Blood Glycerol Is an Important Precursor for Intramuscular Triacylglycerol Synthesis*

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The utilization of blood glycerol and glucose as precursors for intramuscular triglyceride synthesis was examined in rats using an intravenous infusion of [2-14C]glycerol and [6-3H]glucose or [6-14C]glucose. In 24-h fasted rats, more glycerol than glucose was incorporated into intramuscular triglyceride glycerol in soleus (69 ± 23 versus 4 ± 1 nmol/μmol triglyceride/h, respectively, \( p = 0.02 \) glycerol versus glucose) and in gastrocnemius (25 ± 5 versus 9 ± 2 nmol/μmol triglyceride/h, respectively, \( p = 0.02 \)). Blood glucose was utilized more than blood glycerol for triglyceride glycerol synthesis in quadriceps. In fed rats, the blood glycerol incorporation rates (4 ± 2, 8 ± 3, and 9 ± 3 nmol/μmol triglyceride/h) were similar (\( p > 0.3 \)) to those of glucose (5 ± 2, 8 ± 2, and 5 ± 2 nmol/μmol triglyceride/h for quadriceps, gastrocnemius, and soleus muscle, respectively). Glucose incorporation into intramuscular triglycerides was less with [6-3H]glucose than with [6-14C]glucose, suggesting an indirect pathway for glucose carbon entry into muscle triglyceride. The isotopic equilibrium between plasma and intramuscular free glycerol ([U-13C]glycerol) was complete in quadriceps and gastrocnemius, but not soleus, within 2 h after beginning the tracer infusion. We conclude that blood glycerol is a direct and important precursor for muscle triglyceride synthesis in rats, confirming the presence of functionally important amounts of glycerol kinase in skeletal muscle.

It is a biochemistry precept that the glycerol moiety of triacylglycerols and phospholipids in non-hepatic mammalian tissues is primarily derived from glucose via glycolysis (1). Dihydroxyacetone phosphate (DHAP), originating from glucose, is reduced to glycerol 3-phosphate (G3P) by the action of glycerophosphate dehydrogenase. G3P then undergoes sequential acylation steps to incorporate three fatty acids to form a triacylglycerol (TG) (2). DHAP can also take a different path (DHAP pathway) via 1-acyldihydroxyacetone phosphate and then 1-acylglycerol 3-phosphate, but this appears to be a quantitatively minor reaction (3, 4). Glycerol can be converted directly to G3P by glycerol kinase, but this is believed to occur primarily, if not solely, in the liver and kidney because the glycerol kinase activity in these tissues is sufficient to permit large quantities of blood glycerol to be used for gluconeogenesis and TG synthesis. The direct conversion of free glycerol to G3P is thought to be negligible in skeletal muscle and adipose tissue because of their low activities of glycerol kinase (5–8). A corollary to this supposition is that glycerol generated by the hydrolysis of TG in skeletal muscle quantitatively enters the circulation. These assumptions form the basis for using systemic glycerol appearance rate, measured by isotope dilution techniques, as a quantitative measure of whole body lipolysis (9, 10).

The presence of small but measurable glycerol kinase activity in skeletal muscle of various animal species, including rat (5) and humans (6), raises concerns as to the validity of these assumptions, however. For example, although low in specific activity, skeletal muscle glycerol kinase could be important in the metabolism of circulating glycerol considering the mass of this tissue. Reports of tracer-determined glycerol uptake across human forearm (11, 12) suggest skeletal muscle may utilize glycerol, although it has been argued that this represents isotope disequilibrium rather than true uptake. Whether the glycerol kinase activity that is observed in mammalian skeletal muscle is functionally important has not been addressed, nor has the downstream intracellular fate of the G3P generated from blood glycerol.

These studies were designed to test the hypothesis that skeletal muscle utilizes blood glycerol for intracellular TG glycerol synthesis. We also measured the rate of glycerol incorporation into TG by muscle groups of different fiber types and compared glycerol to blood glucose as a substrate for TG glycerol biosynthesis.

