Determination of Skeletal Muscle Triglyceride Synthesis Using a Single Muscle Biopsy

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A novel stable isotopic technique for the determination of triglyceride synthesis in skeletal muscle by using a single muscle biopsy has been developed and evaluated in rats. In previous studies using ^{13}C-tracers, muscle triglyceride synthesis is usually determined using at least 2 biopsies, the first of which serves as the baseline sample for the measurement of natural ^{13}C abundance. In the present studies, the baseline biopsy has been eliminated by making the use of the isotopic information of a nontraced fatty acid in the muscle triglyceride pool. This is based on the fact that the source and, hence, the natural ^{13}C abundance of fatty acids in the same triglyceride pool is similar. To demonstrate and validate the method, a series of rat studies have been conducted to have established that (1) the natural ^{13}C abundance of 4 major fatty acids in the muscle triglyceride pool is similar; (2) there are no ^{13}C-label exchanges between fatty acids in the lipid pool; and (3) the incorporation of ^{13}C-palmitate into muscle triglycerides determined using this technique favorably compared with that determined by the traditional method. This approach makes stable isotope studies possible in which more than 1 muscle biopsy is difficult or impossible. Therefore, it has the potential to facilitate investigation of triglyceride metabolism in the skeletal muscle.

INTRAMYOCELLULAR TRIGLYCERIDE (imcTG) of skeletal muscle is an important energy source for muscle work. However, its metabolism remains poorly understood due primarily to technical difficulties involved with the collection of muscle samples. The difficulties stem from 2 sources. The first is contamination of imcTG by adipocytes associated with skeletal muscle,[1] which has the potential to introduce large errors. However, it has been established that this contamination issue can be resolved by using microdissection techniques.[1] The second source of difficulty relates to muscle biopsying itself. Unlike other tissues, such as blood or fat, the biopsying of muscle tissue triggers significant immediate or postbiopsy pains and has the potential to affect muscle functions. For example, in small animals, biopsying may introduce stress that will affect the results. This can potentially compromise a 2-biopsy experiment in which all measurements are made after the first biopsy. In humans, muscle biopsying often results in complaints by the subjects after the procedure despite careful anesthetization. Although the 2-biopsy approach has been used in many studies,[2,3] it is estimated that the concerns and resulting complications have hindered the investigation of muscle metabolism. One possible way to circumvent this problem would be to use 2 separate study groups, each used for collecting 1 of the 2 muscle biopsies, while both subjecting to an identical experimental protocol. However, this approach inevitably necessitates doubling the sample size. Even more important, this approach loses the greater statistical power inherent with collecting both samples from the same individuals. Therefore, to reduce the number of biopsies will enable one to simplify study protocols and improve the reliability of data. More importantly, this can facilitate studies of muscle metabolism.

Because the first muscle biopsy (the baseline) in the 2-biopsy protocol is only used to obtain the knowledge of natural ^{13}C abundance of the traced fatty acid (eg, palmitate when ^{13}C-palmitate is used as the tracer), other nontraced fatty acids in the same imcTG pool can be used for the same purpose provided that they have the same natural ^{13}C abundance. The chemical nature of TG (containing multiple component fatty acids) makes this uniquely possible. For example, if stearate, oleate, or linoleate (nontraced) of muscle TG have the same natural ^{13}C abundance as that of palmitate (traced), and if there is no ^{13}C-label transfer from ^{13}C-palmitate to these nontraced fatty acids, the ^{13}C enrichment of the nontraced fatty acids during or after isotope infusion will reflect that of palmitate that was before the infusion (its baseline). Thus, the baseline biopsy is eliminated.

Because the validity of this assumed single biopsy approach depends on the 2 preconditions, their confirmation by experimentation is critical. Therefore, the present studies were designed to determine whether the natural ^{13}C abundance of various fatty acids in imcTG pool is similar and whether ^{13}C label exchange occurs between the traced fatty acid and other nontraced in the same lipid pool.

THE PRINCIPLE AND HYPOTHESIS

A baseline biopsy that is traditionally required for the determination of imcTG synthesis is used solely for the purpose of knowing the initial ^{13}C enrichment of the traced fatty acid (usually at the natural abundance or 1.1%). This value is then subtracted from the ^{13}C enrichment of the same fatty acid from the second biopsy after being exposed to the tracer (thus higher enrichment). Because the baseline sample is only used as a reference, we thought alternatives of using other fatty acids instead to the baseline biopsy may exist. This is because all fatty acids in a lipid pool have the same origin and hence similar natural ^{13}C abundance, and the ^{13}C abundance of other nontraced fatty acids could remain unchanged during isotope infusion if label exchange is absent. If so, a nontraced fatty acid in the imcTG pool from the second muscle biopsy, collected after being exposed to the isotope, could be used as a surrogate

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to serve the same purpose and, therefore, the first biopsy can be spared. For example, when ^{13}C-palmitate is the tracer, other fatty acids could be used as the surrogate. To be qualified as a surrogate fatty acid, however, 2 conditions must be met: first, it must have the same or similar ^{13}C abundance as the traced one prior to the isotope administration; and second, during the isotope infusion, the label does not exchange with the surrogate fatty acid. Although conceptually fatty acid elongation or desaturation may complicate the matter, skeletal muscle is considered an 'inactive' tissue in these functions.[4,5] Although the liver could be a significant source of fatty acid carbon exchange, this would not be expected to be an issue for typical tracer studies, because such tracer infusion is usually in relatively short term (minutes to hours).

MATERIALS AND METHODS

Animals

Sprague Dawley male rats (body weight, 300 to 400 g) were used throughout the studies. The animals were purchased from Harlan Sprague Dawley (Indianapolis, IN). Upon receiving, the animals were fed standard rat chow (Purina, Richmond, IN) ad libitum. After at least 2 weeks, the rats were killed for muscle biopsy from quadriceps, gastrocnemius, and soleus muscle either under the normal feeding regimen (fed, n = 5) or after a fast for 24 hours (fasted, n = 5) (Protocol 1). In a separate validation study (Protocol 2), additional fasted rats (n = 4) of the same breed were used in experiments in which stable isotope-labeled fatty acids were infused to determine fatty acid incorporation into imcTG. All the animal study protocols were approved by Mayo Foundation Institutional Animal Care and Use Committee.

