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Oxidation of Intracellular and Extracellular Fatty Acids in Skeletal Muscle:

Application of kinetic modeling, stable isotopes and liquid chromatography/electrospray ionization ion-trap tandem mass spectrometry technology

J. Xu¹, L. Zhou¹, X-M. Persson², P. Balagopal³, M.D. Jensen¹, and ZK. Guo^{1,*}

¹Endocrine Research Unit, Mayo Foundation, Rochester, MN, USA

²Mass Spectrometry Core Facility, Mayo Foundation, Rochester, MN, USA

³Nemours Children's Clinic, Jacksonville, FL, USA

Abstract

Fatty acids are a major fuel for many tissues and abnormal utilization is implicated in diseases. However, tissue fatty acid oxidation has not been determined reliably in vivo. Furthermore, fatty acid oxidation has not been partitioned into intracellular and extracellular components. In this report, a one-pool model is described that enables direct quantitation of fluxes of intracellular and plasma fatty acids to mitochondria in skeletal muscle using dual stable isotopes and liquid chromatography/electrospray ionization ion-trap tandem mass spectrometry (LC/ESI-itMS²) technology. It is validated by the determination of palmitate oxidation by skeletal muscle in lean and obese rats and the regulation by insulin. Resting postabsorptive intramyocellular and plasma palmitate oxidation by gastrocnemius muscle was determined to be 3.47 ± 0.8 and 2.06 ± 0.5 nmol/g min in lean and 6.96 ± 1.8 and 1.34 ± 0.2 nmol/g min in obese rats, respectively. In obese rats, hyperinsulinemia (1 nmol/l) suppressed intramyocellular (by $59 \pm 5\%$ to 2.88 ± 0.3 nmol/g min $P < 0.05$) but not plasma (1.41 ± 0.14 nmol/g min, $P > 0.05$) palmitate oxidation. The fractional turnover rate of palmitoylcarnitine ($0.34 \pm 0.1/\text{min}$ vs. $0.83 \pm 0.2/\text{min}$, $P < 0.05$) was also suppressed by insulin. In obese and lean rats, there are 83% and 51%, respectively ($P = 0.08$), of plasma fatty acids traverse triglyceride pool before being oxidized. The results demonstrated that the methodology is feasible and sensitive to metabolic alterations and thus can be used to study fatty acid utilization at tissue level in a compartmentalized manner for the first time.

Keywords

beta-oxidation; intracellular; extracellular; skeletal muscle; compartmentalization; stable isotope; LC-MS

INTRODUCTION

Fatty acids are a major fuel for many tissues especially skeletal muscle, heart and liver. Dietary fat typically contributes 30–40% of daily energy intake or even more [1]. Therefore, the study of fatty acid utilization is important for nutrition. Abnormality in lipid metabolism is implicated in many diseased conditions and thus it is critical for understanding and treatment of diseases. This is exemplified by the intense studies of fatty acid metabolism over the past decades in relation to insulin resistance, a characteristic of a number of

*Correspondence author: 5-194 Joseph, Mayo Foundation, Rochester, MN 55905, Phone: (507) 255-6461, Fax: (507) 255-4828, guo.zengkui@mayo.edu.

diseases such as obesity, type 2 diabetes and other complications [2–7]. A focus of these studies is the role of fatty acid oxidation in the impairment of glucose metabolism and insulin resistance, stimulated by the discovery of glucose-fatty acid cycle by Randle and associates in 1963 (hence also termed Randle cycle). The theory was synthesized based on the finding in heart and diaphragm muscle in vitro that fatty acid oxidation causes accumulation of acetyl CoA and citrate acid which inhibit pyruvate dehydrogenase and phosphofructokinase, respectively, and thus glucose metabolism [8]. The importance of this experimental observation on the understanding of glucose-fatty acid interactions and insulin resistance can not be overstated as it provided the first explanation of muscle insulin resistance at the substrate level. On the other hand, the glucose-fatty acid interactions remain not well understood on both qualitative and quantitative terms. For example, Randle cycle has been demonstrated to operate under many physiologic and metabolic conditions [9–11] but not in others [12; 13] and controversy remains [11; 14; 15]. The controversy stems to a large extent from the fact that fatty acid oxidation at the tissue level can not be measured in vivo due to the lack of an effective method. As a result, the role of fatty acid oxidation in insulin resistance can not be studied directly and has to rely on in vitro studies. This technical difficulty significantly hindered the advances in the investigation of mechanism underlying insulin resistance [2; 10; 16]. Ironically, Randle cycle theory has not been directly confirmed in vivo. Over the recent years, an alternative, but not necessarily conflicting, theory has emerged that signal transduction is implemented in insulin resistance [17; 18]. This may complement Randle theory.