MATERIALS AND METHODS

Animals and Experiments

Experiment 1—Male Harlan Sprague-Dawley rats (body weight 350 g) were fed ad libitum (n = 5) or fasted for 24 h (n = 5) before the studies. The rats were conditioned in a rat restraint cage with wire floor for 30 min prior to study. [2-14C]Glycerol and [6-3H]glucose (DuPont) were infused for 3 h via a tail vein at ~0.04 μCi/min (the exact infusion rate in each animal was determined) using a Terumo infusion set with a 25-gauge × 3/4-inch needle (Terumo Medical Corp., Elkhart, IN). After 3 h of the tracer infusions, pentobarbital (30 mg/kg) was injected through the infusion line to lightly anesthetize the animal. Arterial blood samples were collected by cardiopuncture, and the plasma was separated by centrifugation at 3000 rpm for 15 min. Immediately after the blood was taken, more pentobarbital was injected (200 mg/kg) to euthanize the rats. Quadriceps, lateral gastrocnemius, and soleus muscles were quickly removed and washed of blood elements in 0.9% saline. The muscle samples were placed in 2-ml polyethylene microcentrifuge tubes, immediately merged in liquid N₂ and stored at ~80 °C for later analysis.

Experiment 2—to assess the suitability of [6-3H]glucose as a tracer for glycerogenesis, separate experiments were performed in which [6-14C]- and [6-3H]glucose were infused simultaneously without [2-14C]glycerol in three fasted rats and [6-14C]glucose was infused alone in three fed rats. Muscle and blood samples were collected and processed as described for Experiment 1.

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The abbreviations used are: DHAP, dihydroxyacetone phosphate; G3P, glycerol 3-phosphate; TG, triacylglycerol; SA, specific activity; HPLC, high performance liquid chromatography; GC, gas chromatograph.
Experiment 3—To evaluate whether glycerol equilibrates between plasma and intramuscular compartments, [U-13C]glycerol was infused intravenously at 0.2 μmol/min for 3 h in five fasted rats using the same protocol, except a base-line blood sample (to measure background enrichment) was collected through a tail vein before beginning the infusion. Additional experiments were conducted to determine the time course of isotopic equilibration between plasma glycerol and intramuscular free glycerol pools. Three rats were infused with [U-13C]glycerol for 1 h and another three rats for 2 h. The 13C enrichment of plasma and muscle free glycerol were measured on samples collected at the end of the tracer infusion.

Determination of Plasma Glycerol and Glucose specific Activities (SA)

Plasma was deproteinized with 0.3 M BaOH and 0.3 M ZnSO4 (1:1:1, v/v/v) and the supernatant loaded on ion-exchange column (AG 1X8, hydroxide form, 200–400-mesh) (Bio-Rad). Glycerol was eluted with 4 ml of deionized water and then glucose with 4 ml of 1 N NaCl. The [13C]Glycerol SA was measured by HPLC (13) or by an enzymatic method (14). Plasma glycerol concentrations were determined using a glucose analyzer (Beckman, Palo Alto, CA), and glucose SA was determined by counting the glucose samples on a liquid scintillation counter.

Evaluation of Glycerol Equilibration between Plasma and Muscle Compartments

Plasma samples from the [U-13C]Glycerol infusion experiments were processed chromatographically as described above. Muscle free glycerol was obtained from aqueous phase of a Folch lipid extract. The aqueous phase was air-dried and dissolved in water, loaded onto the change column, and glycerol eluted with water. Purified plasma and muscle free glycerol was derivatized to triacetyl glycerol in 200 μl of pyridine and acetyl anhydride (1:1, v/v) at 70 °C for 60 min. The glycerol derivative was analyzed by gas chromatography/combustion/isotope ratio mass spectrometry for 13C enrichment. The gas chromatograph (GC; Hewlett Packard, model 5890) was equipped with a DB-5 MS capillary column (30 m x 0.32 mm inner diameter, 0.25 μm film) (J&W Scientific, Folsom, CA). The GC oven temperature was programmed initially at 80 °C for 0.5 min, and increased to 200 °C at 20 °C/min and then to 300 °C at 35 °C/min and stayed at 300 °C for 3 min. The GC was electronically controlled for constant pressure and humidity. Details of isotope ratio mass spectrometry operation were as described previously (17). 13C enrichment was expressed as atom % excess. The ratio of 13C enrichment of muscle free glycerol to that of plasma glycerol was calculated and used as an index of glycerol equilibration between these two compartments (a ratio of 1 indicates complete equilibration).

