Measurement of precursor enrichment for calculating intramuscular triglyceride fractional synthetic rate

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Abstract Our goal was to assess the validity of the enrichments of plasma free palmitate and intramuscular (IM) fatty acid metabolites as precursors for calculating the IM triglyceride fractional synthetic rate. We infused U13C16-palmitate in anesthetized rabbits for 3 h and sampled adductor muscle of legs using both freeze-cut and cut-freeze approaches. We found that IM free palmitate enrichment (0.70 ± 0.07%) was lower (P < 0.0001) than IM palmitoyl-CoA enrichment (2.13 ± 0.17%) in samples taken by the freeze-cut approach. The latter was close (P = 0.33) to IM palmitoyl-carnitine enrichment (2.42 ± 0.16%). The same results were obtained from the muscle samples taken by the cut-freeze approach, except the enrichment of palmitoyl-CoA (2.21 ± 0.08%) was lower (P = 0.02) than that of palmitoyl-carnitine (2.77 ± 0.17%). Plasma free palmitate enrichment was ~2-fold that of IM palmitoyl-CoA enrichment and palmitoyl-carnitine enrichment (P < 0.001). These findings indicate that plasma free palmitate overestimated IM precursor enrichment owing to in vivo IM lipid breakdown, whereas IM free palmitate enrichment underestimated the precursor enrichment because of lipid breakdown during muscle sampling and processing. IM palmitoyl-carnitine enrichment was an acceptable surrogate of the precursor enrichment because it was less affected by in vitro lipid breakdown after sampling.

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Supplementary key words stable isotopes • mass spectrometry • rabbits • fatty acyl-coenzyme A • fatty acyl-carnitine

The possible physiological importance of intramuscular (IM) triglyceride (TG) metabolism in a variety of circumstances has attracted great attention in recent years. IM TG not only serves as a fuel source to provide energy for muscle contraction, but it also plays a role in metabolic regulation (1, 2). Increased TG deposition in the skeletal muscle is often associated with insulin resistance, which is particularly true in sedentary individuals (3–7). IM TG concentrations are also increased in endurance-trained individuals, who are very sensitive to the action of insulin (7, 8). The response in endurance athletes suggests that elevated IM TG in the context of periodic accelerated demands for energy substrates in muscle may play an important role in the regulation of substrate metabolism during exercise. Quantification of IM TG kinetics is therefore necessary to further elucidate the physiological significance of the responses in both sedentary individuals and endurance athletes.

The investigation of IM TG kinetics has been limited by the lack of a generally accepted method with which to measure the rate of synthesis. Isotopic techniques represent the most practical approach to measuring IM TG synthesis, but the difficulty in measuring the precursor for IM TG synthesis has been a significant obstacle. It is generally accepted that fatty acyl-CoA is the true precursor for intracellular lipid synthesis. In practice, however, either plasma FFA (9) or IM FFA (10–13) has been used as a surrogate of the precursor when a labeled fatty acid is infused to measure the fractional synthetic rate (FSR) of IM TG. The use of either plasma or IM palmitate as precursor for IM TG synthesis reflects the difficulty of accurately measuring the enrichment of IM palmitoyl-CoA (PalCoA). This in part can be attributed to rapid decay after excision, most likely due to continuous lipid breakdown that dilutes the PalCoA enrichment (14). We have proposed the use of palmitoyl-carnitine (PalCn) enrichment to represent the PalCoA enrichment for calculating IM lipid synthesis (14). PalCn is a downstream metabolite of PalCoA, and therefore, theoretically less affected by tracee dilution from in

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Abbreviations: FSR, fractional synthetic rate; IM, intramuscular; PalCn, palmitoyl-carnitine; PalCoA, palmitoyl-CoA; TG, triglyceride.

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vitro lipid breakdown during muscle biopsy and processing. We have established the stable isotope and mass spectrometry methods of measuring both PalCoA and PalCn, as well as more conventionally used surrogates, as precursors for the measurement of IM TG synthesis (15, 16). The current study was performed to assess the relationship of the potential surrogates for precursor enrichment with the measured enrichment of PalCoA, which we considered the true value.

