 Intramuscular fatty acid metabolism evaluated with stable isotopic tracers

ZENGKUI GUO AND MICHAEL D. JENSEN
Endocrine Research Unit, Mayo Clinic, Rochester, Minnesota 55905

Guo, ZengKui, and Michael D. Jensen. Intramuscular fatty acid metabolism evaluated with stable isotopic tracers. J. Appl. Physiol. 84(5): 1674–1679, 1998.—We evaluated the applicability of stable isotopic tracers to the study of intramuscular fatty acid metabolism by infusing both [U-13C]palmitate and [1-13C]oleate intravenously for 4 h into fasted conscious rats. Skeletal muscles were sequentially biopsied, and the concentration and 13C enrichment of fatty acids were measured by gas chromatography/combustion/isotope ratio mass spectrometry. Throughout the study, the 13C enrichment of plasma palmitate and oleate remained substantially greater than intramuscular nonesterified palmitate and oleate enrichment, which in turn was greater than intramuscular triglyceride palmitate and oleate enrichment. Fractional synthesis rates of intramuscular triglycerides in gastrocnemius and soleus were 0.267 ± 0.075 and 0.100 ± 0.030/h (P = 0.04), respectively, as determined by using [U-13C]palmitate, and were 0.278 ± 0.049 and 0.075 ± 0.013/h (P = 0.02), respectively, by using [1-13C]oleate. We conclude that plasma free fatty acids are a source for intramuscular triglycerides and nonesterified fatty acids; the latter are likely the synthetic precursors of the former. Uniformly and singly labeled [$^{13}$C]fatty acid tracers will provide an important tool to study intramuscular fatty acid and triglyceride metabolism.

Methods

Animal Preparation

Male Sprague-Dawley rats (body weight ~400 g), fed on standard rat chow ad libitum with free access to water, were used after a 24-h fast. The morning of the study the animals were anesthetized for a brief period (~1 h) by an intraperitoneal pentobarbital sodium (30 mg/kg) injection. A 19-gauge flexible plastic catheter was inserted into the left carotid artery for blood sampling and was kept patent by infusing 0.9% NaCl (5 µl/min). After a quadriceps muscle biopsy was taken, the animals were transferred into a restraining cage that prevented free movement and kept them in a normal standing position. A baseline blood sample was collected, and the tracer infusions were begun after the rats recovered from anesthesia.

Tracer Preparation and Infusion

[U-13C]palmitate and [1-13C]oleate (both 99% pure) were purchased from Isotech (Miamisburg, OH), bound to bovine serum albumin, and diluted in 0.9% NaCl to an appropriate concentration. The tracers were infused into a tail vein by using a 23-gauge infusion needle at a rate of 0.013 (n = 7) or 0.049 (n = 3) µmol·kg⁻¹·min⁻¹ for [U-13C]palmitate and 0.017 (n = 7) or 0.068 (n = 3) µmol·kg⁻¹·min⁻¹ for [1-13C]oleate. The infusions were nonprimed and continuous for 4 h.

Sample Collection

Blood samples were collected at 90, 105, 120, 210, 225, and 240 min after the tracer infusions were begun. The plasma was separated by centrifugation (3,000 revolutions/min for 15 min) and then stored at −80°C. Before the tracer infusions were begun, the skin over the left quadriceps was anesthetized by a local lidocaine injection, after which an incision was made to allow a biopsy (<0.1 g wet wt) to be taken. After 2 h of tracer infusion, the area of the left gastrocnemius-plantaris-soleus bundle was anesthetized with lidocaine and incised, and the muscle bundle exposed by gentle blunt dissection. The covering fascia and epimysium were dissected to expose separate gastrocnemius and soleus. A small piece of gastrocnemius (~0.1 g wet wt) and the entire soleus muscle were removed, immediately merged in liquid N₂, and transferred to a freezer (~−80°C) for later analysis. Occasionally it was necessary to rinse the gastrocnemius sample in iced 0.9% NaCl to completely remove small amounts of blood. The muscle and skin wounds were suture closed. At 4 h the same procedure was carried out on the right side, after which the tracer infusions were stopped.

