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A liquid chromatography/tandem mass spectrometry method for measuring the in vivo incorporation of plasma free fatty acids into intramyocellular ceramides in humans

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Abstract

RATIONALE—Sphingolipids are important components of cell membranes that serve as cell signaling molecules; ceramide plays a central role in sphingolipid metabolism. De novo ceramide biosynthesis depends on fatty acid availability, but whether muscle uses circulating free fatty acids or pre-existing intracellular stores is unknown. Our goal was to develop a method to detect the incorporation of intravenously infused [U-¹³C]palmitate into intramyocellular ceramides.

METHODS—We used a liquid chromatography/tandem mass spectrometry (LC/MS/MS) to measure the concentrations of different sphingolipids species and ¹³C isotopic enrichment of 16:0-ceramide. Chromatographic separation was performed using ultra performance liquid chromatography. The analysis was performed on triple quadrupole mass spectrometer using positive ion electrospray ionization source with selected reaction monitoring (SRM).

RESULTS—The sphingolipids ions, except enriched ceramide, were monitored as [M+2+H]⁺. The [¹³C₁₆]16:0-ceramide was monitored as [M+16+H]⁺. By monitoring 2 different transitions of the [¹³C₁₆]16:0-Ceramide (554/536 and 554/264) we could indirectly measure enrichment of the palmitate that is not a part of the sphingoid base. Concentration and enrichment could be measured using 20 mg of muscle obtained from volunteers receiving a low dose [U-¹³C]palmitate infusion.

CONCLUSIONS—LC/MS/MS can be used to detect the incorporation of plasma palmitate into muscle ceramides in humans, in vivo.

Introduction

Sphingolipids are an important lipid class because of their role in cell signal transduction. Ceramide, a sphingosine-based lipid, is a central molecule in sphingolipid metabolism because it acts as a second messenger for cellular functions ranging from proliferation and differentiation to growth arrest and apoptosis.^[1–4] The variety of ceramide actions can be attributed to its ability to alter the activity of kinases, phosphatases and transcription factors. Sphingolipids have been implicated in insulin resistance; a negative relationship between ceramides content in skeletal muscle and insulin sensitivity has been reported.^[5]

The ceramide content of cells is dependent on the rate of its production and degradation. Ceramide is generated via de novo biosynthesis and by the hydrolysis of sphingomyelins (SM) via the action of sphingomyelinases. The first and rate limiting step in de novo ceramide synthesis is catalyzed by serine palmitoyltransferase (SPT), which condenses serine and palmitoyl-CoA. Another key enzyme in de novo biosynthesis is ceramide

synthase; this step involves the addition of another long chain acyl-CoA to sphinganine to create dihydroceramide. Thus, the availability of fatty acids is essential for de novo ceramides biosynthesis. Whether the fatty acids needed for de novo synthesis are derived from plasma free fatty acids (FFA) in vivo is not known, nor is it clear whether the majority of intramyocellular ceramide is from de novo synthesis or SM hydrolysis. In some circumstances increased FFA availability stimulates lipid oxidation and leads to reduced glucose utilization,^[6] whereas in chronically insulin-resistant states tissue fatty acid oxidation is not increased whereas lipid storage is.^[7] It is now thought that intramyocellular accumulation of fatty acids or their metabolites play an important role in the pathogenesis of human insulin resistance.^[8]

Experiments using stable or radioactive isotopic tracers to discover the fate of fatty acids, glucose and amino acids have been conducted for over 50 years. Using a uniformly labeled [U-¹³C]fatty acid tracer allows investigators to elucidate the fate of plasma FFA within tissue while administering only small amounts of the compound.^[9, 10] Because ceramides are one of the lipids classes whose accumulation in skeletal muscle that may contribute to insulin resistance^[11, 12] and because the potential contributions of circulating FFA to ceramide synthesis is not known, we developed a method to measure sphingolipid concentrations and ceramide enrichment in tissue. Our goal is to understand whether intramyocellular ceramides largely derive from plasma FFA in physiological and pathological states. By relating ceramide enrichment to plasma FFA enrichment it should be possible to understand the relative contribution of de novo biosynthesis from FFA vs. SM hydrolysis/in determining intracellular ceramides concentrations.

Many methods have been used for sphingolipid analysis, from classic methods such as enzymatic assays,^[13] high performance liquid chromatography (HPLC),^[14] and gas chromatography (GC) to mass spectrometry (GC/MS and HPLC/MS). The most recently developed method for sphingolipid analysis employs HPLC/MS. The MS methodology allows users to monitor changes in the composition of all sphingolipids species.^[15–19] Here we report an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) method that can detect the incorporation of [U-¹³C]palmitate from plasma FFA in humans into intramyocellular ceramides, including enrichment sphingolipid concentrations and using selected reaction monitoring (SRM) mode.