MATERIALS AND METHODS

Animal preparation

Adult male New Zealand white rabbits (Myrtle's Rabbitry, Thompson Station, TN), weighing ~4.5 kg, were used for this study. The rabbits were housed in individual cages and were given 150 g/day of unpurified diet (Lab Rabbit Chow 5326, Purina Mills, St. Louis, MO) for weight maintenance. This protocol complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals. The animals were maintained in a 12 h light/12 h dark cycle in individual cages with free access to water. Surgery was performed to insert catheters into the carotid artery and jugular vein under general anesthesia, with free access to water. Surgery was performed to insert catheters into the carotid artery and jugular vein under general anesthesia. The rabbits were housed in individual cages and were given 150 g/day of unpurified diet (Lab Rabbit Chow 5326, Purina Mills, St. Louis, MO) for weight maintenance. This protocol complied with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, incorporated in the Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals, and it was approved by the Animal Care and Use Committee of the University of Texas Medical Branch at Galveston.

The animals were studied after overnight food deprivation with free access to water. Surgery was performed to insert catheters into the carotid artery and jugular vein under general anesthesia (14, 17). The arterial line was used for drawing blood and monitoring arterial blood pressure and heart rate; the venous line was used for infusion of anesthetics and saline. An additional venous line was installed in a marginal ear vein by means of a Teflon-top needle (24 G 3/4 Introcan Safety, B. Braun Medical, Inc., Bethlehem, PA), which was used exclusively for tracer infusion. Tracheotomy was performed for placement of a tracheal tube, which was connected to a hood filled with oxygen-enriched room air.

Stable isotope tracer infusion

After surgery, we observed blood pressure, heart rate, and rectal temperature for 20-30 min to ensure stable physiological conditions before the start of tracer infusion. U-13C16-palmitate (99% enriched; Cambridge Isotope Laboratories), bound to 5% albumin, was infused continuously for 3 h at a dose of ~0.1 µmol/kg/min after a priming dose of 1.0 µmol/kg. The tracer was infused into the marginal ear vein using a Harvard Syringe pump (Harvard Apparatus, Boston, MA) set at 15 ml/h. Blood samples were taken from the carotid artery catheter at 0, 5, 30, 60, 90, 120, 150, and 180 min. After centrifugation, plasma was separated and stored at ~20 ºC for later analysis. The adductor muscle of both legs was sampled at 0, 5, 60, 120, and 180 min. At each sampling time except the zero time (background enrichment), a pair of muscle samples were taken: the first muscle sample was taken using the freeze-cut approach and the second one using the cut-freeze approach. The freeze-cut approach involved the in situ clamp prechilled in liquid nitrogen. The tissue was held with forceps while the frozen muscle was excised. The samples were immediately put in liquid nitrogen. After thorough freezing in liquid nitrogen for >1 min, extra tissue not included in the part of the sample that was freeze clamped was trimmed. Immediately following the freeze-cut, a second piece of muscle was taken using scissors (the cut-freeze approach) from the muscle adjacent to the freeze-cut site. The samples were briefly washed with ice-cold saline if blood was visible. The samples were then frozen in liquid nitrogen using a metal net to dunk the samples. All frozen muscle samples were transferred into cryogenic tubes for storage in a ~80 ºC freezer. The freeze-cut and cut-freeze muscle sampling was performed twice in each leg in the sequence from the distal to the proximal site of the adductor muscle.

Mean arterial blood pressure, heart rate, and rectal temperature were maintained stable by adjusting the infusion rates of anesthetics and physiological saline, and by using a heating blanket. These vital signs were recorded every 30 min during the 3 h tracer infusion. At the end of the experiment, the rabbits were euthanized by intravenous injection of 5 ml saturated KCl under general anesthesia.

Sample analysis

Plasma FFA and TG were processed for palmitate enrichment using GC-MS (MSD, Agilent Technologies, Palo Alto, CA) (14, 17, 18). Ions were selectively monitored at m/z 270, 285, and 286 for palmitate. Heptadecanoic acid and triheptadecanoin were added to the plasma samples as internal standards for calculating palmitate concentrations in plasma FFA and TG. Seven fatty acids in plasma FFA and TG were measured using a GC system with flame ionization (FI) detection (model 6890, Agilent Technologies). The percentage of palmitate in the seven fatty acids was used to convert palmitate concentration to total FFA and TG concentrations in plasma. The seven fatty acids were myristate (14:0), palmitate (16:0), palmitoleate [16:1(n-6)], stearate (18:0), oleate [18:1(n-9)], linoleate [18:2(n-6)], and linolenate [18:3(n-3)].