Sample Analyses

One-half milliliter of plasma was extracted for total lipids (4), from which FFA were separated by high-performance...
liquid chromatography (HPLC) (2). The FFA were derivatized to methyl esters by using acetyl chloride-MeOH methylation kit (Alltech, Deerfield, IL) at 70°C for 60 min and were analyzed by GC/C/IRMS for $^{13}$C enrichment and masses. Frozen muscle samples were lyophilized to dryness over night, weighed, and pulverized in a ground-bottomed glass homogenizer. Heptadecanoic acid (0.1 µmol) was added to the muscle powder to serve as an internal standard for NEFA, after which total lipids were extracted (6) and the TG and FA fractions were purified by HPLC (2). Heptadecanoic acid (0.2 µmol) was added to the TG fraction, and the TG FA (TGFA) were transmethylated by using 2.5% of sulfuric acid in methanol at 70°C for 2 h (13) and were analyzed by GC/C/IRMS for $^{13}$C enrichment and masses. To eliminate any phospholipids that coeluted with the muscle FA fraction, the samples were passed over a silicic acid column (Unisil, Clarkson Chemical, Williamsport, PA), and the FA were eluted with CHCl$_3$ (14). The purified NEFA were converted to their methyl esters and analyzed as described above for plasma FFA.

In separate experiments we assessed the extent of ex vivo hydrolysis of muscle triglycerides during the sample handling procedures by adding [U-13C]triolein to rat muscle before sample processing. We found that 0.2–0.4% of the triglyceride could be recovered in the NEFA fraction described above.

**GC/ C/IRMS Analysis**

The GC/C/IRMS consisted of a Hewlett-Packard 5890 GC and a Finnigan MAT delta S IRMS (Finnigan MAT, San Jose, CA), interfaced with a ceramic microcombustion oxidizer maintained at 960°C. The GC was equipped with a DB-1 column (60 m x 0.25 µm film, J&W Scientific, Folsom, CA). The oven temperature was programmed as follows: the initial temperature was 150°C; it was increased to 190°C at 5°C/min, then increased to 200°C at 1°C/min, and was kept at 200°C for 2 min; and finally it was increased to 290°C at 35°C/min and was kept at 290°C for 4 min. The injector and the flame ionization detector were both set at 250°C. Before sample analysis, the GC/C/IRMS was calibrated with a series of palmitate and oleate standards containing unlabeled palmitate and oleate and different concentrations of [U-13C]palmitate and [1-13C]oleate, respectively, ranging from 0.001 to 0.200 mole percent excess. Regression analysis was performed to obtain the instrument response slopes that were used to correct the raw $^{13}$C enrichment data of samples. $^{13}$C enrichment is expressed as atom percent excess (APE). The detailed procedures of GC/C/IRMS analysis were as described previously (10).

The quantities of palmitate, oleate, linoleate, and stearate of intramuscular TG and NEFA were determined by quantifying the peak-area ratios of CO$_2$ generated from combustion of the FA samples and the internal standard heptadecanoate. First, calibration curves were constructed by analyzing a series of standards containing gradient concentrations of palmitate, oleate, linoleate, and stearate, each containing the internal standard at a constant concentration. Regression analysis was performed on the CO$_2$ peak-area ratios against the molar ratios of the four FA standards to the internal standard, and a calibration slope for each FA standard was obtained. The CO$_2$ peak-area ratios of intramuscular TG and nonesterified palmitate, oleate, linoleate, and stearate to the internal standard were calculated off line, and their masses were determined by using their respective calibration slopes. The pool sizes of intramuscular NEFA and TG were calculated from the determined masses.

**Calculations**

Intramuscular TG fractional synthesis rate (FSR). Calculation of muscle TG FSR was performed by using the intramuscular NEFA as the precursor pool. The numerator for this calculation was the increment in $^{13}$C enrichment of muscle TG palmitate and oleate during the 2- to 4-h interval. For the denominator we used the arithmetic average $^{13}$C enrichment of intramuscular nonesterified palmitate and oleate over the same time interval. For [U-$^{13}$C]palmitate, the formula is as follows:

$$\text{TG FSR} = \frac{(4\text{-h TG palmitate}_{APE} - 2\text{-h TG palmitate}_{APE})}{[2\text{-h NEFA palmitate}_{APE} + 4\text{-h NEFA palmitate}_{APE}]2} \times \frac{1}{2}$$

The formula for TG FSR calculation by using [1-$^{13}$C]oleate is the same except oleate is substituted for palmitate.