Materials and methods

Supplies

Sphingosine (Sph), sphinganine (dhSph), sphingosine-1-phosphate (S1P), ceramides containing myristic acid (C14:0-Cer), ceramides containing palmitic acid (C16:0-Cer), ceramides containing stearic acid (C18:0-Cer), ceramides containing oleic acid (C18:1-Cer), ceramides containing arachidic acid (C20:0-Cer), ceramides containing lignoceric acid (C24:0-Cer), ceramides containing nervonic acid (C24:1-Cer) as well as 3 internal standards, ceramides containing margaric acid (C17:0-Cer), 17C-sphingoid base sphingosine (d17:1-Sph), sphingosine-1-phosphate (d17:1-S1P) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Ceramide containing uniformly labeled [U-¹³C] palmitic acid as the second fatty acid (added to the sphingoid base ([¹³C₁₆]16:0-Cer) was synthesized by the Lipidomics Core, MUSC (Medical University of South Carolina). HPLC grade methanol was purchased from EMD Chemicals, (Gibbstown, NJ). HPLC grade water, isopropanol, ethyl acetate and 4ml-screw cap vials were obtained from Fisher Chemical (Pittsburg, PA). Formic acid, ammonium formate, KCl, Tris, EDTA, essentially fatty acid free albumin, potassium phosphate, potassium biphosphate and ethanol were obtained from Sigma-Aldrich (St. Louis, MO). All caps were lined with Teflon liners (Arthur H. Thomas, Philadelphia, PA). The UPLC column was purchased from Waters Corporation (Milford, MA).

Stock Solutions and Standards

A mixed solution of 10 mM of potassium phosphate and diphosphate were prepared in water. The pH of the phosphate buffer was adjusted to 7.6 using 10 M sodium hydroxide. A 1 % albumin solution was made by slowly adding 1 g of albumin to 100 ml of 10 mM phosphate buffer. Homogenization buffer containing 0.25 M sucrose, 20 mM KCl, 50 mM Tris and 0.5 mM EDTA, pH 7.4 was prepared using HPLC grade water. The extraction solution was made by mixing together 35 ml of isopropanol, 5 ml water and 60 ml ethyl acetate. The stock internal standard (IS) was made in ethanol to contain d17:1-Sph, d17:1-S1P and C17:0-Cer, at 1.125 µg/ml, 0.75 µg/ml and 12.5 µg/ml respectively. The stock concentration standard (CS) comprised of 20.0 µg/ml Sph, dhSph, S1P, C14:0-Cer; 100 µg/ml of C16:0-Cer; 3.0 µg/ml of C18:1-Cer; 400 µg/ml of C18:0-Cer, C24:0-Cer, C24:1-Cer, and 40.0 µg/ml of C20:0-Cer, was also made in ethanol. A ten-point (0.01% – 0.50%) [¹³C₁₆]16:0-Ceramide enrichment curve was prepared in ethanol.

In vivo experiments

Plasma and muscle samples were obtained from five lean and obese volunteers participating in two ongoing research studies of free fatty acid turnover. These protocols were approved by Mayo Institution Review Board, and all participants provided signed, informed consent. On the study day, each volunteer received an infusion of [U-¹³C]palmitate at 2 nmol•kg FFM⁻¹•min⁻¹ for 6 hours. Four participants, 2 lean, 2 obese, were resting in bed, consuming small amounts of fat free food every 20 minutes for 5 hours to maintain steady-state insulinemia (postprandial), which should suppress lipolysis and FFA concentrations. One obese volunteer was walking on a treadmill for 5 hours at ~ 2 miles/hour in order to raise FFA concentrations and engage fatty acid oxidative machinery in muscle. The feeding and exercise started 90 minutes prior to the first muscle biopsy. A series of arterialized venous blood samples were collected over 30 min prior to the biopsies for measurement of plasma palmitate concentration and enrichment.^[20] Quadriceps muscle biopsies were performed under sterile conditions using local anesthesia 2 and 6 hours after starting the [U-¹³C]palmitate infusion. The exercising volunteer was briefly transferred to a bed for the muscle biopsies. The muscle samples were immediately rinsed with ice cold saline to remove any blood residue and stored at –80°C for analysis at a later date.