Five representative frozen muscle samples from concomitant other experiments were thawed and observed under a stereo microscope (10-40×, Konus microscopes). No overt adipose tissue was found under the microscope, so the muscle samples taken by the same procedure were considered free of fat contamination. Frozen muscle samples were pulverized with a mortar and pestle prechilled in liquid nitrogen. Heptadecanoyl-CoA (Sigma Chemical, St. Louis, MO) or d4-PalCn (CIL, Andover, MA) were added as internal standards for calculating the concentration of PalCoA or PalCn, respectively. Fatty acyl-CoA was extracted with KH2PO4 and 2-propanol, and fatty acyl-carnitine was extracted with KH2PO4 and acetonitrile/methanol from 40-50 mg tissue (15, 16). The enrichments of PalCoA and PalCn along with percentage composition of seven fatty acyl-CoAs and seven fatty acyl-carnitines were measured on an Agilent 1100 series liquid chromatograph-1956B SL single quadrupole mass spectrometer (Agilent Technologies) as previously described (15, 16). The seven fatty acids were the same as measured in plasma FFA and TG.

Muscle TG was extracted from 30-50 mg of muscle powder overnight at 4 ºC in 1:2 (v/v) methanol/chloroform solution containing 0.05 mg/ml butylated hydroxytoluene. Heptadecanoic acid was added to the samples as an internal standard for calculating IM FFA concentration. After extraction, the samples were centrifuged, and the supernatant was dried under nitrogen gas. The samples were reconstituted with 50 µl chloroform for TLC (Partisil LKSD, Silica Gel 150 Å, Schleicher and Schuell, Maidstone, United Kingdom) isolation of TG and FFA. The isolation was processed in a tank with a mixture of hexane:ethyl ether acetate (70:30:1 in ml). The isolated TG was hydrolyzed in HNO3 to get fatty acids and glycerol. Fatty acids from the IM FFA fraction were derivatized to their methyl esters for measurement of palmitate enrichment by GC-MS; fatty acid concentrations were measured by GC-FI. Fatty acids from IM TG were derivatized to their methyl esters; palmitate enrichment was measured on a GC-combustion-isotopic ratio MS (Finnigan, MAT, Bremen, Germany). The measured 13C enrichment was multiplied by 17/16 to convert to the enrichment of the U-13C16-palmitate as 16 of 17 total carbons in the palmitate methyl ester molecule have a chance to be labeled.
Calculations

The concentrations of palmitate in plasma and muscle lipids were calculated by the internal standard method. Palmitate (or its metabolites) concentrations were divided by percentage of palmitate in the seven fatty acids to obtain total fatty acid concentrations.

FSR of IM TG was calculated by the tracer incorporation method, which is based on the precursor-product principle (18). The equation is:

\[
\text{FSR} = \frac{E_t2 - E_t1}{EP(t2-t1) \times (t2-t1)} \quad (\text{Eq. 1})
\]

Where \((E_{t2} - E_{t1})\) is the enrichment increment of TG-bound palmitate from \(t_1\) to \(t_2\), and \(E_P\) \((t_2 - t_1)\) is the average precursor enrichment from \(t_1\) to \(t_2\).

The percentage contributions from plasma FFA, plasma TG, and IM lipids to IM precursor were calculated using the following equations (14):

\[
\% \text{ contribution from plasma FFA} = \left(\frac{E_{CoA_P}}{E_{PalP}}\right) \times 100\% \quad (\text{Eq. 2})
\]

\[
\% \text{ contribution from plasma TG} = \left[\left(\frac{E_{CoA_P}}{E_{PalP}}\right) - \left(\frac{E_{CoA_0}}{E_{Pal_0}}\right)\right] \times 100\% \quad (\text{Eq. 3})
\]

\[
\% \text{ contribution from IM lipids} = \left[1 - \left(\frac{E_{CoA_P}}{E_{PalP}}\right)\right] \times 100\% \quad (\text{Eq. 4})
\]

Statistics

Values are expressed as means ± SEM. Differences comparing two parameters from same muscle samples were tested by the paired \(t\)-test. Differences among three parameters were compared with ANOVA. The Tukey adjustment was used to adjust for multiple comparisons. We used SAS®9.2 software (SAS Institute, Cary, NC) for the analysis. A \(P\) value less than 0.05 was considered statistically significant.