To date, calorimetry has been used as the de facto standard technique to measure lipid oxidation in vivo. However, it is not tissue-specific as it only measures whole body including all tissues. Fatty acid oxidation at tissue level is usually determined by muscle strip incubation [19; 20], leg muscle perfusion [21] or by using metabolic surrogates such as enzyme activity/level or transcription factors [22]. As these surrogates are often discrepant from actual substrate flux, they do not reliably represent the state of fatty acid oxidation. Arterial-venous balance is also used to measure fatty acid oxidation by tissues such as heart [23] and skeletal muscle [24]. However, its application is limited by the invasiveness of catheterization across the tissue bed of interest. Recently, PET (Positron Emission Tomography) has been increasingly used to evaluate its ability to measure tissue lipid oxidation [25]. The technique, while extremely costly, only measures fatty acid uptake, not oxidation per se. Another technique relies on the use of 2-bromo-palmitate and another palmitate tracer to derive an index of plasma fatty acid oxidation by tissues [26]. As 2-bromo-palmitate inhibits mitochondrial β -oxidation, the procedure must be carried out briefly (in minutes) which may compromise the accuracy. In addition, it appears to be a complete lack of methods to differentiate intracellular from extracellular oxidation.

Due to the complexity and difficulties involved with the measurement of tissue-specific fatty acid oxidation, both increased [2; 3; 27] and decreased [28] lipid oxidation have been reported for tissues such as skeletal muscle in obesity and type 2 diabetes. In these populations, intramyocellular triglyceride (imcTG) stores are abnormally high and it is significantly associated with insulin resistance [29–31]. Based on the conflicting data, opposing hypotheses have been proposed. Some suggested that decreased fatty acid oxidation in skeletal muscle has lead to the excess imcTG accumulation [28] whereas others claim that the enlarged imcTG pool provides excess fatty acid fuels that compromise glucose utilization [32]. This is also true for other tissues such as heart [33] and pancreas [34] where the role of lipid oxidation in insulin resistance is implicated but can not be quantified. To date, the classical issue that whether fatty acid oxidation contributes to increase in hepatic gluconeogenesis and glucose production has not been directly determined due to the technical inability [35]. Therefore, it is not exaggerated to say that this has created a vacuum of research on fatty acid metabolism in both nutrition and diseases.

In this report, an one-pool kinetic model and the related techniques have been developed and evaluated that enable direct quantitation of long chain fatty acid fluxes to mitochondria from both intracellular and extracellular sources in skeletal muscle in vivo using dual stable isotopes and state-of-the-art liquid chromatography/electrospray ionization ion-trap tandem mass spectrometry (LC/ESI-itMS²) technology.

MATERIALS AND METHODS

The model

The model is designed to quantify the flux of palmitate to acylcarnitine pool as a measure of β -oxidation (β). β is contributed from two sources, intracellular fatty acid pools (m) and plasma fatty acids (p). They converge at acylcarnitine pool (Fig. 1) before entering mitochondria [36]. Once formed, acylcarnitine esters are obligated to enter mitochondria and, thus, unidirectional. Therefore, the flux represents the rate of mitochondrial β -oxidation. The model is based on that interactions among intracellular fatty acid pools (imcTG and NEFA) are irrelevant and thus ignored. In other words, all intracellular fatty acid source pools are treated as a single black box. The only parameter the model is designed to measure is the efflux of fatty acids from this box to acylcarnitine (m). As the largest intracellular pool, imcTG-fatty acids contribute the bulk of m . In this report, the term imcTG is used to represent this black box, unless as indicated otherwise.

Both m and p are determined at the end of a pulse-chase protocol using dual ¹³C-palmitate isotopes. β is determined using [¹³C₄]palmitate and p using [U-¹³C]palmitate. m is calculated ($\beta - p$). Fig. 2 depicts the isotopic techniques and concepts, as detailed below.

Determining β

Protocol—The entire experiment lasts 180 min. The first 120 min is pulse period (–120 min to time t_0). It is immediately followed by chase period (60 min, from t_0 to t). During pulse, [¹³C₄]palmitate is intravenously infused to prelabel imcTG. During chase, the tracer is replaced by [U-¹³C]palmitate to trace plasma palmitate flux. In the end, rats are euthanized and muscle samples collected (time t). M+4 and M+16 isotopomer enrichment (molar % excess, MPE) and content of palmitoylcarnitine, imcTG-palmitate, non-esterified palmitate and plasma palmitate are analyzed by mass spectrometry (below).

