Supplemental information for:

Factors to consider in using [U-^{13}C]palmitate for analysis of sphingolipid biosynthesis by liquid chromatography-tandem mass spectrometry

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This document includes supplemental experimental procedures, Tables I to IV, and Supplemental Fig. 1 and 2

Experimental procedures

Reagents

The internal standard cocktail (catalog number LM-6002, Avanti Polar Lipids, Alabaster, AL) was obtained in sealed ampoules and certified (1) to be > 95% pure and within 10% of the specified concentration (250 μM). It was comprised of the 17-carbon chain length sphingoid base analogs: C17-sphingosine, (2S,3R,4E)-2-aminoheptadec-4-ene-1,3-diol (d17:1-So); C17-sphinganine, (2S,3R)-2-aminoheptadecane-1,3-diol (d17:0-Sa); C17-sphingosine 1-phosphate, heptadecasphing-4-enine-1-phosphate (d17:1-S1P); and C17-sphinganine 1-phosphate, heptadecasphinganine-1-phosphate (d17:0-Sa1P); and the C12-fatty acid analogs of the more complex SP(s) C12-Cer, N-(dodecanoyl)-sphing-4-enine (d18:1/12:0-Cer); C12-Cer 1-phosphate (Cer-1-P), N-(dodecanoyl)-sphing-4-enine-1-phosphate (d18:1/12:0-Cer1P); C12-sphingomyelin, N-(dodecanoyl)-sphing-4-enine-1-phosphocholine (d18:1/12:0-SM); C12-glucosylceramide, N-(dodecanoyl)-1-β-glucosyl-sphing-4-eine (d18:1/12:0-GlcCer); and C12-lactosylceramide, N-(dodecanoyl)1-β-lactosyl-sphing-4-eine (d18:1/12:0-LacCer); as well as one very-long-chain Cer analog, C25-Cer, N-(pentacosanoyl)-sphing-4-enine (d18:1/25:0-Cer). Fatty acyl-CoA internal standards (pentadecanoyl-CoA, heptadecanoyl-CoA, tricosanoyl-CoA, and pentacosanoyl-CoA) were from Avanti Polar

Lipids (Alabaster, AL) and individually supplied as 0.5 mg dry powder in sealed ampoules.

The HPLC grade solvents (acetonitrile, # EM-AX0145; chloroform, # EM-CX1050; hexane, # JT9304-33; and methanol, # EM-MX0475, as well as ACS grade formic acid, # EM-FX0440-7) were obtained from VWR (West Chester, PA), and acetic acid (ACS grade, # A38C-212) was obtained from Fisher (Pittsburg, PA).

Cell culture

For analysis of SP biosynthesis by a mammalian cell line, HEK293 cells were obtained from the American Tissue Culture Collection (Manassas, VA) (cat# CRL-1573). The cells were grown in 60-mm plastic culture dishes in DMEM supplemented with 10% FBS, 4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 100 U/ml penicillin and 0.1 mg/ml streptomycin in Steri-cult CO_2 incubators (Thermo Fisher Scientific Inc., Waltham, MA) with 5% CO_2 and 90% relative humidity at 37°C. In labeling experiments cells were incubated for 0 to 6 h with 0.1 mM [U-^{13}C]palmitate in a 1:1 molar complex with fatty acid-free bovine serum albumin prepared as described in reference (2) for preparation of the sphingosine-BSA complex.

Liquid chromatography-electrospray ionization-tandem mass spectrometry

The SP were extracted from the cells and LC-ESI-MS/MS analysis of the SP was performed as previously described (3). Prior to selecting MRM pairs for analysis of HEK293 cells, the variation in N-acyl chain length was determined, which allowed MRM transitions to correspond to the observed N-acyl species. This was accomplished by precursor ion scans (*m/z* 184.4) of the extracts containing N-acyl SPs, which indicate the molecular species of SM present in the extract. SM was selected because they are typically abundant and are indicative of both sphingosine (So) and sphinganine (Sa)

species. For example, in HEK293 cells, the major SM molecular species are d18:1/16:0, d18:1/18:0, and d18:1/24:1. This nomenclature (4) indicates the number of hydroxyl moieties (d for dihydroxy), the number of carbon atoms in the sphingoid base (eighteen), the number of double bonds (one), and also the number of carbon atoms and double bonds in the N-acyl chain (e.g. sixteen and zero, respectively).