RESULTS

The body weight of rabbits was 4.5 ± 0.1 kg. During the 3 h tracer infusion, the rabbits were maintained under stable physiological conditions and sufficient depth of anesthesia; mean rectal temperature was 37.7 ± 0.4°C; heart rate was 153 ± 2 beats/min; and mean arterial blood pressure was 63 ± 2 mmHg. Plasma concentrations of FFA and TG were 335 ± 71 and 191 ± 31 nmol/ml, respectively; palmitate accounted for 42 ± 7% and 47 ± 6% of the seven fatty acids measured, respectively.

The enrichment of plasma free palmitate increased rapidly after the start of U-13C16-palmitate infusion, and it reached a plateau after 1 h of tracer infusion (Fig. 1). Plasma enrichment of TG-bound palmitate increased gradually during tracer infusion (Fig. 1). The mean plasma free palmitate enrichment was 4.56 ± 0.95 mol% excess (MPE), which was calculated from the 5, 30, 60, 90, 120, 150, and 180 min enrichments.

The mean enrichments of IM free palmitate, PalCoA, and PalCn were calculated from the 5, 60, 120, and 180 min muscle samples. Because the enrichment of plasma free palmitate was much greater \((P < 0.0001)\) than that of the enrichment of IM free palmitate, IM PalCoA, or IM PalCn (Table 1), the plasma free palmitate was not an acceptable surrogate of IM precursor for lipid synthesis. The following comparisons were made among the three IM metabolites using ANOVA. In the muscle samples taken by the freeze-cut approach, the enrichment of PalCoA was lower \((P = 0.33)\) than that of PalCn (Fig. 2A and Table 1). In the muscle samples taken using the cut-freeze approach, the enrichment of PalCoA was lower \((P = 0.02)\) than that of PalCn (Fig. 2B). In both cases, IM free palmitate enrichment was much lower \((P < 0.0001)\) than

![Fig. 1. Isotopic enrichments of plasma free palmitate and plasma TG-bound palmitate. After the start of U-13C16-palmitate infusion, plasma free palmitate enrichment increased rapidly and reached a plateau after 1 h of tracer infusion. Plasma TG-bound palmitate enrichment was not detectable during the first 15-20 min of tracer infusion, but then increased gradually over time, indicating a time delay for the liver to take up labeled palmitate, synthesize it into TG, and secrete it as very low density lipoprotein.](image)

### Table 1. Intramuscular TG FSR calculated from different precursors

| Precursor | MPE (mol%) | Product MPE (mol%) | FSR (%/h) |
|-----------|------------|--------------------|-----------|
| **Freeze-cut** |            |                    |           |
| IM Palmitate | 0.70 ± 0.07* | 0.0124 ± 0.0045 | 0.62 ± 0.28* |
| IM PalCoA | 2.13 ± 0.17 | 0.0124 ± 0.0045 | 0.20 ± 0.02 |
| IM PalCn | 2.42 ± 0.16 | 0.0124 ± 0.0045 | 0.17 ± 0.02 |
| **Cut-freeze** |            |                    |           |
| IM Palmitate | 0.82 ± 0.10* | 0.0124 ± 0.0045 | 0.52 ± 0.04* |
| IM PalCoA | 2.21 ± 0.08* | 0.0124 ± 0.0045 | 0.19 ± 0.01 |
| IM PalCn | 2.77 ± 0.17 | 0.0124 ± 0.0045 | 0.15 ± 0.01 |

Values are means ± SEM. IM Palmitate, IM PalCoA, and IM PalCn indicate using IM free palmitate enrichment, IM PalCoA enrichment, and IM PalCn enrichment as precursor enrichment for calculating TG FSR, respectively. MPE, mol% excess.

*\(P < 0.0001\) versus PalCn and PalCoA.

*\(P < 0.05\) versus PalCoA.