During chase, [¹³C₄]palmitate decays from the prelabeled imcTG pool exponentially (e^{-kt}). The decay is reflected in palmitoylcarnitine. From the decay slope, turnover rate (k') is determined:

$$k' = [\ln([\text{¹³C}_4\text{]palmitoylcarnitine MPE}_t) - \ln([\text{¹³C}_4\text{]palmitoylcarnitine MPE}_{t_0})] \div 60 \quad \text{Eq. (1)}$$

This k' in fact reflects the decay of imcTG-[¹³C₄]palmitate because now it is the source pool for the label and therefore dictates the decay (slope). Palmitoylcarnitine, on the other hand, is the receiving pool and thus is not a determinant of the slope. It is only used as a window to 'see' the slope (compare the similar curvilinear slope in both pools in Fig 2). Note that only does the label decay, not the entire pool. The entire pool of palmitate (labeled and unlabeled) turns over at a constant rate (m). This means that during chase, there is less and less of labeled palmitate and more and more of unlabeled palmitate. As m traverses palmitoylcarnitine pool, which is smaller in size than imcTG, it must turn over faster to allow m to go through it in a timely fashion so that m can continue at its constant rate. As an example, assume imcTG contains 1000 palmitate molecules and that of palmitoylcarnitine 100. If 50 palmitate are released from imcTG per minute (m), imcTG turns over at a rate of 0.05/min. But for this flux to go through palmitoylcarnitine pool, the pool must turn over at

0.5/min, 10 time faster. Using Eq 1, the rate of 0.05/min (k'), not 0.5/min (k), is obtained because palmitoylcarnitine 'mirrors' imcTG-palmitate in label kinetics. Therefore, k' is adjusted based on the difference in pool size of imcTG-palmitate and palmitoylcarnitine to obtain k which is greater than k' (Table 1). Indeed, palmitoylcarnitine turns over faster by 2 orders of magnitude [37]. Unlabeled palmitate from plasma does not affect this (by simulation, not shown).

Then, β is calculated using the corrected k :

$$\beta \text{ (nmol/g/min)} = \text{pool size of palmitoylcarnitine (nmol/g)} \times k \quad \text{Eq. (2)}$$

Determining p

During chase, [U- ^{13}C]palmitate is infused to trace plasma palmitate oxidation (Fig. 2). Because this tracer is subject to the same turnover kinetics as the parent palmitoylcarnitine pool, the flux of plasma [U- ^{13}C]palmitate label is calculated using the same k :

$$[\text{U-}^{13}\text{C}]\text{palmitoylcarnitine label flux (nmol/g/min)} = \text{label amount in pool (nmol/g)} \times k$$

As plasma fatty acids turnover rapidly ($t_{1/2} = \sim 2$ min in rats), the tracer reaches equilibrium in plasma fatty acid and palmitoylcarnitine pool ($t_{1/2} < 1$ min) after minutes into the infusion (mathematically obvious, intensive experimental evidence as well, e.g. ref. 37). Therefore, steady state equation is used to calculate p using plasma [U- ^{13}C]palmitate as the denominator (precursor) based on isotope dilution principle (a classical technique):

$$p \text{ (nmol/g/min)} = [\text{U-}^{13}\text{C}]\text{palmitoylcarnitine label flux} \div \text{plasma } [\text{U-}^{13}\text{C}]\text{palmitate MPE.} \quad \text{Eq. (3)}$$

The calculation of p only relies on palmitoylcarnitine and plasma palmitate but not imcTG. Thus, the non-steady state of the new tracer in imcTG (slower equilibration) is irrelevant. This is in contrast to the case of the 1st tracer where the determination of k relies on imcTG and palmitoylcarnitine (but not plasma palmitate).

During chase some plasma palmitate traverses imcTG before entering acylcarnitine pool. It is termed indirect flux and is estimated by comparing [U- ^{13}C]/[$^{13}\text{C}_4$] ratio of imcTG-palmitate and palmitoylcarnitine. This is based on the fact that if *all* plasma palmitate takes this path, the ratios for the 2 pools should be the same: [U- ^{13}C]/[$^{13}\text{C}_4$]_{imcTG-palmitate} $\div$ [U- ^{13}C]/[$^{13}\text{C}_4$]_{palmitoylcarnitine} = 1. On the other hand, if *all* are direct, the same ratio = 0 (i.e. [U- ^{13}C]/[$^{13}\text{C}_4$]_{imcTG-palmitate} = 0). The actual ratios fall between 0 and 1 indicating that this indirect path is partial, as expected.

Determining m

$$m = \beta - p.$$

STUDY DESIGN

Fatty acid oxidation in gastrocnemius and insulin regulation in lean and high fat-fed obese rats were determined using the model. For comparison, fatty acid oxidation was also determined independently (production of $^3\text{H}_2\text{O}$ from [9,10- ^3H]palmitate in vivo and of $^{14}\text{C}_2\text{O}$ from ^{14}C -palmitate by muscle strip incubation ex vivo), as described below.