**Rates of palmitate and oleate incorporation into intramuscular TG.** These values were calculated as the product of TG FSR and the respective TG pool size.

**Statistics**

All values are expressed as means ± SE. Comparisons between gastrocnemius and soleus, 2 and 4 h and between the two tracers, were made by using paired Student's t-test when the number of samples was equal. When numbers were not equal, unpaired Student's t-test with equal variance was used. Analysis of variance was used for comparisons among gastrocnemius, soleus, and quadriceps. When the test is significant, Bonferroni's multiple-comparison method was used for pairwise comparisons among the three muscle groups.

**RESULTS**

**Intramuscular TGFA and NEFA Pool Sizes**

Table 1 provides the quadriceps, gastrocnemius, and soleus intramuscular TGFA pool sizes of the four major individual FA. The TGFA pools in the soleus are three to seven times larger than those in the quadriceps or gastrocnemius. Similarly, the combined TGFA pool in

<table>
<thead>
<tr>
<th></th>
<th>Quadriceps (n = 6)</th>
<th>Gastrocnemius (n = 12)</th>
<th>Soleus (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitate</td>
<td>4.50 ± 0.93</td>
<td>4.13 ± 0.68</td>
<td>17.61 ± 3.02†</td>
</tr>
<tr>
<td>Oleate</td>
<td>4.85 ± 1.40</td>
<td>3.71 ± 0.78</td>
<td>19.52 ± 4.83‡</td>
</tr>
<tr>
<td>Linoleate</td>
<td>5.00 ± 1.45</td>
<td>5.83 ± 1.51</td>
<td>35.81 ± 9.67§</td>
</tr>
<tr>
<td>Stearate</td>
<td>0.25 ± 0.13</td>
<td>0.67 ± 0.09</td>
<td>2.44 ± 0.62§</td>
</tr>
<tr>
<td>Total</td>
<td>15.17 ± 3.81</td>
<td>14.34 ± 2.87</td>
<td>75.38 ± 17.31‡</td>
</tr>
<tr>
<td>NEFA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitate</td>
<td>0.78 ± 0.47</td>
<td>0.19 ± 0.02</td>
<td>0.36 ± 0.06</td>
</tr>
<tr>
<td>Oleate</td>
<td>0.53 ± 0.28</td>
<td>0.14 ± 0.03</td>
<td>0.30 ± 0.06</td>
</tr>
<tr>
<td>Linoleate</td>
<td>0.18 ± 0.06</td>
<td>0.12 ± 0.04</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>Stearate</td>
<td>0.25 ± 0.13</td>
<td>0.06 ± 0.01</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Total</td>
<td>1.74 ± 0.92</td>
<td>0.51 ± 0.11</td>
<td>0.96 ± 0.15*</td>
</tr>
</tbody>
</table>

Values are means ± SE given in µmol/g dry muscle; n, no. of samples. TGFA, triglyceride fatty acid; NEFA, nonesterified fatty acid. There were no significant differences in individual fatty acids of NEFA pool among the 3 muscle groups. *P < 0.01 and †P < 0.05; gastrocnemius vs. soleus. ‡P < 0.01 and §P < 0.05; quadriceps vs. soleus.
the soleus is five times larger (P < 0.01) than those in the gastrocnemius or quadriceps. There were no differences (P > 0.4) in the TGFA pool sizes in muscle samples collected at 2 and 4 h (not shown) of the study.

Table 1 also provides the NEFA pool sizes of the three muscle groups. The individual NEFA pool sizes are similar (P > 0.05) among the three muscles; however, the combined NEFA pool size in the soleus is greater (P < 0.01) than that in gastrocnemius. The absolute difference (0.45 µmol/g dry muscle) is small compared with the difference in intramuscular TGFA pools between these same two muscles (~61 µmol/g dry muscle). Note that the NEFA content of the quadriceps was greater and more variable than those of the other two muscles, which perhaps relates to the prevailing conditions at the time of the biopsy (during recovery from anesthesia immediately after the insertion of the vascular catheters).