Procedures

On the day the muscle samples were processed, an eight-point concentration standard curve was constructed by diluting the stock concentration solution with the 1% albumin solution to yield the ranges shown in table 2. The sphingolipid extraction from muscle was performed as previously described^[15] with minor modifications. Briefly, 20 mg of muscle was homogenized 4 times in 200 µl of chilled homogenization buffer while keeping the mixture on an ice bath. Ten µl of diluted stock internal standard solution (1:25 with 1% albumin solution) and 1.5 ml of extraction solution were added to each muscle homogenate and concentration standards. The mixture was vortexed, sonicated and then centrifuged for 10 min at 4000 rpm. The supernatant was transferred to a new vial and pellet was extracted once more. The combined supernatants were evaporated under nitrogen on chilled blocks until dryness for UPLC/MS/MS analysis.

UPLC/MS/MS condition

The concentration of the 10 sphingolipids and isotopic enrichment of [¹³C₁₆]16:0-ceramide in muscle were simultaneously measured against an extracted concentration standard curve as well as an enrichment standard curve on a Thermo TSQ Quantum Ultra mass spectrometer (Waltham, MA) coupled with a Waters Acquity UPLC system (Milford, MA). The sphingolipids were separated on the UPLC with a Waters Acquity C8 UPLC BEH

column 2.1×150 mm, $1.7 \mu\text{m}$ at 43°C using two buffers. Buffer A was methanol, 2 mM ammonium formate, 0.1% formic acid; buffer B was water, 1 mM ammonium formate, 0.1% formic acid. The flow rate was 0.4 ml/min, and the gradient conditions were as follows: 0 min at 20% B, 0–1.5 min 20–10% B, 1.5–2.3 min isocratic at 10% B, 2.3–9.3 min 10–1% B, 9.3–11 min isocratic at 1% B, 11–11.3 min 1–20% B, 11.3–13 min isocratic at 20% B. Standards and samples were re-suspended in 50 μl buffer A prior to injecting 5 μl onto the UPLC/MS/MS. Figure 1 shows the separation of all species in the standards (panel A) and muscle (panel B).

The mass spectrometer was equipped with an electrospray ionization interface. The following conditions were used: the spray voltage set at 4000V, sheath gas at 0.675 L/min, ion sweep gas at 0.6 L/min, aux gas at 1.2 L/min, and transfer capillary at 275°C . The collision gas was set at 1.2 mTorr. All sphingolipids, except C16:0-Cer, were monitored as $[\text{M}+\text{H}]^+$ in positive mode. The C16:0-Cer and $[\text{C}_{16}^{13}\text{C}]16:0\text{-Cer}$ were monitored as $[\text{M}+2+\text{H}]^+$ and $[\text{M}+16+\text{H}]^+$ respectively. Transition of masses and collision energy are shown in Table 1. The entire analysis was performed in SRM mode.

Results

Muscle sphingolipid concentrations

Prior to analyzing the samples from the volunteers who had received the $[\text{U-}^{13}\text{C}]$ palmitate tracer infusion, we tested our method for measuring concentration using a pooled muscle tissue on five separate days. Each day 5 replicate muscle samples were worked up together with a concentration standard curve. The intra- and inter-assay CV's for these measurements are provided in table 2; they were generally below 1%.

The extraction recovery was measured using homogenized muscle samples that were kept at room temperature for one day followed by heating three times at 45°C for 1 h. Part of the homogenate was extracted to ensure that sphingolipids were not detectable. The remaining homogenate was divided for 5 aliquots to which a known amount of a sphingolipid mixture was added; this allowed us to calculate the extraction recovery. The extraction efficiency ranged from 85–95% for each measured sphingolipid.

Ceramide isotopic enrichment measurement

A ten point standard enrichment curve with $[\text{C}_{16}^{13}\text{C}]16:0\text{-Cer}$ molar percentage excess (MPE) ranging from 0.01% to 0.5% is shown in Figure 2. The observed $[\text{C}_{16}^{13}\text{C}]16:0\text{-Cer}/\text{C}16:0\text{-Cer}$ area ratio and theoretical MPE showed an excellent linear relationship ($R^2 = 0.9997$). Accurate measures of total enrichment to as low as 0.01 – 0.02% are feasible.