Tracers

Stable isotopes [U-^{13}C]palmitate and [1-^{13}C]oleate (both 99% isotopic purity) were purchased from Isotec (Miamisburg, OH).

EXPERIMENTAL PROTOCOLS

Protocol 1: Determination of Natural ^{13}C Abundance of Fatty Acids

In the morning, the rats were placed in a wire-bottomed rat-restraining cage and conditioned for at least 1 hour. A blood sample of 0.5 to 1 mL was then collected from a tail vein, gently but without anesthesia. Plasma was separated from the whole blood by centrifugation (3,000 rpm for 15 minutes) and saved at -80°C for free fatty acid extraction. The animals were then euthanized by an intraperitoneal (IP) injection of a lethal dose of pentobarbital. Soleus, gastrocnemius, and quadriceps muscles were excised, dissected of associated fat, and saved immediately in liquid N_2 and then transferred to a freezer (-80°C) for later lipid extraction and analysis.

Protocol 2: Testing of Label Exchange During Isotope Infusion

In the morning, rats were anesthetized by pentobarbital (IP, 50 mg/kg) and a carotid catheter was placed surgically. The surgical sites were immediately closed using sterile techniques. Then, while the rats were still anesthetized, a small (≈ 50 mg) muscle biopsy was taken from quadriceps (0 minute, the baseline biopsy). The wound was immediately closed with sutures and covered with a piece of sterile gauze and taped. After the rats woke up, they were allowed to recover for at least 30 minutes. Then, after a blood sample was collected via the carotid catheter, a unprimed, simultaneous infusion of [U-^{13}C]palmitate (0.05 μmol/kg/min) and [1-^{13}C]oleate (0.08 μmol/kg/min) was started via a sterile infusion line into a tail vein. After 2 hours, while the tracer infusions were continued, a small biopsy was taken from soleus and gastrocnemius from 1 leg under local anesthesia (lidocaine infiltration). The wounds were immediately closed and covered using sterile techniques. Three blood samples were collected from the carotid catheter during a 30-minute period immediately before this muscle biopsy. The tracer infusion continued for another 2 hours. Immediately before ending the tracer infusion at 240 minutes, another 3 blood samples at the same intervals were collected. The animals were then euthanized by a lethal dose of pentobarbital. Entire soleus and a large gastrocnemius biopsy were collected on the contralateral leg. Blood samples were placed on ice upon collection and shortly plasma was separated by centrifugation and saved in a freezer. All muscle samples were chilled in liquid N_2 immediately upon biopsy and transferred to a freezer for later analysis.

Lipid analysis. Frozen muscle samples were thawed and dissected of adipocytes using microdissection techniques.[1] The dissected muscle samples were thoroughly pulverized into fine powder on dry ice by a stainless steel mortar and pestle. The muscle powder was then extracted for total lipids by the method of Folch.[6] TGs and nonesterified fatty acids (NEFA) were purified from the extracted muscle lipids by high-performance liquid chromatography (HPLC).[7] The NEFA fraction was further subjected to silicic acid to remove phospholipids.[8] The purified NEFA were derivatized into methyl esters,[8] and the TGs (imcTG) were directly transmethylated.[8] The fatty acid methyl esters were analyzed by a gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) to determine ^{13}C content.[9]

Plasma total lipids were extracted by the method of Dole[10] and subjected to the same HPLC procedures as described above to purify free fatty acids (FFA). The purified plasma FFA were derivatized to methyl esters using the same methods as above for purified muscle NEFA and analyzed by GC/C/IRMS.

GC/C/IRMS Operation

The detailed procedures for the determination of ^{13}C enrichment of fatty acids by high precision GC/C/IRMS have been described previously.[9] Briefly, a high precision GC/C/IRMS instrument (delta S series, Finnigan, San Jose, CA) was used to determine the isotope ratio of ^{13}C/^{12}C of fatty acids in the form of CO_2 by means of online oxidation. The instrument has a precision (standard deviation, SD) of 0.0002 atom percent (AP), equivalent to 2 ppm of ^{13}C.[11] The lower detection limit is therefore less than 0.001 AP (10 ppm). The fatty acid methyl esters were injected into the GC (split-less) by an auto sampler and separated by a DB-23 capillary column. The GC program is as follows: initial temperature 80°C for 0.5 minute, then increased to 185°C at 2°C/min, and immediately ramped up again at 25°C/min to 250°C and maintained at the temperature for 3 minutes. The injection port was at 250°C. Palmitate, oleate, stearate, and linoleate peaks were selectively fed into the online oxidizer where the fatty acids were combusted into CO_2 gas. The CO_2 was quantitatively transferred into an online IRMS where the ^{13}C/^{12}C ratio was determined. The ^{13}C enrichment was expressed in δ ^{13}C per mil (‰) notation, and it was converted to AP offline.[9]

Determination of imcTG synthesis rate. The synthesis rate of imcTG is expressed by the incorporation of [U-^{13}C]palmitate into imcTG pool at 120 and 240 minutes of the tracer infusion. For the determination of tracer incorporation using the traditional 2-biopsy method, the ^{13}C enrichment of imcTG-palmitate from gastrocnemius and soleus collected at 120 and 240 minutes was subtracted by that of imcTG-palmitate from the baseline sample quadriceps. For the new method, the ^{13}C abundance of imcTG-stearate from the muscle biopsy collected at 120 and 240 minutes was subtracted from that of imcTG-palmitate from the same biopsy. The differences resulting from all of these

Table 1. Variability of ^{13}C Analysis by GC/C/IRMS and Muscle imcTG Fatty Acid ^{13}C Abundance Among Animals