**Intercompartmental Tracer Equilibration**

The 13C enrichment in palmitate and oleate in the plasma FFA, intramuscular NEFA, and intramuscular TGFA pools is provided in Table 2. The 13C enrichments are the highest in plasma FFA, lowest in TG, and intermediate in NEFA. The APE values of NEFA palmitate in both muscles are greater (P < 0.05) in the 4-h than in the 2-h biopsy, as are those of TG palmitate (P < 0.05). All APE values of TG palmitate are less than the corresponding NEFA palmitate values, and the pattern of change in the two pools is similar. The 13C enrichment in oleate is much less than that in palmitate, reflecting the fact that the oleate tracer is singly labeled. However, like palmitate, the APE of TG oleate is greater at 4 than at 2 h and is less than the corresponding NEFA oleate enrichment.

**FA Profiles**

The percent contributions of linoleate, palmitate, oleate, and stearate to plasma FFA, intramuscular NEFA, and intramuscular TGFA are depicted in Fig 1. These four FA represent >90% of the total FA in these lipid pools. The relative FA composition of intramuscular NEFA is similar in the three muscle groups. The same is true for the intramuscular TGFA composition. In contrast, there are distinct differences in the FA composition between these intramuscular lipid pools and plasma FFA, especially with regard to the relative amounts of linoleate and stearate. Linoleate increased, whereas stearate decreased, progressively in the order of plasma FFA, intramuscular NEFA, and intramuscular TG pools, the probable pathway of plasma FFA flow to intramuscular lipid pools. The difference in the abundance of linoleate between intramuscular TG and NEFA pools is significant (P < 0.05) in all three muscles. The lesser abundance of intramuscular TG stearate compared with that of NEFA stearate appears to reflect a true decrease, not merely a reflection of the greater amount of linoleate, because the ratio of stearate to palmitate or oleate (not shown) is also decreased.

**Intramuscular TG FSR**

The FSR of intramuscular TG determined by using [U-13C]palmitate were 0.267 ± 0.075 and 0.100 ± 0.030/h (P = 0.04) for gastrocnemius and soleus, respectively (Fig 2). The TG FSR of the same muscles determined by using [1-13C]oleate were 0.278 ± 0.049 and 0.075 ± 0.013/h, respectively (P = 0.02). The FSR calculated by using the two tracers were not significantly different in either muscle group (P = 0.92 for gastrocnemius and P = 0.37 for soleus).

The absolute rates of palmitate and oleate incorporation into TG pools of gastrocnemius and soleus are presented in Fig. 2, inset. The incorporation rates of palmitate into gastrocnemius and soleus were 0.68 ± 0.21 and 1.13 ± 0.16 µmol·g dry muscle⁻¹·h⁻¹, respectively (P < 0.05). The corresponding values for oleate were 1.1 ± 0.26 and 1.27 ± 0.25 µmol·g dry muscle⁻¹·h⁻¹ (P > 0.05).

**DISCUSSION**

To our knowledge, this is the first study to assess the relationship among FA in the intramuscular NEFA, intramuscular TG, and plasma FFA pools. We infused 13C-labeled FA tracers intravenously and found evidence for active incorporation of plasma FFA into the intramuscular NEFA and TG. If plasma FFA were rapidly and directly incorporated into intramuscular TG, without mixing with the intracellular NEFA pool,

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**Table 2. 13C enrichment in plasma FFA and intramuscular TGFA and NEFA pools**