Calculation of Glycerol and Glucose Appearance Rate

The infusion rate (dpm/min) of [2-14C]glycerol and [6-3H]glucose was divided by SA (dpm/μmol) of plasma glycerol and glucose, respectively, to give the appearance rates (μmol/min).

Calculation of Blood Glycerol and Glucose Incorporation Rates into Muscle TG

Incorporation rates were calculated by dividing the SA of plasma glycerol or glucose (dpm/μmol) by the total radioactivity (dpm) of the intramuscular TG glycerol moiety. In experiment 1, appreciable amounts of [13C]Glycerol were converted to [14C]glucose (Table II) via gluconeogenesis, implying that the 13C percent in muscle TG glycerol could originate from both glucose and glycogen. We initially planned to use [6-3H]glucose to trace glucose incorporation into TG glycerol; however, this approach was found to be unsatisfactory (see below). The indirect glycerol incorporation into muscle TG glycerol via glucose was therefore calculated by multiplying the plasma glucose [14C] SA in experiment 1 (Table I) by the glucose incorporation rate determined using [6-14C]glucose (experiment 2; conducted under identical conditions). The results were then subtracted from the apparent glycerol incorporation rates to determine those directly attributable to glycerol. The incorporation rates of blood glycerol are given as those after the correction for indirect contribution via glucose. Glucose incorporation rate is given as that determined using [6-14C]glucose as well as using [6-3H]glucose. Each glucose molecule can potentially contribute 2 molecules of TG glycerol; therefore, to compare glycerol and glucose on a carbon equivalent basis, it is necessary to double the apparent glucose values. Because blood was sampled only at the end of tracer infusion, it was necessary to assume that the SA of plasma glycerol and glucose were at steady state for the duration of infusion. This seems a reasonable assumption, considering the very brief half-life of circulating glycerol (18); however, it is unlikely that the [13C]Glycerol SA in experiment 1 was at steady state because of the gradual incorporation of [14C]glycerol into [14C]glucose. Therefore, the rates of indirect [14C]glycerol incorporation into intramuscular TG glycerol via glucose in experiment 1 (above) are likely to be overestimates. The incorporation rates are expressed on a per hour basis assuming the incorporation was linear during the 3-h tracer infusion.

Calculation of Loss of 3H Label from Glucose (Indirect Glycerogenesis)

This is calculated as the difference from unit in the ratio of glucose incorporation rate determined by [6-3H]glucose to that by [6-14C]glucose (1 – glucose incorporation[6–3Hglucose]/glucose incorporation[6–14C]glucose). All values are expressed as mean ± S.E. Analysis of variance was used to detect difference between three muscle groups, and paired, one-tailed Student’s t test for comparison between two muscles, if appropriate, to test for differences in a specific direction based upon previous work (17). Two-tailed, unpaired Student’s t test was used for comparisons between glycerol and glucose parameters.

RESULTS

Glycerol and Glucose Kinetics—The infusion rates of [2-14C]glycerol and [6-3H]glucose, the plasma glycerol and glucose concentration and SA values, and glycerol and glucose appearance rates for experiment 1 are presented in Table I. Plasma glycerol concentrations in fasted rats were 33% greater, but significantly (p < 0.05) lower in fed rats, indicating active glycerol to glucose production via gluconeogenesis. Therefore, the rates of indirect [14C]glycerol incorporation into intramuscular TG glycerol via glucose in experiment 1 (above) are likely to be overestimates. The incorporation rates are expressed on a per hour basis assuming the incorporation was linear during the 3-h tracer infusion.