Chemicals and animals

[U-¹³C]palmitoylcarnitine was custom-synthesized by and [1,2,3,4-¹³C₄]- and [U-¹³C]palmitate were purchased from Isotec (Miamisburg OH, USA). [U-¹⁴C]- and [9,10-³H]palmitate were purchased from PerkinElmer (Waltham, MA). Male Sprague Dawley rats (400–500 g), purchased from Harlan fed on regular rat chow (lean) or a high fat diet (55% calories from lard) (obese), were used throughout the studies using the protocols as described below. More details on animals and materials are provided elsewhere [38].

In vivo experiments

Pre-study—After an overnight fast, the rats were catheterized in a tail artery using the procedures established earlier [39] for glucose monitoring and blood collections. When glucose returned to basal level, experiments were initiated by inserting a flexible plastic i.v. infusion line with a 25G needle into a lateral tail vein by venipuncture and secured. The following pulse-chase protocols were then started immediately.

Pulse-chase—The one-pool model was applied in these studies using pulse-chase technique. The technique was the same as detailed previously [38], except that [¹³C₄]- and [U-¹³C]palmitate were infused intravenously at 0.5 μmol/kg/min during pulse and chase, respectively. During chase, lean (n=6) and obese (n=6) rats were infused with saline as control (basal state) and another 4 obese rats were studied using euglycemic clamp technique to determine insulin regulation of fatty acid oxidation. Plasma fatty acids, glycerol, triglycerides and glucose were maintained by substrate infusion during the clamp so that insulin was the only variable. All these rats were only sampled at the end of chase (time t), not pulse. A separate group of lean and obese rats (both n=7) were only pulsed with [¹³C₄]palmitate, without chase, to determine the extent of the labeling in imcTG, NEFA and palmitoylcarnitine pools (time t₀). All rats were awake. Blood samples were taken awake and gastrocnemius collected after euthanization. The details of the protocols and procedures are provided elsewhere [38].

Experiments using [9,10-³H]palmitate—Another group of the obese rats (n=4) were infused with [9,10-³H]palmitate at 0.05 μCi/kg/min for 120 min. Blood samples and gastrocnemius were then collected at the end to estimate fatty acid oxidation in muscle based on the rate of production of ³H₂O in muscle.

Sample processing and analysis

Stable isotopes: Sample processing and lipid extractions and purification are as previously described [40]. MPE of M+4 and M+16 isotopomers and mass of palmitoylcarnitine was determined by LC/ESI-ITMS² (TermoFinnigan, San Jose, CA; model LCQ-deca, as previously described, and LTQ with minor modifications in operation procedures) as previously described [41]. MPE of the same isotopomers of imcTG-palmitate and non-esterified palmitate were determined by GC-MS. Pentadecanoate, tripentadecanoin and pentadecanoylcarnitine were used as the internal standards for mass determination in conjunction with the use of standard curves.

Radioisotopes: ³H-specific activity of imcTG-fatty acids was determined by liquid scintillation counting. The aqueous phase of muscle extracts was divided into two parts, one directly counted for ³H radioactivity and the other air blow-dried before counting. The difference was taken as ³H₂O produced from oxidation of ³H-palmitate in muscle. Total fatty acid oxidation by muscle (nmol/g min) was calculated as the rate of ³H₂O production divided by the specific activity of imcTG-fatty acids. Palmitate is assumed to account for 1/3 of total fatty acids.

Ex vivo experiments

Additional rats (n=6) were used to determine palmitate oxidation ex vivo. After an overnight fast, gastrocnemius was collected under anesthesia and muscle strips were prepared to determine palmitate oxidation according to Fruebis et al [19] using ^{14}C -palmitate. imcTG-palmitate oxidation was quantified as the rate of $^{14}\text{CO}_2$ production divided by ^{14}C -specific activity of imcTG-palmitate.

RESULTS

Plasma substrate homeostasis

The high fat-fed obese rats used in this study is mildly insulin resistant as indicated by lowered insulin-stimulated systemic glucose uptake and modestly elevated plasma fatty acids [42]. Along with enlarged imcTG pool size, imcTG synthesis is also increased markedly [31]. Nonetheless, imcTG metabolism appears to remain sensitive to insulin [40]. During the euglycemic clamp experiments, plasma fatty acids, glycerol and triglycerides in addition to glucose were kept relatively stable at or near their basal levels by substrate co-infusions, as shown previously (34). This eliminated much of potential confounding effects by drastic changes in these metabolites that would surely incur without the coinfusions. Thus, insulin was the predominant, if the only, variable.

Turnover kinetics of palmitoylcarnitine and imcTG

Table 1 shows the enrichment and content of imcTG-palmitate and palmitoylcarnitine and the determined k' and k of palmitoylcarnitine in lean and obese rats and insulin effects in obese rats. Palmitoylcarnitine turnover was comparable in obese and lean rats and was suppressed by insulin in obese rats ($P=0.04$). Insulin also tended to increase the pool size of palmitoylcarnitine ($P=0.05$).