Fatty Acid	GC/C/IRMS	Interanimal
Palmitate	0.17 ± 0.03	0.25 ± 0.03
Oleate	0.19 ± 0.02	0.19 ± 0.02
Linoleate	0.23 ± 0.03	0.30 ± 0.06
Stearate	0.35 ± 0.06	0.50 ± 0.05

NOTE. Values (mean ± SEM) are in δ ^{13}C (‰). The data for GC/C/IRMS variability are the mean of standard deviations for plasma FFA, muscle NEFA, and imcTG fatty acid ^{13}C abundance in fasted rats. The means are equivalent to 0.0001 to 0.0004 AP. The data for interanimal variation are calculated from the average standard deviations of ^{13}C abundance of fatty acids in quadriceps, soleus, and gastrocnemius imcTG of fasted rats. The means are equivalent to 0.0002 to 0.0005 AP. The values for plasma FFA and muscle NEFA (not shown) are in similar ranges (n = 5).

Abbreviations: NEFA, nonesterified fatty acid; FFA, free fatty acid; AP, atom %.

subtractions were taken as the incorporation of [U-^{13}C]palmitate into the imcTG pool. The [U-^{13}C]palmitate incorporations were presented as both AP, as well as the international standard δ ^{13}C notation (‰).

Statistical Analysis

Analysis of variance (ANOVA) was used to compare differences among more than 2 fatty acids. Paired Student's *t* test was used to compare 2 fatty acids. Values are mean ± SEM unless otherwise indicated.

RESULTS

Instrumental Precision of ^{13}C Analysis and Interanimal Variation of Natural ^{13}C Abundance

Table 1 depicts the variability of ^{13}C analysis by GC/C/IRMS and interanimal variability of fatty acid natural ^{13}C abundance for 3 muscles from fasted rats without isotope administered. As shown in Table 1, the instrumental variability of ^{13}C analysis by GC/C/IRMS for biologic compounds is typical of the instrument, usually $<0.5‰$.[11] This corresponds to less than 0.0005 AP or less than 5 ppm. The average variability of natural ^{13}C abundance for 4 major muscle TG fatty acids was slightly higher than the instrumental variation, up to 0.5‰ or 0.0005 AP.

Natural ^{13}C Abundance of imcTG Fatty Acids

Table 2 shows the natural ^{13}C abundance of imcTG-palmitate, -oleate, -linoleate, and -stearate in quadriceps, gastrocnemius, and soleus muscle from fasted and fed rats. In fasted rats, the natural ^{13}C abundance of palmitate, oleate, linoleate, and stearate are similar across all 3 muscles with a maximum difference (between oleate, the highest, and stearate, the lowest) of 0.0040, 0.0038, and 0.0040 AP for quadriceps, gastrocnemius, and soleus, respectively. The differences among the 3 fatty acids other than oleate were much smaller, 0.0015, 0.0016, and 0.0017 AP for the same muscles, respectively. These differences represent only less than 2% of the ^{13}C enrichment that is achieved with the infusion of a ^{13}C fatty acid at a low rate.[8] Although the maximum differences between oleate and stearate were statistically significant ($P < .05$), chiefly because of the high precision of ^{13}C analysis by GC/C/IRMS (Table 1), the magnitude of the differences represents less than 5% of the enrichment that is achieved with the infusion of the same ^{13}C fatty acid.[8]

The natural ^{13}C abundance of imcTG-fatty acids in the fed rats was approximately 0.003 AP higher than that in the fasted rats (Table 2). Presumably, this difference likely reflects incorporation of dietary fatty acid carbons that had a greater concentration of ^{13}C. However, the difference between the 4 imcTG fatty acids remained small (even less than that seen in the fasted rats). The maximum difference was 0.0016 and 0.0004 AP for quadriceps and soleus, respectively, and 0.0047 AP for gastrocnemius. However, none of the differences in ^{13}C abundance of the imcTG fatty acids was significant ($P > .05$). The variations in ^{13}C abundance for the same fatty acids from fed rats were noticeably larger than those from the fasted rats, probably reflecting chemical/isotopic nonequilibrium in the midst of fatty acids inflows after food ingestion. Overall, however, the ^{13}C abundance of the 4 major imcTG fatty acids was markedly similar.

Table 2. The Natural ^{13}C Abundance of Muscle TG Fatty Acids in Rats on Regular Chow

		Fasted		Fed	
Muscle	Fatty Acid	δ ^{13}C, ‰	AP	δ ^{13}C, ‰	AP
Quadriceps	Palmitate	−27.6 ± 0.6	1.0808 ± 0.0003	−24.3 ± 0.1	1.0844 ± 0.0012
	Oleate	−25.4 ± 0.5	1.0833 ± 0.0002	−24.0 ± 0.1	1.0848 ± 0.0012
	Linoleate	−29.1 ± 0.4	1.0793 ± 0.0002	−25.5 ± 0.6	1.0832 ± 0.0007
	Stearate	−29.1 ± 1.2	1.0793 ± 0.0006	−25.3 ± 1.5	1.0834 ± 0.0017
Gastrocnemius	Palmitate	−27.8 ± 0.6	1.0806 ± 0.0003	−22.8 ± 0.9	1.0861 ± 0.0010
	Oleate	−25.8 ± 0.3	1.0828 ± 0.0002	−22.3 ± 1.9	1.0867 ± 0.0021
	Linoleate	−28.2 ± 0.9	1.0802 ± 0.0004	−26.6 ± 1.3	1.0820 ± 0.0015
	Stearate	−29.4 ± 1.2	1.0790 ± 0.0006	−24.7 ± 1.0	1.0840 ± 0.0011
Soleus	Palmitate	−27.3 ± 0.4	1.0812 ± 0.0002	−24.7 ± 1.8	1.0840 ± 0.0020
	Oleate	−27.3 ± 0.4	1.0833 ± 0.0002	−25.1 ± 2.0	1.0836 ± 0.0022
	Linoleate	−27.8 ± 0.7	1.0806 ± 0.0004	−25.1 ± 2.0	1.0836 ± 0.0022
	Stearate	−29.0 ± 0.9	1.0793 ± 0.0004	−25.0 ± 2.2	1.0837 ± 0.0024

NOTE. The fatty acid methyl esters for each muscle, prepared as described in Materials and Methods, were analyzed by GC/C/IRMS. The results, in δ ^{13}C (‰), were transformed to AP offline. The abundance was not corrected for the negligible isotopic dilution caused by the added methyl carbon in the derivative. N = 5 for each group.