TABLE I

|                      | Fed (n = 5) | Fasted (n = 5) |
|----------------------|------------|---------------|
| Infusion rate (dpm/rat/min) | 0.76 × 10^9 | 0.78 × 10^9 |
| [2-14C]Glycerol | 0.97 × 10^9 | 1.04 × 10^9 |
| [6-3H]Glucose  | 0.11 ± 0.01 | 0.16 ± 0.02 |
| Glycerol            | 5.3 ± 0.2   | 4.8 ± 0.1    |
| Plasma SA (dpm × 10^3/μmol) | 13.8 ± 3.3  | 10.2 ± 0.6   |
| Glycine            | 9.1 ± 0.6   | 9.4 ± 0.7    |
| Rate of appearance (μmol/min)                             |
| Glycerol            | 5.5 ± 1.1   | 7.8 ± 0.4    |
| Glucose             | 10.8 ± 1.8  | 11.3 ± 1.9   |

Statistics

Calculation of Blood Glycerol and Glucose Incorporation Rates into Muscle TG

Incorporation of Glycerol and Glucose into Skeletal Muscle TG Glycerol Moiety—Table II provides the rates of blood glycerol
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**Table II**

Rates of incorporation of plasma glycerol and glucose into muscle triglyceride glycerol

Values are mean ± S.E. Rates are given as nanomoles/μmol of triglyceride/h. Tracers were infused intravenously for 3 h prior to the muscle biopsies. Glycerol incorporation was measured using [2-14C]glycerol, together with [6-2H]glucose to measure incorporation of glucose. Gluconeogenesis from [1-14C]glycerol resulted in [14C] in muscle TG-glycerol that may have been incorporated via glucose rather than from blood glycerol directly (indirect glycerol incorporation). Both direct and indirect glycerol incorporation rates are provided (see “Materials and Methods” for calculation). Glucose incorporation was measured in additional studies using [6-14C]glucose alone (fasted) or together with [6-2H]glucose (fasted). Statistical comparisons of incorporation rates of glycerol (direct incorporation only) and glucose ([6- 14C]glucose-measured rates only) is on a carbon-equivalent basis (glucose incorporation rate × 2).

| Muscle      | Direct Indirect | p (direct vs. indirect) | [6-14C]Glucose | [6-2H]Glucose | p (14C)glucose vs. 2H-glucose | p (glycerol vs. glucose) |
|-------------|-----------------|------------------------|----------------|---------------|-------------------------------|-------------------------|
| **Fasted**  |                 |                        |                |               |                               |                         |
| Quadriceps  | 7.3 ± 2.3       | 9.9 ± 3.2              | 0.22           | 8.6 ± 1.4     | 0.13 ± 0.2                   | <0.0001                 | 0.02                     |
| Gastrocnemius| 24.5 ± 5.1      | 10.0 ± 3.3             | 0.08           | 9.3 ± 1.6     | 0.05 ± 0.1                   | <0.0001                 | 0.2                      |
| Soleus      | 65.7 ± 23       | 4.8 ± 1.6              | 0.04           | 4.4 ± 0.7     | 0.5 ± 0.4                    | 0.0001                  | 0.03                     |
| **Fed**     |                 |                        |                |               |                               |                         |
| Quadriceps  | 3.7 ± 1.7       | 2.3 ± 0.7              | 0.30           | 4.7 ± 1.9     | 1.8 ± 0.8                    | 0.17                    | 0.18                     |
| Gastrocnemius| 8.3 ± 2.6       | 3.8 ± 1.1              | 0.15           | 7.7 ± 1.9     | 0.8 ± 0.2                    | 0.01                    | 0.15                     |
| Soleus      | 8.5 ± 2.5       | 2.2 ± 0.7              | 0.02           | 4.6 ± 2.4     | 1.4 ± 0.6                    | 0.19                    | 0.9                      |
| **p (fasted vs. fed)** | | | | | | |
| Quadriceps  | 0.25            | 0.04                   |               | 0.14          | 0.02                         |                         |
| Gastrocnemius| 0.02            | 0.1                    |               | 0.8           | 0.0001                       |                         |
| Soleus      | 0.03            | 0.2                    |               | 0.8           | 0.2                          |                         |