When analyzed in positive-ion mode the most specific common fragment ion for ceramides is the sphingoid base backbone minus two water molecules, which has an m/z 264 (Figure 3A, CE 25eV).^[16, 21, 22] In addition to measuring concentration, we wished to measure the enrichment found in skeletal muscle ceramides during a low dose $[\text{U-}^{13}\text{C}]$ palmitate infusion and to determine in which position the labeled palmitate had been incorporated. To accomplish this we: 1) used $[\text{M}+2+\text{H}]^+$ (correspond to second isotopic peak) as our base peak for analyzing unlabeled species; 2) monitored the enriched ceramide as 2 product ions, one containing both fatty acid moieties, the second containing only the sphingosine. The later tactic allowed us to determine in which of the two positions an enriched palmitate from plasma FFA was incorporated using a subtraction strategy. By monitoring 2 different transitions of the $[\text{C}_{16}^{13}\text{C}]16:0\text{-Ceramide}$ (554/536 and 554/264) we could indirectly measure enrichment of the palmitate that is not a part of the sphingoid base. A product ion scan of the $[\text{C}_{16}^{13}\text{C}]16:0\text{-Ceramide}$ is shown in Figure 3B. As expected, the enrichment of the $[\text{C}_{16}^{13}\text{C}]16:0\text{-Cer}$ in muscle tissue was very low. By using $[\text{M}+2+\text{H}]^+$ as our base peak for

analyzing unlabeled species rather than the $[M+H]^+$ we could increase the relative abundance of $[M+16+H]^+$ species.

All ceramides, sphingosine, sphinganine, and sphingosine-1-phosphate in the concentration standard mixture were separated not only by their mass transitions but also by elution time on the gradient LC (Figure 1).

The concentration and enrichment data from human skeletal muscle samples collected during a continuous intravenous infusion of $[U-^{13}C]$ palmitate are provided in Table 3. Plasma $[^{13}C_{16}]$ palmitate enrichment was measured according to Persson et al.^[20] Sphingolipid concentrations were not significantly different between the samples collected 2 and 6 h after beginning the $[U-^{13}C]$ palmitate infusion (data not shown except the C16:0-Cer concentrations). The results were obtained with three replicate human muscle samples analyzed over 3 days. We detected enrichment in the total ceramide molecule (transition m/z 554 to m/z 536) 3 out of the 4 samples collected two hours after starting the $[U-^{13}C]$ palmitate infusion. For the 2 hour samples with detectable enrichment, the majority of enrichment was in the sphingoid base. From the volunteers with detectable enrichment at 2 hours, there was increased ceramide enrichment in the samples collected at hour 6. The samples from the exercising volunteer had no detectable enrichment after 2 hours and after 6 hours the only detectable enrichment was in the sphingoid base.

Discussion

Our goal was to establish a method that would allow us to measure both the tissue sphingolipid concentrations and to detect incorporation of labeled palmitate from an intravenous infusion of a low dose of $[U-^{13}C]$ palmitate. By using UPLC/MS/MS in SRM mode we were able to reproducibly measure muscle concentrations of sphingolipids with a relatively short (13 min) run time and to assess the net incorporation of plasma FFA palmitate into $[^{13}C_{16}]16:0$ -Cer total and $[^{13}C_{16}]16:0$ -Cer – sphingoid base. We employed the well-established strategy of monitoring the $[M+2+H]^+$ species as a base peak^[20, 23, 24] to increase the relative abundance of the $[M+16+H]^+$ ions for the enriched sphingolipids.

Our results suggest that under fed conditions 10–30% of muscle ceramides derive from de novo synthesis from plasma FFA within 2 h. In contrast, with exercise a lesser proportion of muscle ceramide contains fatty acids derived from plasma, consistent with the concept that physical activity will shunt fatty acids into oxidative pathways. We have observed that the enrichment in intramyocellular triglycerides and long chain acyl-carnitines^[9] is ~5% of plasma FFA enrichment after 6 hours of tracer infusion. Thus, the enrichment we found in ceramide is somewhat closer to that of plasma palmitate than that of intramyocellular triglycerides and palmitoyl-carnitine^[9]. There is a significant time delay in the appearance of tracer from plasma in intramuscular ceramides, suggesting this may be a slowly turning over pool or that the plasma FFA are first incorporated into other lipid pool, subsequently liberated and then incorporated into ceramides.

Our approach to measuring the positional $[U-^{13}C]$ palmitate content of ceramides differs in several respects from that of Hayes et al,^[18] who incubated HEK293 cells with 0.1mM of $[U^{13}C]$ palmitate for 0 to 6 hours. We purchased a custom made $[^{13}C_{16}]16:0$ -Cer in order to create the standard curve and use SRM approaches rather than enzymatic cleavage of sphingomyelins to determine the position of the labeled fatty acid moiety.