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Plasma [U-13C]palmitate (n = 6)</th>
<th>Gastrocnemius NEFA</th>
<th>TGFA</th>
<th>Soleus NEFA</th>
<th>TGFA</th>
<th>SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>0.265 ± 0.027</td>
<td>0.042 ± 0.009</td>
<td>0.013 ± 0.009</td>
<td>0.065 ± 0.005</td>
<td>0.005 ± 0.004</td>
<td>0.002</td>
</tr>
<tr>
<td>240</td>
<td>0.245 ± 0.022</td>
<td>0.067 ± 0.013</td>
<td>0.044 ± 0.009</td>
<td>0.099 ± 0.004</td>
<td>0.024 ± 0.013</td>
<td>0.004</td>
</tr>
<tr>
<td>120</td>
<td>0.030 ± 0.009</td>
<td>0.007 ± 0.001</td>
<td>0.001 ± 0.001</td>
<td>0.010 ± 0.002</td>
<td>0.000 ± 0.000</td>
<td>0.002</td>
</tr>
<tr>
<td>240</td>
<td>0.033 ± 0.007</td>
<td>0.006 ± 0.002</td>
<td>0.004 ± 0.001</td>
<td>0.010 ± 0.002</td>
<td>0.002 ± 0.004</td>
<td>0.000</td>
</tr>
</tbody>
</table>

Values are means ± SE in 13C atom percent excess; n, no. of rats. Data are from lower infusion rate experiments only. Plasma samples from higher infusion rate experiments were not analyzed because of too high 13C enrichment of plasma palmitate. For both time points, plasma samples were collected at 15-min intervals during a 30-min period immediately preceding muscle biopsies, and averages are used. Single sample from each muscle was collected at each time point. FFA, free fatty acid.
and if intramuscular TGFA replenish intracellular NEFA, the TGFA enrichment should equal the NEFA enrichment. In contrast, the $^{13}$C enrichment of intramuscular NEFA was substantially greater than intramuscular TGFA enrichment, suggesting the former serves a precursor pool function for the latter. This pattern was the same in both slow-twitch red (soleus) and mixed fast-twitch white and red (gastrocnemius) muscle types, which were readily differentiated by both TGFA pool size and FSR. We conclude that $^{13}$C FA tracers can be used for studies of intracellular NEFA and TG metabolism in skeletal muscle.

The $^{13}$C enrichment of intramuscular TGFA pools increased during the second one-half of tracer infusion, indicating continued plasma FFA incorporation. This is direct evidence that plasma FFA are an important source for intramuscular TGFA. The tracer equilibration pattern in the intramuscular NEFA and TGFA pools (Table 2) resembles a precursor-product relationship, suggesting that intramuscular NEFA is an (the) immediate precursor for TG synthesis. This supposition cannot be verified by the present study; however, additional studies utilizing the methods described in this report could readily test this hypothesis. The large decrease in APE values from plasma FFA to intramuscular NEFA (Table 2) could be caused by intracellular TGFA/NEFA cycling, contribution of FA from other sources (e.g., plasma TG) (15, 16), or a relatively slowly turning over intramuscular NEFA pool. A tracer equilibrium between plasma FFA and intramuscular NEFA pools seems unlikely even over an extended period of time.
The distinct FA profiles among intramuscular TG, intramuscular NEFA, and plasma FFA pools suggest different metabolic kinetics among individual FA, especially linoleate and stearate. The greater percentage of linoleate and lesser percentage of stearate in intramuscular TG than in intramuscular NEFA and plasma FFA strongly imply some selectivity or differentiation in the individual FA flows from plasma to their intracellular destinations. The implication of our observation is that linoleate is discriminated against for oxidation or is preferably esterified.

