-13C₃]glycerol, [U-13C₆]glucose, or [U-13C₃]lactate in the citric acid cycle prior to incorporation into hepatic acylglycerols. White circles, 12C; black circles, 13C; gray circles, 12C or 13C. D, doublet; S, singlet; Q, quartet.
arise only from the natural abundance endogenous glycerol backbone, so it was not included in further calculations. In this particular spectrum, $D/(D/H_{11001}T)$ was 22%, whereas $T/(D/H_{11001}T)$ was 78%. The doublet component reflects the sum of $[1,2-^{13}C_2]$glycerol and $[2,3-^{13}C_2]$glycerol isotopomers and hence could arise only from the indirect pathway of $[U-^{13}C_6]$glucose via the citric acid cycle. The triplet component reflects only $[U-^{13}C_3]$glycerol, but this isotopomer could arise from either the direct or indirect pathway. As noted above, because fed animals given glucose/glycerol/$[U-^{13}C_3]$lactate showed a constant $T/D$ ratio (36/64 = 0.56) in the glycerol C2 resonance, the fraction of the triplet resulting from the indirect pathway in the $[U-^{13}C_6]$glucose experiment was then estimated at 12% (22% × 0.56). Consequently, the triplet portion from the direct pathway is $78 - 12\% = 66\%$, whereas the indirect contribution of $[U-^{13}C_6]$glucose is 34%. The contributions of all other substrates to total glycerol production were determined similarly and normalized to 100%.

**Contributions of Exogenous Substrates to Liver Acylglycerols in Fed Animals**—The $^{13}$C NMR spectra of extracts of the aqueous layer obtained after hydrolysis of liver fats show well resolved resonances from glycerol (Fig. 4). The $^{13}$C enrichment
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FIGURE 5. Effect of fasting on direct and indirect contributions of glucose and free glycerol to the glycerol moiety of acylglycerols in liver. A, for fed animals, rats given [U-13C6]glucose/glycerol/lactate had 35 ± 4% (n = 6) indirect contribution of glucose through the citric acid cycle, and rats given glucose/[U-13C6]glycerol/lactate had 17 ± 1% (n = 8) indirect contribution. B, for fasted animals, rats given [U-13C6]glucose/glycerol/lactate had 71 ± 4% (n = 5) indirect contribution, and rats given glucose/[U-13C6]glycerol/lactate had 24 ± 1% (n = 5) indirect contribution. The calculation was based on 13C NMR analysis of the C2 resonance of the glycerol moiety. The presence of doubly labeled glycerol of acylglycerols is evidence of carbon cycling through the citric acid cycle (glycolysis → pyruvate → the citric acid cycle → the gluconeogenic pathway → G3P → the glycerol moiety). Singlets (S) represents [2,13C]glycerol, doublets (D) represent [1,2-13C2]glycerol and [2,3-13C2]glycerol, and triplets (T) represent [U-13C3]glycerol hydrolyzed from acylglycerols.

in the glycerol moiety of acylglycerols was estimated using two approaches by analysis of the multiplet areas of the C1 and C3 resonance, which do not distinguish between the direct versus indirect pathway, the C2 resonance is more informative (Fig. 5). Here, the triplet component largely reflects direct formation of acylglycerol-[U-13C3]glycerol from either [U-13C6]glucose or [U-13C6]glycerol, whereas the doublet component can reflect only the indirect formation of the glycerol moiety after passage of the labeled substrate through the citric acid cycle. The results reported from the glucose/glycerol/[U-13C3]lactate experiment in fed animals (Fig. 4) showed 64% doublet and 36% triplet in the glycerol C2 resonance. This demonstrates that a small amount of the triplet component also arose from metabolism in the citric acid cycle. This amount was considered in the calculation of the direct versus indirect contribution of [U-13C6]glucose or [U-13C3]glycerol as described above. Given this correction, 35 ± 4% of the [U-13C6]glycerol carbons passed through the citric acid cycle prior to formation of the glycerol moiety.