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IM free palmitate enrichment as the precursor enrichment were greater ($P < 0.0001$) than the corresponding values measured using PalCoA enrichment or PalCn enrichment as the precursor enrichment in muscle samples taken by both the freeze-cut and cut-freeze approaches (Table 1). However, the FSR values calculated using IM PalCn enrichment as the precursor enrichment were not significantly ($P = 0.64$ for freeze-cut and $P = 0.94$ for cut-freeze) different from the corresponding FSR values calculated using IM Pal-CoA as the precursor enrichment.

In the muscle samples taken by the cut-freeze approach, IM FFA concentration was greater than that in muscle samples taken by the freeze-cut approach (Table 2). The concentrations of IM fatty acyl-CoA and fatty acyl-carnitine were comparable between the muscle samples taken by the freeze-cut and cut-freeze approaches (Table 2).

The enrichments of PalCn were $53.3 \pm 2.3\%$, $58.9 \pm 2.9\%$, and $60.8 \pm 3.8\%$ of the corresponding plasma free palmitate enrichment at 1, 2, and 3 h, respectively. The increasing percentages of IM PalCn enrichment to plasma free palmitate enrichment over time were explained by the gradual increase of plasma TG-bound palmitate enrichment (Fig. 1), which contributed labeled palmitate to the IM precursor via the action of lipoprotein lipase. If we extrapolate to 0 h using linear curve fitting, the percentage contribution of IM PalCn enrichment to plasma free palmitate enrichment was $50.2\%$, which represented the contribution of plasma FFA to IM precursor (see Eq. 2). At 3 h, the percentage of IM PalCn enrichment to plasma free palmitate enrichment was $60.8\%$, so plasma TG contribution was $10.6\%$ ($60.8\% - 50.2\%$) at 3 h. However, plasma TG-bound palmitate enrichment did not reach a plateau at 3 h. Based on exponential curve fitting, the plateau enrichment should be $3.88\%$, which was $69\%$ greater than the enrichment at 3 h ($2.29\%$). Thus, the maximal contribution of plasma TG to IM precursor was estimated to be $17.9\%$, which was the sum of $10.6\%$ at 3 h plus a further $69\%$ increase at the plateau of plasma TG-bound palmitate enrichment (see Eq. 3). The contribution of IM lipids to precursor was therefore $31.9\%$ (see Eq. 4).

![Figure 2](image1.png)

**Fig. 2.** IM free palmitate, PalCoA, and PalCn enrichment. A: In muscle samples taken by the freeze-cut approach, IM free palmitate enrichment was lower ($P < 0.0001$) than were PalCoA and PalCn enrichments. IM PalCoA enrichment was not significantly ($P = 0.33$) lower than PalCn enrichment. B: In muscle samples taken by the cut-freeze approach, the same results were obtained, except the enrichment difference between PalCoA and PalCn was significant ($P = 0.02$).

The enrichment of IM TG-bound palmitate, which was measured from the muscle samples taken by the freeze-cut approach, increased over time (Fig. 3). The enrichment increase appeared to be faster later than earlier during tracer infusion. FSRs calculated using the enrichments of IM free palmitate, PalCoA, and PalCn as the precursor enrichment were compared using ANOVA. The FSRs calculated using IM free palmitate enrichment as the precursor enrichment were greater ($P < 0.0001$) than the corresponding values measured using PalCoA enrichment or PalCn enrichment as the precursor enrichment in muscle samples taken by both the freeze-cut and cut-freeze approaches (Table 1). However, the FSR values calculated using IM PalCn enrichment as the precursor enrichment were not significantly ($P = 0.64$ for freeze-cut and $P = 0.94$ for cut-freeze) different from the corresponding FSR values calculated using IM Pal-CoA as the precursor enrichment.

In the muscle samples taken by the cut-freeze approach, IM FFA concentration was greater than that in muscle samples taken by the freeze-cut approach (Table 2). The concentrations of IM fatty acyl-CoA and fatty acyl-carnitine were comparable between the muscle samples taken by the freeze-cut and cut-freeze approaches (Table 2).