Fatty acid oxidation and insulin regulation

Fig. 3 shows imcTG and plasma palmitate oxidation by gastrocnemius in lean and obese rats and the effects of insulin. Total palmitate oxidation by the muscle at basal state determined using ^3H -palmitate was not significantly different from that determined using the model ($[^{13}\text{C}_4]\text{palmitate}$) ($P=0.5$). Total palmitate oxidation by muscle was not significantly different between obese and lean rats ($P=0.2$). However, imcTG-palmitate oxidation in obese rats tended to be higher than in lean rats ($P=0.1$). In obese rats, imcTG-palmitate oxidation accounted for $79\pm 4\%$ of the total, higher than that in the lean ($59\pm 8\%$, $P=0.05$).

Insulin suppressed the oxidation of total palmitate by $48\pm 4\%$ ($P<0.05$) and that of imcTG-palmitate by $59\pm 5\%$ ($P<0.05$) in obese rats. However, insulin did not affect the oxidation of plasma palmitate ($P>0.05$), likely due to the maintained stable plasma fatty acid level [43].

Plasma palmitate oxidation was lower than imcTG oxidation and was similar between lean and obese groups ($P=0.3$). In obese rats, 83% and in lean rats 51% ($P=0.08$) of plasma palmitate traversed imcTG before entering palmitoylcarnitine pool (indirect path). Under insulin effect, 104% of plasma palmitate took this indirect path in obese rats ($P=0.5$, vs. basal).

Ex vivo experiments

Using the traditional muscle strip incubation technique and the same pulse-chase technique ex vivo, palmitate oxidation by gastrocnemius was determined to be 0.27 ± 0.04 nmol/g/min, grossly lower ($P<0.01$) than that determined in vivo using the model.

DISCUSSION

Palmitoylcarnitine

Quantifying fatty acid turnover and oxidation by analyzing palmitoylcarnitine has several advantages. It avoids lipid contamination, a common problem with skeletal muscle that distorts imcTG results. It also eliminates concern for TG-FA recycling which complicates k calculation [38]. Equally important, the approach automatically takes into account all source pools that contribute to palmitoylcarnitine flux.

The one-pool model and related techniques are a combination of kinetic modeling, dual tracer, pulse-chase technique and LC/ESI-itMS² technology. These techniques are either classical or has been established recently (i.e. LC/ESI-itMS², ref. 41). The kinetic modeling is the only new component and discussed here.

Comparison to other methods

To validate the model using different techniques, several comparisons are made. First, the traditional ³H-palmitate method was employed in a pulse protocol. As the radiotracer does not establish equilibrium in imcTG pool until the late stage, the results calculated using the specific activity of imcTG-fatty acids as the denominator is likely an underestimate. However, this is offset to some extent by the influx of plasma ³H₂O. The comparability of the results to that obtained using the model with ¹³C-palmitate isotopes is evident indicating this new methodology is quantitatively valid. Also, the observed resting muscle palmitate oxidation in lean rats (3.47 nmol/g/min) determined using the model is in similar range to that we determined previously for healthy humans exercising at 45% VO_{2max} using a method that is related to the present model, 2.3 nmol/g/min [44]. The difference likely reflects higher metabolic rate in rats and the different metabolic conditions (resting vs. exercising). Using the more reliable, but very invasive, A-V balance technique, total fatty acid oxidation by pig heart was determined to be 25 nmol/g/min [23]. This is compared to the calculated rate for lean rat muscle, 10.4 nmol/g/min (assuming palmitate accounts 1/3 of all fatty acids), using the present results. The difference includes species and tissue (heart is much more lipid oxidative).

The more common ex vivo method of muscle strip incubation produced remarkably lower results as already reported previously [19] [20] [45]. This is surprising given the method having been utilized as the seemingly only way of measuring tissue-specific fatty acid oxidation. Usually using ¹⁴C-palmitate or ¹⁴C-oleate as the tracer, fatty acids oxidation by various rat and mouse muscles has been determined and the results vary widely. The lower end of the results [19] [20] [45] are similar to that observed in the present study with muscle incubation. The higher values (but not the highest) [46] [47] are comparable to the results of the present study obtained using the model with ¹³C-palmitate tracers. Using muscle homogenate, the results were even lower [48]. Therefore, it appears that these ex vivo/in vitro methods are associated with alterations of tissue intrinsic metabolic setting so that fatty acid oxidation is affected, such as by the lack of endocrine/paracrine factors.