Discussion

These studies examined whether blood glycerol is a direct precursor for intramuscular TG glycerol synthesis in a rat model. It has been long thought that triglycerides acquire their glycerol moiety primarily from glucose via DHAP (1, 4). Although there have been debates over relative contributions of G3P pathway versus DHAP pathway to triglyceride and phospholipid synthesis (3, 4), the possibility that glycerol itself is a significant precursor of intramuscular TG glycerol synthesis does not appear to have been explored. Glycerol kinase directly
activates glycerol to G3P for acylation, and it seemed reasonable to believe that tissues low in glycerol kinase activity, such as skeletal muscle, rely on glucose for the synthesis of TG glycerol. However, results from the present study suggest this is not the case. Even in fed rats, where glycerol is less available and glucose is more abundant, the glycerol incorporation rate into muscle triglyceride was comparable to that of glucose on a carbon-equivalent basis. In fasted rats, the rate of blood glycerol incorporation into muscle TG glycerol was much greater than that of glucose, especially in soleus, a primarily oxidative muscle. On the other hand, glucose was the predominant precursor for muscle TG glycerol synthesis in quadriceps, consistent with conventional belief as described above. Thus, in fasted rats, a pattern was observed that the preference of blood glycerol over blood glucose for TG glycerol synthesis in skeletal muscle is consistent with the muscles’ oxidative capacity (soleus > gastrocnemius > quadriceps).

The rat soleus muscle has greater intracellular TG content (17) and oxidative capacity (20) than gastrocnemius and quadriceps muscles. We found that soleus can also utilize greater amount of blood glycerol. In contrast, quadriceps, a fast twitch, glycolytic muscle (21), has a triglyceride content one-fifth of that of soleus (17, 22), and its utilization of blood glycerol for fatty acid esterification appears limited. Since quadriceps relies on glycolysis to a greater extent for ATP production, it seems logical for it to use more blood glucose than glycerol as a precursor for TG glycerol. The rat lateral gastrocnemius contains both glycolytic and oxidative muscle fibers (21), and has an intermediate capacity to utilizing blood glycerol for fatty acid esterification. We conclude that utilization of blood glycerol for intramuscular TG glycerol synthesis is related to the ability of muscle to oxidize and store fatty acids. The preference of glycerol over glucose for muscle TG glycerol synthesis in type 1 fiber-rich muscle suggests that the glycerol kinase activity is higher than in other muscle types (5).

Another possible reason for the greater use of blood glycerol for TG synthesis by soleus muscle is a lesser activity of glycero-phosphate dehydrogenase, the enzyme responsible for the conversion of DHAP to G3P. This might explain the limited flow of glucose carbons to G3P and hence to TG glycerol we observed in some muscle groups. Indeed, the activity of cytosolic glycerophosphate dehydrogenase in skeletal muscle is at least 2–3 times lower than that of liver in rats (23, 24). In addition, due to oxidative shift of redox status as a result of fasting (25), a high NAD+/NADH ratio in fasted rats may have inhibited glucose incorporation into muscle TG glycerol because it favors the equilibrium from G3P to DHAP.