The enrichments of PalCn were $53.3 \pm 2.3\%$, $58.9 \pm 2.9\%$, and $60.8 \pm 3.8\%$ of the corresponding plasma free palmitate enrichment at 1, 2, and 3 h, respectively. The increasing percentages of IM PalCn enrichment to plasma free palmitate enrichment over time were explained by the gradual increase of plasma TG-bound palmitate enrichment (Fig. 1), which contributed labeled palmitate to the IM precursor via the action of lipoprotein lipase. If we extrapolate to 0 h using linear curve fitting, the percentage contribution of IM PalCn enrichment to plasma free palmitate enrichment was $50.2\%$, which represented the contribution of plasma FFA to IM precursor (see Eq. 2). At 3 h, the percentage of IM PalCn enrichment to plasma free palmitate enrichment was $60.8\%$, so plasma TG contribution was $10.6\%$ ($60.8\% - 50.2\%$) at 3 h. However, plasma TG-bound palmitate enrichment did not reach a plateau at 3 h. Based on exponential curve fitting, the plateau enrichment should be $3.88\%$, which was $69\%$ greater than the enrichment at 3 h ($2.29\%$). Thus, the maximal contribution of plasma TG to IM precursor was estimated to be $17.9\%$, which was the sum of $10.6\%$ at 3 h plus a further $69\%$ increase at the plateau of plasma TG-bound palmitate enrichment (see Eq. 3). The contribution of IM lipids to precursor was therefore $31.9\%$ (see Eq. 4).

**Table 2. Concentrations of intramuscular fatty acid metabolites**

|                  | Freeze-cut | Cut-freeze |
|------------------|------------|------------|
| **Intramuscular free fatty acids** |            |            |
| Palmitate        | 27.2 ± 2.0 | 44.8 ± 1.7 |
| % Pal            | 42.3 ± 0.9 | 46.2 ± 2.8 |
| Fatty acids      | 63.9 ± 3.7 | 99.0 ± 9.3 |
| **Intramuscular fatty acyl-CoA** |            |            |
| PalCoA           | 2.79 ± 0.19| 3.00 ± 0.19|
| % PalCoA         | 17.8 ± 1.2%| 17.9 ± 1.2%|
| Fatty acyl-CoA   | 15.8 ± 1.1 | 17.2 ± 1.8 |

**Table 3. Concentrations of intramuscular fatty acid metabolites**

|                  | Freeze-cut | Cut-freeze |
|------------------|------------|------------|
| **Intramuscular fatty acyl-carnitine** |            |            |
| PalCn            | 0.39 ± 0.05| 0.37 ± 0.08|
| % Pal            | 22.5 ± 1.1%| 22.0 ± 1.1%|
| Fatty acyl-Cn    | 1.77 ± 0.23| 1.45 ± 0.22|

Values are means ± SEM. Unit is nmol/g for concentrations. Palmitate in the seven fatty acids measured is expressed as percentage.

*P < 0.05 versus freeze-cut by paired t-test.
DISCUSSION

We found that IM PalCoA and PalCn enrichments were approximately 50% of plasma free palmitate enrichment. This reflects that there must have been active intracellular lipid breakdown whereby released unlabeled palmitate diluted the precursor enrichment. IM lipid breakdown was calculated to account for 31.9% of IM precursor, and plasma FFA and TG accounted for 50.2% and 17.9% of precursor, respectively. Because the palmitate enrichment in IM TG could be regarded as fundamentally unlabeled in relation to the precursor enrichment (Table 1), and palmitate enrichment in plasma TG was lower than that of plasma free palmitate (Fig. 1), the lower enrichment of the IM precursor can only be attributed to intracellular lipid breakdown. Thus, the use of intracellular free palmitate enrichment as the precursor enrichment would have overestimated TG FSR by a minimum of approximately 300%, and perhaps even more, because of the difference in the free IM palmitate enrichment as compared with the PalCn enrichment.

The lower enrichment of IM free palmitate as compared with those of PalCoA and PalCn indicates that there was continuous lipid breakdown during muscle sampling and processing. Muscle sampling instantly stopped the blood supply (and delivery of tracer) to the tissue. Whereas entry of labeled palmitate into the tissue was stopped with excision of the tissue, the process of lipid breakdown and release of unlabeled palmitate into the IM compartment was not necessarily stopped. Continued lipolysis could explain a greater reduction in free palmitate enrichment than either PalCoA or PalCn due to the more rapid turnover time of the free IM palmitate pool. It is also possible that if PalCoA and PalCn continued to be formed after sampling, then the enrichment of those compounds would also have been diluted.