There have been extensive efforts to probe intramyocellular lipid oxidation. However, to date it has not been possible to partition tissue fatty acid oxidation into intracellular and extracellular components. The classical method of measuring intracellular fatty acid oxidation by the difference between total lipid oxidation and plasma fatty acid oxidation only provides the total that is contributed by all tissues and is not specific to any particular tissue. Therefore, the ability to do so is a major advantage of the present methodology that can be used to study the role of imcTG metabolism in muscle insulin resistance where imcTG is enlarged and robustly associated with, and likely contributes to, insulin resistance. It is equally advantageous that direct and indirect paths of plasma fatty acid oxidation can be

estimated directly in vivo using the methodology. The combination of dual ^{13}C -palmitate isotopes and LC/ESI-ITMS² technology enables this to be quantified based on the isotopic signatures in imcTG and acylcarnitine pools. In fact, the instrument is able to measure much lower isotopic enrichment as it has a precision as low as 0.03 MPE, impossible with single quadrupole MS (41). This implies that the same task can be performed in humans where isotopic enrichment is usually low.

The results showed that more than a half of plasma palmitate (probably other fatty acids as well) takes indirect path before oxidation. This is similar to previous finding that all plasma fatty acids are esterified into imcTG before being oxidized. This appears to be the first direct demonstration in vivo that the classical fatty acid tracer technique overestimates plasma oxidation. The non-equilibrium of the 2nd tracer in imcTG pool only causes modest underestimation of the parameter as this is also reflected in palmitoylcarnitine pool. With the ability to differentiate intracellular vs. extracellular components and direct vs. indirect fluxes of fatty acid oxidation at tissue level, the methodology is useful for the studies of fuel economy and plasma fatty acid metabolism in different nutritional and metabolic conditions and diseases.

Insulin regulation

Data on insulin regulation of fatty acid oxidation in skeletal muscle in vivo is virtually lacking. In vitro, insulin suppresses intramyocellular fatty acid oxidation by 2/3 and plasma oxidation by 1/3 in incubated rat soleus muscle strips [49]. In humans, insulin infusion (20 mU/m²/min) suppressed total lipid oxidation by 62% and 88%, respectively (indirect calorimetry), when plasma fatty acids were maintained by heparin infusion or allowed to fall. But plasma fatty acid oxidation (^{14}C -palmitate) was not affected when plasma fatty acids were maintained but was decreased when allowed to fall [43]. In a group of young and lean healthy subjects, plasma fatty acid oxidation ([1- ^{13}C]palmitate) was suppressed by insulin only if no heparin or triglycerides was infused, but total lipid oxidation (IC) was suppressed by insulin regardless [50]. These observations suggested that insulin mainly inhibits intracellular fatty acid oxidation but its effect on plasma fatty acid is a function of substrate availability. The results from the present study exactly duplicated these earlier findings, in a more direct way, by showing that insulin did not affect plasma palmitate oxidation where basal plasma fatty acids were maintained by compensatory fatty acid infusion. In contrast, intracellular palmitate oxidation was suppressed by 59% and total oxidation by 48%. This illustrated that the methodology is sensitive to compartmentalized changes in fatty acid oxidation.

Fatty acid balance

As another way of validating the method, a crude daily fatty acid balance was performed for lean rats (body wt 370 ± 18 g, $n=31$) over a period of 8 weeks. At the end of the period, the average body wt gain was 1.1 g and food intake (regular chow with 4.5% fat) was 23.7 ± 0.6 g. Metabolizable energy from dietary fat is estimated to be 92% [51]. Various body components that utilize fatty acids as fuel are estimated: muscle 130 g (40% of body wt), liver (3% of body wt) and heart 1.2 g. Liver, heart and oxidative red muscle (5% of total skeletal muscle mass [52]) are assumed to utilize fatty acids at a rate twice that for gastrocnemius as determined using the present method. Fatty acid oxidation is assumed to be 50% lower during night time when rats eat (12/12 light-dark cycle). Daily fatty acid deposition is estimated based on the body weight gain and body fat content of 18%. Fatty acids synthesized from glucose are calculated to be 0.11 g based on the same 12/12 cycle (higher when feeding [53]). From these data and estimations, metabolizable energy intake from dietary fat was 0.98g. Thus, the daily fatty acid input was 1.09 g ($0.11+0.98$) and the output (utilization) was 1.12 g (oxidation 0.92 g and fat deposition 0.2 g). Thus, the daily

fatty acid balance was -0.03 g (i.e. an error of 3%). However, using the lower rate determined by muscle strip incubation, this value would have been $+0.85$ g (a 78% error). The results support the model against the muscle strip incubation method.

Assumptions

Single-pool kinetics—It is assumed that acylcarnitine is a kinetically single pool, collectively. This is the case even if multiple sub-pools exist because at steady state they turn over at a constant rate on average with a stable combined pool size. This is illustrated below using a hypothetical acylcarnitine pool consisting of 2 sub-pools.