Although glycerol kinase activity in skeletal muscle (5, 6) is much less than that in the liver, this appears to be only a relative limitation. The mammalian intramuscular TG pool size is generally small, usually up to a few micromoles/g of wet muscle (17, 26). The enzyme activity in rat muscle, 7 nmol/g of wet muscle/min (5), could account for substantial glycerol utilization, i.e., 0.42 μmol/g of wet muscle/h. This rate of utilization is almost the same as the size of the entire pool of intramuscular triglycerides in some predominantly fast twitch, glycolytic muscles such as gastrocnemius and quadriceps. The enzyme activity in human muscle is similar to the rat, or 9 nmol/g/min (6), and thus could provide 15 mmol/h of G3P for TG synthesis (assuming 40% of a 70-kg body mass as skeletal muscle). This value exceeds the glycerol appearance rate in adult humans (~150 μmol/min). Thus, glycerol kinase could play a greater role in skeletal muscle lipid metabolism than previously realized.

In present studies, the rate of incorporation of blood glycerol into intramuscular TG glycerol in fasted rats was ~0.4 and ~6 nmol/g of wet muscle/min for gastrocnemius and soleus muscle, respectively. In a previous study using the same experimental conditions (17), we found the synthesis rates of intramuscular TG for gastrocnemius and soleus to be 2.3 and 3.8 nmol/g of wet muscle/min, respectively, using fatty acid tracers. This suggests that, for the soleus muscle, blood glycerol provides the vast majority of the backbone of intramuscular TG. It appears that blood glycerol and glucose make a substantial (~20%) contribution to intramuscular TG synthesis in the gastrocnemius. The source of the remaining TG glycerol is not clear, but glycerolysis of muscle glycogen-derived glucose may provide TG glycerol carbons. Another possibility is glycerogenesis from other three carbon intermediates. The virtually complete loss of 3H label relative to 14C from blood glucose in muscle TG glycerol implies that glucose had gone through pyruvate carboxylation catalyzed reactions (27, 28), and therefore gluconeogenic precursors could have made their way to triglyceride glycerol as well.

We obtained evidence for indirect glycerogenesis from blood glucose, as indicated by the extensive loss of 3H label from [6-3H]glucose. This suggests that the majority of bone glucose destined for TG glycerol arrived at G3P from steps further down the glycolytic pathways. Presumably, glucose has gone through pyruvate/lactate stage before making its way to G3P via gluconeogenesis, during which hydrogen atoms at the 6th carbon position are lost (19, 27, 28). This is the only explanation for the loss of 3H labels from [6-3H]glucose. In the interconversion of pyruvate and alanine, although 6-3H of glucose is lost (19), the three-carbon metabolite has to undergo gluconeogenesis via oxaloacetate in order to return to DHAP because phosphoenolpyruvate → pyruvate reaction is irreversible (1). This indirect pathway has been established for glycogen synthesis by the liver (29) but not in skeletal muscle.

We observed a moderate delay in isotopic equilibration between plasma and muscle free glycerol pools. In quadriceps and gastrocnemius muscles, the equilibration was complete by 2 h of tracer infusion. In the soleus, on the other hand, a full isotopic equilibrium was not observed even after 3 h of tracer infusion. At 1 and 2 h of infusion, the 13C enrichment of soleus free glycerol was significantly lower than that in other two muscles. The difference in isotopic equilibrium between this oxidative muscle and other more glycolytic muscles suggests that TG-fatty acid cycling in soleus is active (30). Free glycerol
generated from such cycling may have diluted incoming \([^{13}\text{C}]\text{glycerol}\) because TG content (thus the amount of glycerol generated) in soleus is 5 times higher than that in other two muscles (17). Overall, glycerol isotopic equilibration between plasma and intramuscular pools appears slow. For a small molecule like glycerol, this seems unexpected. Nonetheless, this is consistent with the reported direction of the concentration gradient transport of glycerol in skeletal muscle (12, 31).

In summary, rat skeletal muscle has a greater capacity for utilizing blood glycerol for intracellular TG synthesis than previously realized. Blood glycerol may be a preferred substrate for TG glycerol synthesis in fasted rats depending on the muscle’s oxidative capacity. In fed rats, blood glycerol and glucose contribute similar amounts of carbons to muscle TG glycerol synthesis. The utilization of blood glycerol for this pathway appears to be substrate-regulated, whereas glucose is probably more of a constitutive substrate.

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