Contrasts of Exogenous Substrates to Liver Acylglycerols in Fasted Animals—In fasted animals, enrichments in glycerol from hepatic acylglycerols based on C1 and C3 resonance analysis were 1.63 ± 0.21% in rats given [U-13C6]glucose/glycerol/lactate, 3.19 ± 0.50% in rats given glucose/[U-13C6]glycerol/lactate, and 0.84 ± 0.08% in rats given glucose/glycerol/[U-13C6]lactate (Fig. 4B, left bar). The enrichment from [U-13C6]glycerol was higher compared with the contribution from either [U-13C6]glucose or [U-13C6]lactate. A similar trend was found in glycerol C2 resonance analysis, except again...
higher enrichments compared with the data based on the C1 and C3 resonance analysis: 1.63 → 2.62% in rats given [U-13C6]glucose/glycerol/lactate, 3.19 → 3.81% in rats given glucose/[U-13C6]glycerol/lactate, and 0.84 → 1.31% in rats given glucose/glycerol/[U-13C6]lactate (Fig. 4B, right bar).

As noted above, the percentages of doubly labeled and uniformly labeled acylglycerols from fasted rats provided glucose/glycerol/[U-13C6]lactate were 64 and 36%, respectively. Given the correction using this ratio, the multiplet data of the glycerol C2 resonances derived from fasted animals show that 71 ± 4% of all glucose carbons contributing to the glycerol moiety first passed through the citric acid cycle, whereas 24 ± 1% of all labeled glycerol contributing to the glycerol moiety first passed through the cycle (Fig. 5B).

**DISCUSSION**

In either fed or fasted animals given a mixture of glucose, glycerol, and lactate, the majority of glycerol in hepatic acylglycerols was derived from glucose and free glycerol. Significant portions of glucose and glycerol contributions occurred after entry into the citric acid cycle, and this fraction was sensitive to the nutritional state. Fasting caused a 2-fold increase in the fraction of acylglycerols derived from glucose via the indirect pathway compared with the fed state. The contribution of lactate to the glycerol moiety was trivial in fed animals, and although it increased somewhat in fasted rats, lactate remained a minor contributor to the glycerol moiety in liver.

Previous studies using the water tracer method to determine the sources of triglyceride-glycerol in liver have noted the possibility of overestimation of glyceroenogenesis as a consequence of metabolism of glucose to pyruvate, followed by synthesis to the glycerol moiety (6). Therefore, Nye et al. (9) complemented the use of [3H2O] with the addition of [U-13C6]glucose to allow correction for the contribution of glucose arising through the citric acid cycle. Triglyceride-glycerol labeled at C2 and C3 was considered in the calculation of cycled glucose. However, other labeling patterns in the glycerol moiety could also arise with passage of glucose carbons through the oxaloacetate pool. [U-14C4]Pyruvate in liver from glycolysis of [U-14C4]glucose also results in triglyceride-[U-14C4]glycerol and triglyceride-[1,2-14C3]glycerol through the metabolic network involved in the citric acid cycle. Furthermore, [2,3-14C2]G3P formed from [U-14C3]pyruvate is in exchange with glycerol, a symmetric molecule, which consequently can become [1,2-14C2]G3P and eventually triglyceride-[1,2-14C2]glycerol. The relative amounts of these isotopomers will be sensitive not only to the fraction of glucose carbons entering the cycle via pyruvate carboxylase but also the extent of “backward” scrambling into the symmetric four-carbon intermediates. In this study, we used [U-13C6]lactate to correct for these pathways, which allowed us to measure the fraction of glucose that passed through the citric acid cycle during glycerol moiety formation. With this correction, the total contribution of glucose to the glycerol moiety was found to be the major source among these three exogenous contributors in fed animals and also an important source in fasted animals, whereas the contribution of exogenous lactate was small in both fed and fasted animals.