Table 3. The Natural ^{13}C Abundance of Plasma FFA in Fasted and Fed Rats on Regular Chow

	Fasted		Fed	
Fatty Acid	δ ^{13}C, ‰	AP	δ ^{13}C, ‰	AP
Palmitate	−29.1 ± 0.4	1.0793 ± 0.0004	−26.3 ± 0.3	1.0823 ± 0.0003
Oleate	−26.6 ± 0.2	1.0819 ± 0.0002	−25.9 ± 0.6	1.0827 ± 0.0006
Linoleate	−28.9 ± 0.3	1.0794 ± 0.0003	−29.7 ± 1.0	1.0786 ± 0.0011
Stearate	−28.8 ± 0.3	1.0796 ± 0.0004	−27.4 ± 0.8	1.0810 ± 0.0008

NOTE. The plasma FFA were purified and derivatized to methyl esters as described in Materials and Methods. Isotopic analysis and data treatment are the same as in Table 2. Data are from the same animals.

Of particular note is that the natural ^{13}C abundance of every imcTG fatty acid across the 3 muscles was extremely similar, especially in the fasted rats. The standard error for the natural ^{13}C abundance between quadriceps, gastrocnemius, and soleus in fasted rats was 0.00015 AP for palmitate and oleate, 0.0004 AP for linoleate, and 0.00016 AP for stearate, and the corresponding values for fed rats were 0.0006, 0.0009, 0.0005, and 0.0002 AP.

Natural ^{13}C Abundance of Plasma FFA

Table 3 shows the natural ^{13}C abundance of plasma palmitate, oleate, linoleate, and stearate from fasted and fed rats. These plasma samples were taken from the animals described in Table 2. The natural ^{13}C abundance of palmitate, linoleate, and stearate in plasma FFA of fasted rats was almost identical, whereas that of plasma oleate was approximately 0.002 AP higher. The difference between oleate and the other plasma FFA was statistically significant ($P < .001$), again, due mainly to the high precision of GC/C/IRMS analysis (Table 1). In the fed rats, the ^{13}C abundance of plasma palmitate, oleate, and stearate was very similar with a maximum difference of 0.0016 AP, whereas linoleate was approximately 0.004 AP lower. The difference among the 4 acids was statistically significant ($P = .02$).

Natural ^{13}C Abundance of Intramyocellular NEFA

To gain insight into the isotopic dynamics among fatty acids in different muscle lipid pools, the ^{13}C abundance of muscle NEFA pool in fed rats was also analyzed. As seen in Table 4, the magnitude of the difference in the natural ^{13}C abundance of muscle NEFA was similar to that observed for the imcTG counterparts, with the maximum difference of 0.0036, 0.0036, and 0.0034 AP for quadriceps, gastrocnemius, and soleus, respectively. Excluding palmitate (the highest), the difference among other 3 NEFAs was only 0.0022, 0.0017, and 0.0024 AP for the same muscles, respectively.

Table 4. The Natural ^{13}C Abundance of Muscle NEFA in Fed Rats

Muscle	Fatty Acid	δ ^{13}C, ‰	AP
Quadriceps	Palmitate	−26.3 ± 0.8	1.0823 ± 0.0009
	Oleate	−27.6 ± 0.2	1.0809 ± 0.0002
	Linoleate	−29.6 ± 0.4	1.0787 ± 0.0005
	Stearate	−27.8 ± 0.2	1.0806 ± 0.0002
Gastrocnemius	Palmitate	−25.9 ± 0.3	1.0827 ± 0.0007
	Oleate	−27.8 ± 0.5	1.0807 ± 0.0005
	Linoleate	−29.2 ± 0.5	1.0791 ± 0.0005
	Stearate	−27.6 ± 0.4	1.0808 ± 0.0003
Soleus	Palmitate	−26.0 ± 0.1	1.0826 ± 0.0001
	Oleate	−26.9 ± 0.5	1.0816 ± 0.0006
	Linoleate	−29.1 ± 0.2	1.0792 ± 0.0003
	Stearate	−27.7 ± 0.2	1.0807 ± 0.0003

NOTE. The sample preparation and analyses and data treatment are the same as in Table 2. Data are from the same animals.

Abbreviation: NEFA, nonesterified fatty acids.

Like that seen for imcTG fatty acids, the ^{13}C abundance of the same fatty acids in the muscle NEFA pool across the 3 muscles was remarkably similar. The standard error was 0.00013, 0.00029, 0.00016, and 0.00006 AP for palmitate, oleate, linoleate, and stearate, respectively.

13C Label Exchange

[U-^{13}C]palmitate and [1-^{13}C]oleate were simultaneously infused in fasted rats for 4 hours to test whether the carbon labels exchange with other untraced fatty acids in the imcTG and NEFA pools. Muscle samples were collected at 2 and 4 hours of the tracer infusion to monitor changes in ^{13}C abundance of linoleate and stearate (nontraced). It was hypothesized that if ^{13}C accumulates in these nontraced fatty acids during the tracer infusion, label exchange was operating between the tracer(s) and linoleate or stearate. However, if ^{13}C accumulation is absent in these acids, that would be a strong indication that there is no label exchange from the ^{13}C labeled carbon(s) in oleate and palmitate to linoleate or stearate.