Sub-pool A and B of size a and b , respectively ($a + b$), each with a turnover rate of k_1 and k_2 , respectively ($k_1 + k_2$). Therefore, the combined flux of A and B, f , is

$$f = ak_1 + bk_2$$

Because at steady state a and b and k_1 and k_2 are all stable, f remains constant despite $a + b$ and $k_1 + k_2$. Therefore, f can be described by single pool kinetics:

$$f = ck$$

where $c = a + b$ and $k = k_1(a/c) + k_2(b/c)$. Relating to the one-pool model, c is the pool size of palmitoylcarnitine and k is its turnover rate constant. As seen, the one-pool model describes the combined pool size and the overall turnover kinetics making no reference to the individual sub-pools. As such, the existence of the sub-pools, if any, does not affect the model. As shown in Fig. 4, palmitoylcarnitine is indeed a single pool, at least kinetically.

Unidirectionality—The biochemical reactions dictate that acylcarnitines are destined to entering mitochondria for oxidation upon formation as it can not revert back to acyl CoA without the intra-mitochondrial enzyme CPT-2 [36]. Therefore, the flux of palmitoylcarnitine represents the rate of β -oxidation. The pathways are shown in Fig. 5.

Limitations

Peroxisomal β -oxidation—The technique does not measure this pathway. However, based on available information, this pathway is considered minor in muscle and other tissues as it is not for energy production and the main substrates are very long chain fatty acids of >20 carbons [54; 55].

Incomplete oxidation—This has been reported for long chain fatty acids from some but not other studies. When this is the case, however, the extent can be estimated by the accumulation of $[^{13}\text{C}_4]/[\text{U-}^{13}\text{C}]$ acetylcarnitine/-CoA, the predominant intermediate of mitochondrial β -oxidation. This is used to correct for the determined m and p .

Heterogeneity—Skeletal muscle heterogeneity increases the variability of results. However, this issue may have been exaggerated somewhat by, e.g. attributing analytical variability to biological. It is possible to minimize the problem. For instance, similar metabolite concentration and isotopic specific activity were obtained from different aliquots of same or different (e.g. left vs. right limb) muscle when proper cares are exercised in sample processing [56].

Export of fatty acids from mitochondria?—The reversal of long chain fatty acids to exit mitochondria is possible only under pathological conditions [57]. However, if the reported export of fatty acids from mitochondria by UCP3 [58] proves correct, its quantitative impact on the model requires further investigation.

CONCLUSION

A one-pool model has been examined for the quantification of fatty acid oxidation by skeletal muscle in vivo. It has the ability to differentiate intracellular vs. extracellular fatty acid sources for oxidation and to trace intracellular fatty acid fates. It has been tested by quantifying postabsorptive palmitate oxidation and insulin regulation in resting rats. The results are overall comparable with those obtained using independent methods and consistent with reported results obtained using various independent techniques. It is concluded that the methodology provides a relatively easy way for the studies of tissue-specific and compartmentalized fatty acid metabolism in vivo.

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Figure 1.

Schematic representation of the one-pool model for quantifying mitochondrial β -oxidation. Intramyocellular fatty acid pool NEFA (non-esterified fatty acids) and imcTG undergo recycling constantly [44]. There might be also fatty acid exchanges between NEFA and PL (phospholipids). Palmitate released from imcTG may directly enter palmitoylcarnitine pool or via NEFA. These interactions are complex but irrelevant to this model. The model treats these intracellular fatty acid pools as a single, composite entity (black box) with the interactions within it irrelevant and thus ignored. Only is the net efflux of fatty acids from this box considered and quantified (m). The efflux of m comes out of this black box and then goes through the much smaller palmitoylcarnitine pool. Thus, the latter pool must turn over faster as described in the text. p represents plasma FFA flux, direct and indirect, to palmitoylcarnitine pool. m and p , and β , are unidirectional as acylcarnitines do not recycle ($m+p=\beta$). The glycerol moiety of imcTG and PL is not directly involved in β -oxidation and thus not shown.

Figure 2.