In summary, we describe an LC/MS/MS method for simultaneous measurement of sphingolipid concentration and positional ceramide enrichment from plasma FFA. Despite using a low dose intravenous $[U-^{13}C]$ palmitate infusion and only 20 mg of muscle for analysis, we were able to detect the enrichment of $[^{13}C_{16}]16:0$ -Cer, finding it to be 10–30 %

of the plasma palmitate enrichment in postprandial humans. We found that most of the labeled FFA tracer is in the sphingoid base, the reaction catalyzed by SPT. Unfortunately, we did not measure the SPT activity and thus cannot comment on whether the observed ceramide enrichment is a result of increased FFA uptake by skeletal muscle in some circumstances or increased activity of enzymes responsible for ceramide de novo biosynthesis. Our method uses ultra performance liquid chromatography coupled with triple quadrupole mass spectrometer in positive electrospray ionization to monitor selected product ions of sphingolipid species (SRM mode). The method is simple, rapid, reproducible and robust.

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Figure 1.

Total ion chromatogram (TIC) of sphingolipids extracted from human skeletal muscle (A) and sphingolipids standards mix (B) in SRM. Panel C shows an enlarged section of the TIC from panel B in the time from 5.1 min to 6.1 min, demonstrating good separation of dihydroceramides from ceramides.

Figure 2. Standard curve of [¹³C₁₆]16:0-Cer enrichment expressed as total MPE (transition m/z 554 to m/z 536).

Figure 3.

The upper panel depicts a product ion spectrum of C16:0-Cer (precursor ion m/z 538.7) and the lower panel depicts a product ion scan of $[^{13}\text{C}_{16}]16:0\text{-Ceramide}$ (precursor ion m/z 554.7). * denotes ^{13}C .

Table 1

Quantitative parameters for sphingolipid analysis.

Compound	precursor ion	product ion	Collision energy (V)	LOD (fmol) on column
d17:1 Sph *	288.3	270.3	14	5.5
Sph	302.3	284.3	14	5.2
dhSph	304.3	286.3	18	5.2
d17:1 S1P *	368.3	252.3	17	4.3
S1P	382.3	266.3	18	4.1
C14:0-Cer	512.7	494.5	12	3.1
C16:0-Cer	540.7	522.5	14	2.9
[¹³ C ₁₆]16:0-Cer	554.7	536.5	14	2.8
[¹³ C ₁₆]16:0-Cer	554.7	264.5	25	2.8
C17:0-Cer *	554.7	536.6	14	2.8
C18:1-Cer	566.7	548.6	13	2.8
C18:0-Cer	568.6	550.7	14	2.8
C20:0-Cer	596.8	578.8	15	2.6
C24:1-Cer	650.8	632.8	17	2.4
C24:0-Cer	652.8	634.8	16	2.4

* internal standards.

Table 2

Reproducibility of sphingolipid concentration measurements from human skeletal muscle.

Compound	Intra-assay n=5		Inter-assay n=5	
	ng/100mg muscle	% CV	ng/100mg muscle	% CV
Sph	12.5	0.4	12.6	1.0
dhSph	2.5	0.6	2.52	0.7
S1P	0.7	1.1	0.8	1.9
C14-Cer	3.7	0.4	3.7	1.0
C16-Cer	81.0	0.6	81.7	0.7
C18:1-Cer	147.0	0.3	147.5	0.8
C18-Cer	457.5	0.3	458.7	0.3
C20-Cer	29.8	0.8	30.0	0.7
C24:1-Cer	231.5	0.1	232.0	0.6
C24-Cer	298.5	0.5	300.0	0.6

The average (Ave) and coefficient of variation (CV) from 5 day replicate measures of sphingolipid concentrations from 5 human skeletal muscle samples are provided.

Table 3

Muscle C16:0-ceramide concentration and enrichment, and plasma U¹³C-palmitate enrichment in human subjects.

Subject	T	Group	ng/100 mg muscle	Total MPE (measured)	sphingoid base MPE (measured)	Fatty acid MPE (calculated)	Plasma [U ¹³ C]palmitate MPE
S1	6h	L P	91	0.029±0.002	0.02±0.001	0.009	0.115
S2	2h	L P	83	0.016±0.002	0.016±0.001	not detected	0.091
S2	6h	L P	88	0.026±0.001	0.017±0.002	0.009	0.098
S3	2h	O P	108	0.032±0.002	0.022±0.001	0.01	0.120
S3	6h	O P	119	0.048±0.002	0.037±0.002	0.011	
S4	2h	O P	104	0.027±0.002	0.019±0.002	0.008	0.100
S4	6h	O P	114	0.043±0.002	0.028±0.002	0.015	
S5	2h	O E	91	not detected	not detected	not detected	0.066
S5	6h	O E	87	0.013±0.001	0.013±0.001	not detected	0.098

T= biopsy time in hours after [U-¹³C]palmitate infusion. L=lean, O=obese, P=postprandial, E=exercise.