The results for imcTG fatty acids are shown in Fig 1. During the tracer infusion, the ^{13}C enrichment of imcTG palmitate and oleate in gastrocnemius and soleus progressively increased from 0 minutes (based on the baseline quadriceps sample) to 120 minutes and further to 240 minutes. In other words, the accumulation of the 2 fatty acid tracers in imcTG pool was continuous during both intervals of the tracer infusion. The magnitude of the increase, however, was much greater for palmitate than that for oleate. This is because [U-^{13}C]palmitate was uniformly labeled (hence there was no intramolecular label dilution when the acid is combusted to CO_2), whereas [1-^{13}C]oleate was singly labeled (hence a label dilution rate of 18).

In contrast, the ^{13}C abundance of imcTG stearate remained unchanged during the same intervals in both muscle (Fig 1). The ^{13}C abundance of imcTG-linoleate in gastrocnemius at 120 minutes appeared higher than the basal (0 minutes) value, but it returned to the basal level at 240 minutes (Fig 1). This suggested that the differences were more likely due to some random variations rather than label exchange. The ^{13}C of

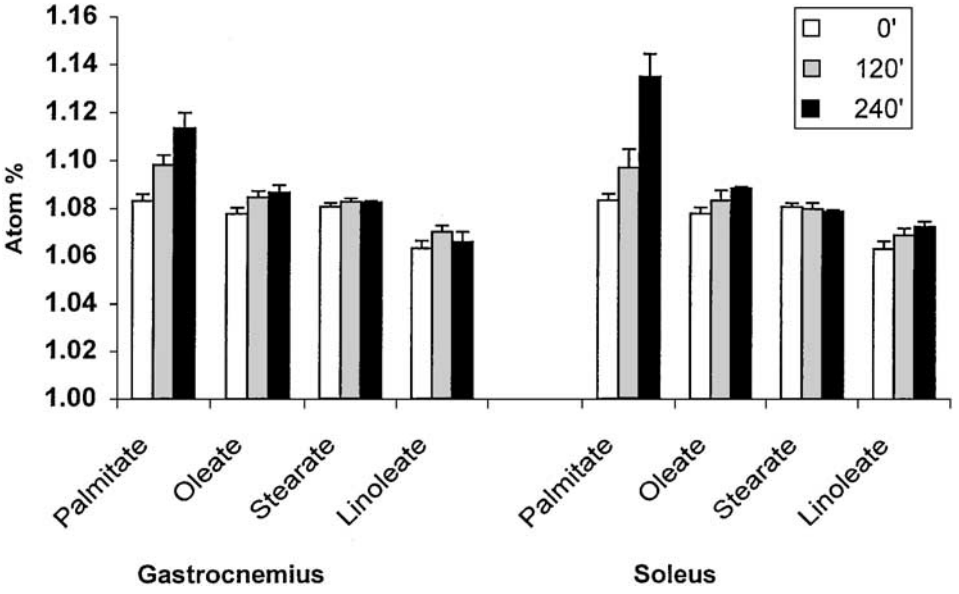

Fig 1. The changes in ^{13}C enrichment of rat imcTG fatty acids during continuous, unprimed infusion of [U-^{13}C]-palmitate and [1-^{13}C]oleate in awake rats. All of the basal values (0 minutes) were taken from quadriceps that were biopsied before the tracer infusion began. Soleus and gastrocnemius were both biopsied at 120 and 240 minutes to monitor the isotopic changes. Data (mean ± SEM) are from 4 rats fasted for 24 hours.

imcTG-linoleate in soleus appeared to have increased continuously at 120 and 240 minutes (see Discussion).

Figure 2 shows the ^{13}C enrichment of the same fatty acids in the muscle NEFA pool. Similarly, the ^{13}C enrichment of palmitate and oleate in this pool increased in the same pattern as that seen for their imcTG counterparts. Again, however, there was no increase in ^{13}C enrichment of linoleate or stearate in gastrocnemius or soleus over the basal level.

The dynamic changes in ^{13}C enrichment during the tracer infusion were more profound in plasma palmitate and oleate (Fig 3). Unlike that seen for intramyocellular fatty acids, on the other hand, the ^{13}C enrichment of these 2 plasma fatty acids reached a plateau at 120 minutes and did not increase further thereafter, reflecting much faster equilibration within the plasma FFA pool. In contrast, the ^{13}C enrichment of plasma stearate and linoleate did not change during the tracer infusion, similar to that seen for their intramyocellular counterparts.

Comparison of Isotope Incorporation Into imcTG Determined by the Two Methods

To test whether the new method will produce the same result as the traditional 2-biopsy method, the rate of incorporation of [U-^{13}C]palmitate into imcTG of gastrocnemius and soleus was determined by using imcTG stearate as the surrogate fatty acid. The result was then compared with that determined by the traditional method by using the baseline sample quadriceps. The results are provided in Fig 4. The tracer incorporation into