Intramuscular TG synthesis has been assessed in a small number of studies by measuring FA tracer incorporation (1, 5, 11). Because the differences in TG contents of different muscle types are enormous (7, 19), determining the fractional synthesis rate and the TGFA pool size provides a more complete metabolic picture of muscle lipid metabolism. However, the accuracy of the results depends on the validity of the assumption that intramuscular NEFA are the direct precursor of intramuscular TG. Our estimated rate of palmitate incorporation into intramuscular TG (Fig. 2, inset) is comparable to that obtained by Hopp and Palmer (11), who incubated rat flexor digitorium brevis muscle in a medium containing palmitate and glucose. The rates of the total FA incorporation into gastrocnemius we observed (2.1 µmol · g dry muscle⁻¹ · h⁻¹, derived from the palmitate incorporation and the FA composition as shown in Fig. 1) are comparable to the value of 1.6 µmol · g fat-free muscle⁻¹ · h⁻¹ as obtained in vivo by Tagliaferro et al. (20). On the other hand, if plasma FFA are used as the direct precursor, the FSR of intramuscular TG for gastrocnemius and soleus would have been ~2.5-fold lower. The similarity of our values to those of others who used different means for calculating FA incorporation rates suggests that intramuscular NEFA are the appropriate precursor pool for TG synthesis. It should be pointed out, however, that the muscle TGFA FSR could reflect the incorporation of both plasma FFA and plasma very-low-density-lipoprotein TGFA, which may have become labeled during the extended [¹³C]FA tracer infusion. In addition, ex vivo hydrolysis of intramuscular TG during sample processing could falsely lower the enrichment of the intramuscular NEFA pool. Our studies indicate that ~8% and ~12%, respectively, of the measured NEFA in the gastrocnemius and soleus muscle samples may have come from ex vivo TG hydrolysis. Thus the intramuscular TG fractional synthesis rates presented should be considered maximum estimates.

The FSR data indicate that the intramuscular FA esterification is a rapid process. This implies that the hydrolysis of this lipid pool must also be rapid to maintain a constant intramuscular TG content. This conclusion is consistent with previous reports that intramuscular TG is continuously synthesized and hydrolyzed at steady state (11, 20, 22) and even during exercises (8). Intramuscular TG may function as a reservoir for intracellular FA, storing them as TGFA when intramuscular NEFA rise and supplying FA when demands increase.

Although a number of studies have determined TG concentration in skeletal muscle (7, 9, 12, 19), few have determined intramuscular NEFA content or directly compared intramuscular TG and NEFA. Our data confirmed previously published data that intramuscular NEFA and TG are drastically different in size (7, 16, 19). In normally fed rats, the NEFA pool size is even smaller (16). It is clear that only a tiny amount of intracellular NEFA is present under normal physiological conditions.

Stable isotopes have not been previously used for direct measurement of intramuscular FA metabolism. To ensure reliable measurement of ¹³C enrichment, we used two different tracers, [U-¹³C]palmitate and [1-¹³C]oleate, each at two different infusion rates to ensure adequate ¹³C signals during MS analysis. With the lower infusion rate, [1-¹³C]oleate generated weak ¹³C signals in TG pool (APE < 0.005) (Table 2). At the higher rate, it generated signals (APE 0.007–0.05) that are adequate for reliable ¹³C measurement. Therefore, the higher infusion rate used in the present study appears to be the minimum for this tracer. The [U-¹³C]palmitate infusion generated stronger ¹³C signals even at the lower infusion rate (Table 2), whereas the higher infusion rate produced excessive ¹³C signals in NEFA pool (APE > 0.3), although it was appropriate for TG (APE < 0.2). An infusion rate similar to the lower rate used in this study should be satisfactory for studies of intramuscular FA metabolism with [U-¹³C]palmitate.

In summary, we have evaluated some basic issues of FA metabolism in skeletal muscle by using a novel, stable isotopic approach. Plasma FFA were found to be quantitatively important lipid sources for intramuscular NEFA and TG. Our data suggest a pathway of FA flow from the plasma FFA to the intramuscular NEFA and finally the TG pool. The absolute rates of FA esterification in slow-twitch red and mixed fast-twitch white and red muscles are similar; therefore, their FSR are reciprocal to their intramuscular TG pool sizes. The relative amounts of the various individual FA differ substantially between plasma FFA and intramuscular lipid pools, suggesting that results from a single FA tracer cannot be readily extrapolated to all FA. Finally, stable isotope techniques are useful for studies of FA metabolism in skeletal muscle, offering the distinct advantage of being able to measure enrichment in the small intracellular NEFA pool. This approach should allow better understanding of the mechanisms of intramuscular TG accumulation under normal and disease conditions.

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Address for reprint requests: M. D. Jensen, Endocrine Research Unit, 5-164 West J oseph, Mayo Clinic, Rochester, MN 55905 (E-mail: jensen.michael@mayo.edu).

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