Theoretically, the true precursor for IM TG synthesis is fatty acyl-CoA (18). When fatty acids enter the intracellular pool, the first step is to be converted to metabolically active fatty acyl-CoA, catalyzed by fatty acyl-CoA synthase and synthetase (19, 20). Our recent results from a complementary experiment demonstrated that the transport of fatty acids into the IM compartment and their subsequent conversion to fatty acyl-CoA and fatty acyl-carnitine occurred very fast, with the peak enrichment within 2 min after a bolus palmitate tracer injection (unpublished data). Thus, the diluted enrichment of IM free palmitate would readily dilute PalCoA enrichment. Because PalCn is a downstream metabolite of PalCoA (21), PalCn is less affected by in vitro lipid breakdown than IM free palmitate. The same explanation applies to the observation that PalCoA enrichment was lower than PalCn enrichment from the muscle samples taken by the cut-freeze approach. The freeze-cut technique decreased the enrichment difference to an insignificant level (Fig. 2 and Table 1), indicating that the in vitro lipid breakdown was responsible for the difference in enrichment in the cut-freeze samples. This finding is consistent with our previous report in which the freeze-cut technique eliminated enrichment difference between PalCoA and PalCn (14).

Tracer dilution could occur at several steps of muscle sampling and processing. When we excised a muscle sample, several seconds were required to submerge it in liquid nitrogen, and freezing was not instantaneous in liquid nitrogen. We used a metal net to freeze the muscle samples when using the cut-freeze approach. This procedure eliminated the time span needed to transfer the sample to a cryogenic tube, and it shortened the time span of being frozen. However, there was still an unavoidable time delay from muscle excision to freezing. Further, when we weighed the muscle powder on a digital scale and added internal standards to the sample vial, the muscle samples might no longer have been in a completely frozen state. Although we made every possible effort to minimize the time span in which the samples were not frozen (such as using dry ice to keep samples cold during the procedure), it was not possible to completely avoid exposing the samples to a temperature change during processing.

The magnitude of dilution of the IM free palmitate can be explained by the large concentration difference between IM lipid-bound palmitate and IM free palmitate. In our previous experiment, IM TG was measured to be 11.6 ± 2.4 μmol/g in muscle from lean rabbits (14). In another experiment, we found that palmitate accounted for ~30% of total fatty acids in muscle TG (unpublished data). Thus, the concentration of IM TG-bound palmitate would be 10.44 μmol/g. If we regard the PalCn enrichment represented in vivo precursor enrichment, the IM free palmitate concentration in Table 2 overestimated the in vivo concentration by the amount of unlabeled palmitate released from in vivo lipolysis. We estimated that the true in vivo IM free palmitate to be ~10 nmol/g [measured free palmitate concentration × (measured free palmitate enrichment / PalCn enrichment)]. This means that the pool size (or concentration) of IM TG-bound palmitate was approximately 1000-fold that of IM free palmitate. If we included the possible in vitro breakdown of muscle phospholipids and diacylglycerol, the difference between total IM lipid-bound palmitate and free palmitate would be even greater. Thus, it is not surprising that in vitro lipid breakdown markedly diluted IM free palmitate enrichment if the lipolytic process was not instantly quenched during muscle biopsy. On the other hand, the concentration difference between IM free palmitate and PalCoA and between PalCoA and PalCn were much smaller (3- to 7-fold, Table 2). Therefore, the dilution effect of free palmitate on PalCoA, and of PalCoA on PalCn decreased in sequence. In the muscle samples taken by the freeze-cut approach, the enrichments of PalCoA and PalCn were not significantly different (Table 1), indicating minimal effect of in vitro lipid breakdown. In the muscle samples taken by the cut-freeze approach, IM PalCoA enrichment was lower than PalCn enrichment (P < 0.05; see Table 1 and Fig. 2). Thus, IM PalCn is an acceptable surrogate for precursor enrichment. Because of severe trauma caused by the freeze-cut approach, the acceptability of PalCn enrichment as the precursor enrichment is meaningful when the freeze-cut technique is not applicable.
Our data do not allow us to exclude the possibility of IM fatty acid compartmentation. If plasma-derived palmitate favors PalCoA formation and if IM lipid-derived palmitate favors IM free palmitate, the IM free palmitate enrichment could be substantially lower than PalCoA and PalCn enrichments. However, compartmentation could not explain the enrichment difference between PalCoA and PalCn when the cut-freeze approach was used (Fig. 2B), nor could it explain the elimination of the enrichment difference by the freeze-cut approach (Fig. 2A). Thus, the tracee dilution from in vitro lipid breakdown during sampling and processing is a reasonable explanation. Fatty acid compartmentation is also possible. Neither of these explanations supports the use of IM free palmitate enrichment as a surrogate of precursor enrichment. The fatty acid metabolic pathways and dilution are depicted in Fig. 4.