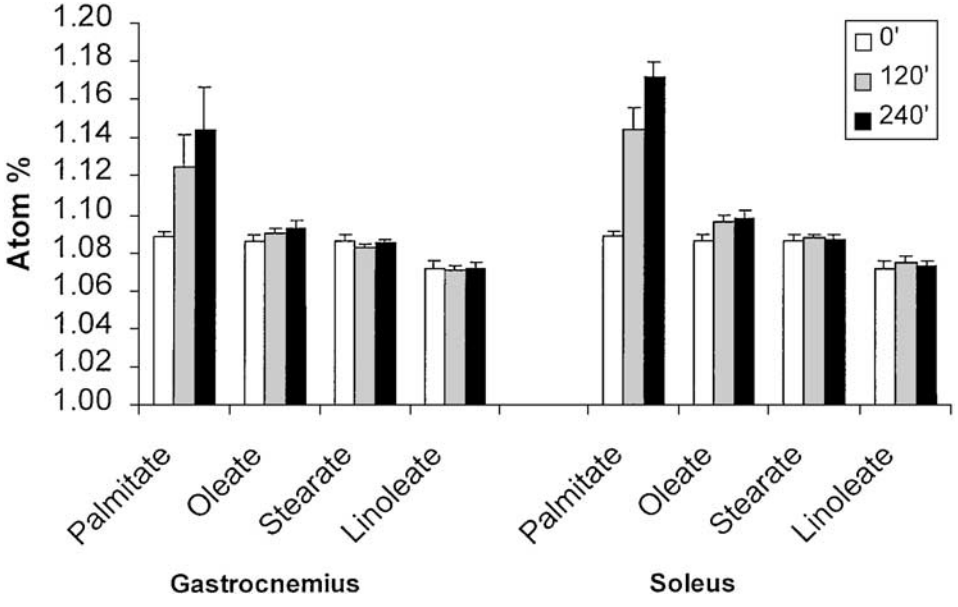

Fig 2. The changes in ^{13}C enrichment of rat muscle NEFA during continuous, unprimed infusion of [U-^{13}C]palmitate and [1-^{13}C]oleate in awake rats. All of the basal values (0 minutes) were taken from quadriceps that were biopsied before the tracer infusion began. Soleus and gastrocnemius were both biopsied at 120 and 240 minutes to monitor the isotopic changes. Data are from the same studies as in Fig 1.

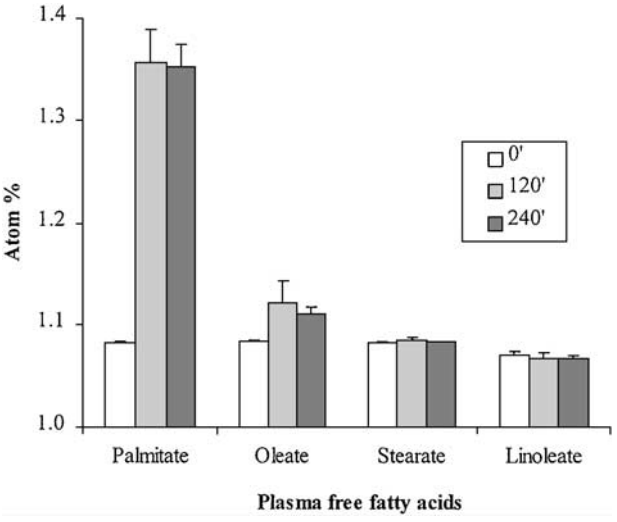

Fig 3. The changes in ^{13}C enrichment of rat plasma FFA during continuous infusion of [U-^{13}C]palmitate and [1-^{13}C]oleate in awake rats. Blood samples were collected via a carotid catheter before (0 minutes) and at 120 and 240 minutes of the tracer infusion. Data are from the same studies as in Fig 1.

imcTG of gastrocnemius and soleus at both 120 and 240 minutes was similar ($P = .10$) as determined by the 2 methods. The relative difference in tracer incorporation between the 2 methods determined at 240 minutes ranged from 1.5% to 8% ($P = .10$).

DISCUSSION

The requirement for a baseline muscle specimen for the calculation of muscle TG synthesis rate with the traditional 2-biopsy method has been a limiting factor for the investigation of imcTG metabolism.[8] The paradigm of collecting at least 2 muscle biopsies introduces 2 limitations. The first is the stress-related hormonal and hence metabolic perturbation to the system triggered by the earlier (eg, the baseline) biopsy. This is especially a problem with children and small animals that are more vulnerable to such stress. The magnitude of such metabolic perturbation and hence the errors introduced can be significant considering that the synthetic activity of imcTG is a process highly regulated by both hormones and substrates.[12] The second limitation is the amounts of time and work involved with the collection of a muscle biopsy. This is significant considering the difficulty associated with the time-consuming muscle biopsy procedures. This is especially true for humans in whom a greater amount of resources and time are required. Because 1 biopsy represents 50% of the total work that is required for the 2-biopsy protocol, the savings of time and resources by eliminating 1 biopsy is significant. In addition, to minimize invasiveness in human and animal research is consistent with ethical considerations. Although this single biopsy technique can be theoretically used with radioactive tracers,[13-15] there are safety concerns for small children and pregnant women. Therefore, this single-biopsy technique offers one the ability to extend investigation to include these populations with the use of nonradioactive, stable isotopes.

The results from the present studies upheld our original hypothesis that fatty acids from the same lipid pools can be used as isotopic reference to each other. The hypothesis was formulated based on the fact that fatty acids in a given lipid pool are most likely from the same origin and thus contain a similar amount of ^{13}C.[16] Although C3 (containing less ^{13}C) and C4 (containing more ^{13}C) plants significantly differ in ^{13}C content naturally,[17] the mixing of C3 and C4 plant components during food preparation affect all fatty acids equally. Therefore, whether dietary fatty acids are from C3 or C4 plants, the ^{13}C balance among fatty acids is not affected. Physiologically, mammals have no known mechanisms to modify or distinguish fatty acid species based on their ^{13}C content at the natural abundance level. Thus, the use of surrogate fatty acids from the same lipid pools is theoretically sound, and in fact, the present finding was expected. Indeed, the isotopic similarity among pool-mate fatty acids was previously observed in humans in whom the ^{13}C abundance of plasma FFA was similar with a difference no more than that seen in the present studies, even under conditions of dynamic nutritional manipulation.[9] Studies also reported that label exchange was not detected among serum fatty acids of humans.[18]

The present studies addressed 3 issues to test these assumptions associated with the technique. First, muscle samples were collected from fasted and fed rats to demonstrate that the natural ^{13}C abundance of the 4 most abundant fatty acids was comparable and, therefore, it is appropriate to use each other as isotopic surrogates. Table 2 showed that this is true in both fed and fasted animals. The maximum difference in the ^{13}C enrichment of the 4 fatty acids was 0.003 to 0.004 AP, equivalent to a δ ^{13}C value of 3 to 4‰. Furthermore, the difference can be minimized by choosing the most appropriate fatty acids. For example, by excluding the more enriched ones (eg, oleate, the highest enriched in fasted rats, Table 2), the difference among