We have also shown that the contribution of exogenous glycerol to the glycerol moiety of acylglycerols was important in liver and that glucose was not the only substrate that passed through the cycle. The free glycerol contribution was similar to glucose in fed animals but was almost 2-fold greater than glucose in fasted animals. The contribution of glyceroenogenesis measured by the [U-13C6]lactate tracer increased in fasted animals, but still, it was the smallest contribution among the sources. Although the indirect contribution of free glycerol was less than that of glucose, it was comparable with the [U-13C6]lactate contribution under fed (1.87 × 0.17 = 0.32% versus 0.23%) and fasted (3.81 × 0.24 = 0.91% versus 1.31%) conditions. The extensive 13C labeling in the citric acid cycle intermediates or molecules in exchange with the intermediates confirmed the involvement of the citric acid cycle in the indirect contribution of glucose or free glycerol to the glycerol moiety.

The indirect contribution observed in this study occurred presumably within the liver itself. However, one cannot exclude the possibility of peripheral metabolism of either glucose or glycerol to lactate, followed by glyceroenogenesis in liver. In the case of rats given glucose/[U-13C6]glycerol/lactate, the 14C enrichment found in blood glucose was only 3% in fed animals and 11% in fasted animals by measured summed enrichments of multiple-labeled glucose isotopomers, including [1,2-13C2]glucose, [2,3-13C2]glucose, [1,2,3-13C3]glucose, [4,5,13C3]glucose, [5,6,13C3]glucose, [4,5,6,13C4]glucose, and [U-13C6]glucose. In rats given [U-13C6]glucose/glycerol/lactate, the 13C enrichment found in blood glucose was 40% in fed animals and 60% in fasted animals. Thus, we further examined the possibility of peripheral lactate contribution to the glycerol moiety of acylglycerols in the livers of fasted rats given [U-13C6]glucose/glycerol/lactate, which had the highest 13C enrichments in blood glucose. Fig. 6 shows C2 resonances of lactate from liver, circulating blood, and skeletal muscle of a fasted rat given a mixture of [U-13C6]glucose, glycerol, and lactate along with the C2 resonance of the glycerol moiety of acylglycerols in liver. The fractions of doublets (produced after cycling) in the glycerol moiety and lactate in liver were much higher than those in either blood or skeletal muscle, indicating that the observed doubly labeled molecules in liver were produced primarily through metabolism in the liver itself. Previ-ously, we observed that skeletal muscle did not produce doubly labeled three-carbon units from [U-13C6]lactate (12). Although [U-13C6]lactate in liver could be derived from either glycolysis in liver or peripheral metabolism, the combination of isotopomers found in glycerol isolated from liver acylglycerols was most consistent with involvement of the citric acid cycle in liver.

13C enrichment in the glycerol moiety of acylglycerols was measured by assuming that the singlet of glycerol C1 and C3 or the singlet of C2 arose from natural abundance 13C. Administration of exogenous 13C-labeled substrates could produce excess singlet, which would cause underestimation of the actual enrichments. In this study, the singlet of glycerol C1 and C3 could arise from exogenous 13C-labeled substrates, whereas the singlet of C2 was essentially only natural abundance 13C in the glycerol moiety of acylglycerols (Fig. 2). This explains why
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FIGURE 6. $^{13}$C NMR spectra of the glycerol C2 resonance of acylglycerols from liver (A) and lactate C2 resonances from liver (B), blood (C), and skeletal muscle (D) of a fasted rat given [U-$^{13}$C$_6$]glucose/glycerol/lactate. Doubly labeled isotopomers of the glycerol moiety and lactate show that $^{13}$C from [U-$^{13}$C$_3$]glucose was metabolized through the citric acid cycle. The doublets (D23 and D12) in lactate are more significant in liver compared with other organs, which suggests that the doubly labeled glycerol moiety of acylglycerols in liver most likely originated from the liver itself rather than from the periphery. $S$, singlet; $D$, doublet; $T$, triplet; $Q$, quartet.

the enrichment based on the C1 and C3 resonance analysis was consistently lower compared with the enrichment based on the C2 resonance analysis (Fig. 4). Nonetheless, compared with the C2 resonance of the glycerol moiety, the simpler multiplet pattern in the C1 and C3 resonance makes it easy to appreciate the degree of enrichments because the singlet reflects mostly natural abundance $^{13}$C, whereas doublets represent signals from all of the multiply labeled glycerol isotopomers (i.e. [1,2-$^{13}$C$_2$]glycerol, [2,3-$^{13}$C$_2$]glycerol, and [U-$^{13}$C$_3$]glycerol), which cannot arise from natural abundance.