The acceptability of PalCn as a surrogate precursor was confirmed by the FSR values calculated using different IM enrichments as the precursor enrichment (Table 1). IM TG FSRs calculated using IM free palmitate enrichment as the precursor enrichment were greater ($P < 0.0001$) than the corresponding value calculated using PalCoA enrichment or PalCn enrichment as the precursor enrichment (Table 1). However, FSRs calculated using PalCoA enrichment were not significantly different ($P = 0.94$ for freeze-cut and $P = 0.64$ for cut-freeze) than the corresponding values from PalCn enrichment. The variable enrichments in the IM TG-bound palmitate (i.e., product enrichment) eliminated the significant difference between IM PalCoA and PalCn enrichments in the muscle samples taken by the cut-freeze approach, so the calculated FSRs were not significantly different ($P = 0.64$).

Note that the enrichments of IM PalCoA and PalCn increased over time during a constant tracer infusion (Fig. 2A, B). Such a precursor enrichment pattern was consistent with that of product enrichment, which increased faster later than earlier during tracer infusion (Fig. 3). This can be explained by the increase in plasma TG-bound palmitate enrichment over time (Fig. 1). At the beginning of tracer infusion, plasma free palmitate was the only source of tracer entering the IM precursor pool. Thereafter, plasma TG-bound palmitate enrichment increased gradually, which also contributed labeled palmitate to the IM precursor pool through the action of lipoprotein lipase (22, 23). Thus, it was proper to calculate the mean precursor enrichment over the 3 h infusion period. In contrast, if only one muscle sample is taken at the end of tracer infusion as some authors reported (10, 24), the measured precursor enrichment would overestimate the precursor enrichment to an unknown extent, which depends on the activity of lipoprotein lipase. This potential error was overlooked in previous publications in which the one biopsy approach was used (10, 24).

A limitation in this experiment was that the IM TG FSR value might reflect mixed TG in both intracellular and interstitial pools. We dissected the adductor muscle before taking biopsies and checked five representative muscle specimens under the stereomicroscope to ensure no adipose tissue contamination occurred during our biopsy procedure. However, we did not dissect the muscle specimens to remove the interstitial tissue when measuring IM TG-bound palmitate enrichment. We did not perform dissection because it would have required a longer period during which the sample was not frozen, thereby inducing rapid changes in intracellular enrichment.

In summary, active breakdown in the muscle complicates the measurement of intracellular concentration and enrichment of lipids and lipid products. Lipid breakdown releases unlabeled palmitate, which results in an enrichment gradient from plasma to the IM compartment. Consequently, plasma free palmitate overestimates the IM precursor enrichment. During muscle sampling and processing, the IM lipid breakdown may continue to release unlabeled palmitate to dilute the precursor enrichment in the sequence of IM free palmitate, PalCoA, and PalCn. The optimal approach to minimizing the in vitro lipid breakdown is to use the freeze-cut technique for muscle sampling and to avoid sample thawing during processing. If the direct measurement of IM PalCoA enrichment is problematic, IM PalCn enrichment is an acceptable surrogate for the precursor enrichment because PalCn is less affected by...
in vitro lipid breakdown. When plasma free palmitate enrichment is used as the precursor enrichment, the resultant FSR represents the use of plasma FFA for synthesizing IM TG, which does not include the portion of TG synthesis that uses fatty acids derived from IM lipid breakdown and from plasma TG breakdown through the action of lipoprotein lipase.

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