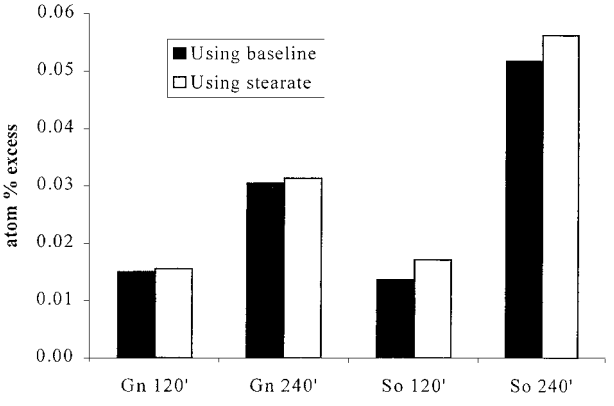

Fig 4. Comparison of [U-^{13}C]palmitate incorporation into imcTG pool of gastrocnemius (Gn) and soleus (So) at 120 and 240 minutes of tracer infusion, as determined by the 1-biopsy method (using stearate) and by the traditional 2-biopsy method (using baseline). The ^{13}C enrichment of imcTG-palmitate in Gn and So at 120 minutes and 240 minutes was subtracted by that of its counterpart in quadriceps (0 minutes) with the using baseline method or by the ^{13}C enrichment of imcTG-stearate in the same muscles (Gn and So) from the same time points with the using stearate method. The tracer incorporations determined by the 2 methods were not different ($P > .1$).

other fatty acids in the same lipid pool was less than 0.002 AP. Because fatty acids in the imcTG pool can be easily labeled to high enrichment (eg, greater than 0.1 AP), even with a tracer amount of [U-^{13}C]palmitate,[8] the difference only represents an insignificant error of 2% in the determination of ^{13}C enrichment and, hence, the incorporation rate of fatty acid. Meanwhile, the marked similarity in natural ^{13}C abundance among fatty acids was not only observed for imcTG pool, but also muscle NEFA (Table 4) and plasma FFA (Table 3). This suggests that this isotopic similarity is a common phenomenon in tissue lipid pools. Therefore, it appears appropriate to use pool-mate fatty acids as the surrogate for the determination of muscle TG synthesis with the use of stable isotopes.

Originally, we anticipated a more unpredictable isotopic dynamic in fed animals due to the influx of newly absorbed fatty acids, which likely differ isotopically from the endogenous ones. The data in Tables 2, 3, and 4 showed that, however, the close isotopic similarity among pool-mate fatty acids was preserved under fed conditions. Therefore, it appeared that this 1-biopsy technique can also be used in fed animals without compromising the results. However, even in the fasted animals, we observed oleate in imcTG and plasma FFA pools to have significantly higher ^{13}C abundance in all 3 muscles (Protocol 1, Tables 2 and 3). If it were used as the surrogate, a higher degree of errors would have been expected. The reason for the relatively large difference is not immediately clear, but we suspect it is likely related to isotopic difference among dietary fatty acids. Given that, the large difference would not have been a concern if it were predetermined in a small pilot study. As long as the same diets are used, the difference should remain the same and can be taken into account in the calculation. This is feasible at least in animals. However, it appears assuring to further evaluate oleate as a surrogate fatty acid.

We noticed a difference in the natural ^{13}C abundance of linoleate between protocol 1 and protocol 2. In protocol 2, the average ^{13}C for imcTG linoleate is 1.0680 AP (Fig 1) and that for muscle nonesterified linoleate is 1.0720 AP (Fig 2). Compared with protocol 1 (Tables 1 through 4), a difference of approximately 0.01 AP was noted. Because the 2 protocols were conducted separately, it is likely that the source of dietary fatty acids (hence likely their ^{13}C content) changed during the interim. Unfortunately, Purina (the vendor for the rat chow) was unable to retrieve or confirm the information. But the discrepancy should not affect the validity of this approach because, in principle, it is independent of the magnitude of the isotopic difference between fatty acids as discussed above. However, this points to an important aspect of using this 1-biopsy method: identical diets should be used during the entire course of a study.

The second issue we examined is label exchange. We found that there was no detectable label exchange from palmitate or oleate to stearate during a relatively prolonged tracer infusion experiment in either imcTG (Fig 1) or muscle NEFA (Fig 2). The ^{13}C abundance of stearate in these lipid pools remained at or fluctuated near the basal level during the infusion intervals. This is also true for imcTG linoleate of gastrocnemius (Fig 1) and nonesterified linoleate of both muscles (Fig 2). Somewhat paradoxically, however, the ^{13}C abundance of imcTG-linoleate in soleus showed an apparently progressive increase during the tracer infusion (Fig 1). At first glance, this appears to suggest a sign of label transfer. But, based on current understanding of this essential fatty acid, this is impossible simply because this acid cannot be synthesized de novo by mammals (hence no carbon exchanges). Therefore, the observed increase in its ^{13}C abundance appeared to have been caused by some random variations, such as nonequilibrium of linoleate originated from different tissues (eg, body fat) with different ^{13}C content. In addition, label exchanges were absent in plasma FFA (Fig 3), suggesting that label exchanges in the liver, if any, have a negligible effect on plasma FFA. Without liver as a concern for the source for label exchange, label exchange within muscle is much less likely, because skeletal muscle is relatively inactive in this regard.

Finally, the rates of fatty acid tracer incorporation into imcTG in soleus and gastrocnemius were determined and compared between the 2 approaches (Fig 4). The results determined using the 1-biopsy method were reasonably comparable with those obtained using the 2-biopsy method with a maximum difference less than 10%. Note that there is no methodologic gold standard for such studies, and thus the errors may be attributable to either method. But, because the new method did not introduce biopsy-related stress during the tracer experiment by eliminating the baseline biopsy, it more likely reflects the true kinetics of imcTG synthesis. The simplification (by saving 50% of the work) of the method, therefore, not only does not compromise the experiment, it may, in fact, benefit the experiment by reducing stress.