In summary, glucose and glycerol are major contributors to the glycerol moiety of acylglycerols in the livers of both fed rats and fasted rats given a mixture of exogenous glucose, glycerol, and lactate. However, significant fractions of both glucose and glycerol contributions occurred by synthesis of the glycerol moiety after metabolism in the citric acid cycle. Interestingly, glycerol contributions occurred by synthesis of the glycerol moiety of acylglycerols in the livers of both fed rats and fasted rats given a mixture of exogenous glucose, glycerol, and lactate. However, significant fractions of both glucose and glycerol contributions occurred by synthesis of the glycerol moiety after metabolism in the citric acid cycle. Interestingly, glycerol contributions occurred by synthesis of the glycerol moiety of acylglycerols in liver most likely originated from the liver itself rather than from the periphery.

REFERENCES

1. Bally, P. R., Cahill, G. F., Jr., Leboeuf, B., and Renold, A. E. (1960) Studies on rat adipose tissue in vitro. V. Effects of glucose and insulin on the metabolism of palmitate-$^{14}$C. J. Biol. Chem. 235, 333–336
2. Margolis, S., and Vaughan, M. (1962) $\alpha$-Glycerophosphate synthesis and breakdown in homogenates of adipose tissue. J. Biol. Chem. 237, 44–48
3. Ballard, F. J., Hanson, R. W., and Leveille, G. A. (1967) Phosphoenolpyruvate carboxykinase and the synthesis of glycero-glycerol from pyruvate in adipose tissue. J. Biol. Chem. 242, 2746–2750
4. Reshef, L., Hanson, R. W., and Ballard, F. J. (1969) Glycerol-glycerol synthesis from pyruvate. Adaptive changes in phosphoenolpyruvate carboxykinase and pyruvate carboxylase in adipose tissue and liver. J. Biol. Chem. 244, 1994–2001
5. Turner, S. M., Murphy, E. J., Neese, R. A., Antelo, F., Thomas, T., Agarwal, A., Go, C., and Hellerstein, M. K. (2003) Measurement of TG synthesis and turnover in vivo by $^2$H$_2$O incorporation into the glycerol moiety and application of MIDA. Am. J. Physiol. Endocrinol. Metab. 285, E790–E803
6. Chen, J. L., Peacock, E., Samady, W., Turner, S. M., Neese, R. A., Hellerstein, M. K., and Murphy, E. J. (2005) Physiologic and pharmacologic factors influencing glyceroneogenic contribution to triacylglycerol glycerol measured by mass isotopomer distribution analysis. J. Biol. Chem. 280, 25396–25402
7. Botin, L. M., Brito, M. N., Brito, N. A., Kettelhut, I. C., and Migliorini, R. H. (1998) Glucose contribution to in vivo synthesis of glycero-glycerol and fatty acids in rats adapted to a high-protein, carbohydrate-free diet. Metabolism 47, 1217–1221
8. Kalhan, S. C., Mahajan, S., Burkett, E., Reshef, L., and Hanson, R. W. (2001) Glyceroneogenesis and the source of glycerol for hepatic triacylglycerol synthesis in humans. J. Biol. Chem. 276, 12928–12931
9. Nye, C. K., Hanson, R. W., and Kalhan, S. C. (2008) Glyceroneogenesis is the dominant pathway for triglyceride glycerol synthesis in vivo in the rat. J. Biol. Chem. 283, 27565–27574
10. Kalhan, S. C., Bugianski, E., McCullough, A. J., Hanson, R. W., and Kelley, D. E. (2008) Estimates of hepatic glyceroneogenesis in type 2 diabetes mellitus in humans. Metabolism 57, 305–312
11. Clark, J. M., Brancati, F. L., and Diehl, A. M. (2002) Nonalcoholic fatty liver disease. Gastroenterology 122, 1649–1657
12. Jin, E. S., Sherry, A. D., and Malloy, C. R. (2009) Evidence for reverse flux through pyruvate kinase in skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 296, E748–E757

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