Isotopic kinetic model of $[^{13}\text{C}_4]$ palmitoylcarnitine and $[\text{U-}^{13}\text{C}]$ palmitoylcarnitine during chase (t_0 — t) for the separation of m and p . During pulse (120 min until t_0), imcTG was labeled by $[^{13}\text{C}_4]$ palmitate. At t_0 , $[^{13}\text{C}_4]$ palmitate infusion stopped and thus $[^{13}\text{C}_4]$ label in imcTG becomes the sole source and starts to decay exponentially according to e^{-kt} kinetics (broken curvilinear descending slope). From this slope, fractional turnover constant of palmitoylcarnitine, k (thick solid descending slope) is derived using **Eq 1** and adjusted as described in the text. Possible loss of the label to other unknown pathways (? sign) does not affect the calculation. Meanwhile, during chase $[\text{U-}^{13}\text{C}]$ palmitate equilibrates quickly in plasma FFA and $[\text{U-}^{13}\text{C}]$ palmitoylcarnitine pools (plateaus in these pools). Solid and broken arrows from plasma represent direct and indirect path, respectively, of plasma palmitate flux to palmitoylcarnitine. The turnover of $[\text{U-}^{13}\text{C}]$ palmitoylcarnitine label is the same as the entire palmitoylcarnitine pool (k). Thus, the flux of $[\text{U-}^{13}\text{C}]$ palmitoylcarnitine label is calculated from which the flux of total plasma palmitate to palmitoylcarnitine pool is determined (**Eq 3**). Note that during chase, $[^{13}\text{C}_4]$ palmitoylcarnitine and $[\text{U-}^{13}\text{C}]$ palmitoylcarnitine are independent of each other with distinct kinetics, namely decaying vs. steady, respectively. This is the basis that enables the segregation of imcTG-palmitate and plasma palmitate fluxes.

Figure 3.

Basal postabsorptive total (β), imcTG (m) and plasma (p) palmitate oxidation in gastrocnemius of lean and higher fat-fed obese rats and the effect of insulin (clamp). From the same studies as in Table 1. β was also estimated using an independent technique with [9,10-³H]palmitate as a tracer in a separate group of obese rats (basal, n=4). For the detailed experimental procedures and data analysis, see METHODS.

*, $P < 0.05$, compared to the same compartments in the obese basal group.

Figure 4.

Turnover of [¹³C₄]palmitoylcarnitine. Decay of [¹³C₄]palmitoylcarnitine label in gastrocnemius of rats determined at 3 sequential time points during chase following prelabeling (pulse). The raw data of [¹³C₄] enrichment (Y-axis) were transformed by natural logarithm plotted against regular scale in X-axis. The linear decay shows that palmitoylcarnitine has single-pool turnover kinetics according to e^{-kt} kinetics. No new labels come in as they have been cleared from the circulation (inset, where $t_0=20$ min, $t=80$ min). It is emphasized that decay curves with or without natural logarithmic transformation of the raw data appear similar at low k 's. However, without transformation, the error is significant, or 14–97% for k at 0.5–10% based on simulation.

Figure 5.

Unidirectional movement of long chain fatty acids (LCFA) into mitochondria. Plasma free fatty acids and imcTG-fatty acids are sequentially activated to acyl CoA by ACS (acyl CoA synthase) and acylcarnitine by extra-mitochondrial CPT1 (carnitine-palmitoyl transferase-1). Upon formation, acylcarnitine is transferred into mitochondria by AT (acylcarnitine translocase) only where can it be reverted back to acyl CoA by the intramitochondrial CPT2 to enter β -oxidation cascade initiated by ACD (acyl CoA dehydrogenase). Note that the extra- and intra-mitochondrial acylcarnitine pools are isotopically identical and that the intra-mitochondrial pool does not accumulate. The thick arrow, or β -oxidation flux, stresses this unidirectionality but is identical stoichiometrically to the upstream and downstream steps.

Enrichment, fractional turnover rate and content of palmitoylcarnitine and imcTG-palmitate in gastrocnemius muscle of lean and obese rats

Table 1

Group (n)	Turnover constant		Palmitoyl-carnitine		imcTG-palmitate		Palmitoylcarnitine	
	Palmitoyl-carnitine, k	imcTG-palmitate, k'	per minute	nmol/g	μmol/g	MPE	Δ [$^{13}\text{C}_4$]	[U- ^{13}C]
Lean basal (6)	0.79±0.2	0.0117±0.001		9.8±1.2	0.53±0.1	1.12±0.11	1.44±0.22	
Obese basal (6)	0.83±0.2	0.0119±0.001		19.4±2	1.33±0.1	1.19±0.07	0.85±0.10	
Obese clamp (4)	0.34±0.1	0.0083±0.0003		26.8±3	1.20±0.1	0.97±0.05	1.17±0.10	
$P_{\text{basal vs clamp}}$	0.04	0.03		0.05	0.4			

Values are mean±SEM. All groups were studied using pulse-chase protocol after overnight fast. At time t, muscle was collected, lipids extracted and content and [$^{13}\text{C}_4$] and [U- ^{13}C] enrichment of palmitoylcarnitine determined (the enrichments are >10 times the isotopic detection limits of the instrument). The magnitude of decline in [$^{13}\text{C}_4$] MPE during chase, Δ [$^{13}\text{C}_4$], is calculated by comparing to [$^{13}\text{C}_4$] at t₀ determined from the pulse group.

$$k = k' \times [\text{imcTG-palmitate}]/[\text{palmitoylcarnitine}] \text{ (see METHODS for detail).}$$