This simplified, single-biopsy technique, however, is not intended to replace other methods for the determination of imcTG synthesis. It is realized that this technique has certain limitations. For example, it cannot be used to measure fractional synthesis rate of imcTG, which requires the knowledge of isotopic steady state in the precursor pool, and thus the use of at least 2 muscle biopsies is mandatory. Rather, this technique is useful in situations where the rate of fatty acid incorporation, rather than fractional synthesis rate, is to be determined, more than 1 muscle biopsy is difficult or impossible, or stress may potentially affect the results. In addition, this method may not work for all tissues. For example, fatty acid tracers can be diluted to a greater extent in adipose tissue, resulting in lower isotopic enrichments in which even a small difference between the surrogate and the traced fatty acid may represent a significant error. The limitations are minor, however, and its advantages over 2- or multiple-biopsy techniques are obvious. To date, measuring the incorporation of fatty acid, instead of the fractional synthesis rate, has been the most common way of determining imcTG synthetic kinetics[13-15,19] because of its simplicity and practical sufficiency of serving the experimental objectives. Moreover, the determined fatty acid incorporation can be converted to the fractional synthesis rate if the pool size of imcTG is also known. This method can be equally useful in time-course studies in which the isotopic data for each time point needs to be corrected for isotopic natural abundance. Therefore, this technique has the potential to become a very useful technique in the studies of muscle lipid metabolism using stable isotopes. In addition to those populations that can benefit the most from using this technique (eg, children, pregnant women), this technique can also offer obvi-

ous advantages to studies involving other human adult populations. Therefore, the applicability of this technique is expected to be relatively broad.

In summary, individual fatty acids in the same lipid pools of skeletal muscle are isotopically similar. Label exchanges among these fatty acids are negligible within the time frame of a common tracer study. Therefore, this single-biopsy technique has the potential to be used for the determination of muscle TG synthesis and thus facilitate muscle metabolism research.

REFERENCES

1. Guo ZK: Sampling the intramyocellular triglycerides from skeletal muscle. J Lipid Res 42:1041-1048, 2001
2. Guo ZK, Burguera B, Jensen MD: Kinetics of intramuscular triglyceride fatty acids in exercising humans. J Appl Physiol 89:2057-2064, 2000
3. Rooyackers OE, Adey DB, Ades PA, et al: Effect of age on in vivo rates of mitochondrial protein synthesis in human skeletal muscle. Proc Natl Acad Sci USA 93:15364-15369, 1996
4. Nugteren DH: The enzymic chain elongation of fatty acids by rat-liver microsomes. Biochim Biophys Acta 106:280-290, 1965
5. St. John LC, Lunt DK, Smith SB: Fatty acid elongation and desaturation enzyme activities of bovine liver and subcutaneous adipose tissue microsomes. J Anim Sci 69:1064-1073, 1991
6. Folch J, Lees M, Sloane-Standley GH: A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497-509, 1957
7. Christie WW: Rapid separation and quantification of lipid classes by high performance liquid chromatography and mass (light-scattering) detection. J Lipid Res 26:507-512, 1985
8. Guo ZK, Jensen MD: Intramuscular fatty acid metabolism evaluated with stable isotopic tracers. J Appl Physiol 84:1674-1679, 1998
9. Guo ZK, Nielsen S, Burguera B, et al: Free fatty acid turnover measured using ultralow doses of [U-^{13}C]palmitate. J Lipid Res 38: 1888-1895, 1997
10. Dole VP: A relation between non-esterified fatty acids in plasma and the metabolism of glucose. J Clin Invest 35:150-154, 1956
11. Guo ZK, Luke AH, Lee WP, et al: Compound-specific carbon isotope ratio determination of enriched cholesterol. Anal Chem 65: 1954-1959, 1993
12. Coyle EF, Jeukendrup AE, Oseto MC, et al: Low-fat diet alters intramuscular substrates and reduces lipolysis and fat oxidation during exercise. Am J Physiol 280:E391-E398, 2001
13. Eaton P, Steinberg D: Effects of medium fatty acid concentration, epinephrine, and glucose on palmitate-1-^{14}C oxidation and incorporation into neutral lipids by skeletal muscle in vitro. J Lipid Res 2:376-382, 1961
14. Shtacher G, Shank M: Uptake and distribution of fatty acids in rat diaphragm and heart muscles in vitro. Arch Biochem Biophys 106:205-213, 1963
15. Tagliaferro AR, Dobbin S, Curi R, et al: Effects of diet and exercise on the in vivo rates of the triglyceride-fatty acid cycle in adipose tissue and muscle of the rat. Int J Obes 14:957-971, 1990
16. Woodbury SE, Evershed RP, Rossell JB: Delta ^{13}C analyses of vegetable oil fatty acid components, determined by gas chromatography-combustion-isotope ratio mass spectrometry, after saponification or regiospecific hydrolysis. J Chromatogr A 805:249-257, 1998
17. Liang Y, White WS, Yao L, et al: Use of high-precision gas isotope ratio mass spectrometry to determine natural abundance ^{13}C in lutein isolated from C3 and C4 plant sources. J Chromatogr A 800:51-58, 1998
18. Metges CC, Kempe K, Wolfram G: Enrichment of selected serum fatty acids after a small oral dosage of (1-^{13}C)- and (8-^{13}C)triolein in human volunteers analysed by gas chromatography/combustion isotope ratio mass spectrometry. Biol Mass Spectrom 23:295-301, 1994
19. Dyck DJ, Peters SJ, Glatz J, et al: Functional differences in lipid metabolism in resting skeletal muscle of various fiber types. Am J Physiol 272:E340-E351, 1997