The SP quantities were normalized to the protein quantity (mg) in the sample as determined by the Pierce BCA assay kit (cat# 23225, Thermo Fisher Scientific, Rockford, IL). It was also observed that 1 x 10^6 HEK293 cells contained 0.59 ± 0.04 mg protein, so 1.0 mg protein corresponds to 1.7 x 10^6 HEK293 cells.

Analysis of backbone labeling of SM by LC-ESI-MS/MS after phospholipase D treatment

To validate this method, an HEK293 cell extract that contained 690 ± 20 pmol of [M + 16] SM (i.e., singly labeled but without knowledge of the position)/mg protein was found to also contained 5 ± 2 pmol of base-labelled Cer-1-P/mg protein, and the same amount of the fatty acid-labeled isotopomer. After treatment with PLaseD, the extract had 70 ± 10 pmol of base-labeled Cer-1-P and 560 ± 20 pmol of fatty-acid labeled Cer-1-P per mg protein. Therefore, the pre-existing Cer-1-P was only a minor fraction, and the sum of 630 ± 20 pmol Cer-1-P/mg protein after PLaseD treatment represents a recovery of 90% of the original singly labeled SM as Cer-1-P. The N-acyl chain length distribution of SM was also preserved with ^{13}C-d18:1/^{12}C-16:0, ^{13}C-d18:1/^{12}C-24:1, ^{12}C-d18:1/^{13}C-16:0 and ^{12}C-d18:1/^{13}C-24:1 Cer-1-P being the most abundant chain lengths (shown in supplemental Fig. 2).

Cell extraction and liquid chromatography-electrospray ionization-tandem mass spectrometry for fatty acyl-CoA quantitation. Fatty acyl-CoAs were extracted from HEK293 cells, and analyzed by LC-ESI-MS/MS as described previously (5).

References

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3. Shaner, R. L., J. C. Allegood, H. Park, E. Wang, S. Kelly, C. A. Haynes, M. C. Sullards, and A. H. Merrill, Jr. 2009. Quantitative analysis of sphingolipids for lipidomics using triple quadrupole and quadrupole linear ion trap mass spectrometers. *J Lipid Res* **50**: 1692-1707.

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Table I. Ceramide precursor and product ion *m/z* values for isotopologues and isotopomers with different N-acyl chain lengths after labeling with [U-^{13}C]palmitate.

Fatty acid[a]	**16:0**	**18:0**	**20:0**	**22:0**	**24:1**	**24:0**	**26:1**	**26:0**
Unlabeled								
MRM *m/z*[b]	538.7 264.4	566.7 264.4	594.7 264.4	622.8 264.4	648.9 264.4	650.9 264.4	676.9 264.4	678.9 264.4
r[c]	0.6817[d]	0.6667	0.6519	0.6382	0.6246	0.6246	0.6109	0.6109
^{13}CFA								
MRM *m/z*[b]	554.7 264.4	582.7 264.4	610.7 264.4	638.8 264.4	664.9 264.4	666.9 264.4	692.9 264.4	694.9 264.4
r[c]	0.8097	0.7924	0.7746	0.7587	0.7418	0.7418	0.7257	0.7257
Dual ^{13}C								
MRM *m/z*[b]	570.7 280.4	598.7 280.4	626.7 280.4	654.8 280.4	680.9 280.4	682.9 280.4	708.9 280.4	710.9 280.4
r[c]	0.9615	0.9407	0.9208	0.9001	0.8811	0.8811	0.8621	0.8621
^{13}C Base								
MRM *m/z*[b]	554.7 280.4	582.7 280.4	610.7 280.4	638.8 280.4	664.9 280.4	666.9 280.4	692.9 280.4	694.9 280.4
r[c]	0.8097	0.7924	0.7746	0.7587	0.7418	0.7418	0.7257	0.7257

a The x:y abbreviation denotes the number of carbon atoms (x) and double bonds (y) in the N-acyl chain.
b Precursor-Product ion *m/z*
c Fraction of total quantity that is measured by the shown MRM pair; for the 12:0 internal standard this value was 0.7123.

Table II. Monohexosyl ceramide precursor and product ion *m/z* values for isotopologues and isotopomers with different N-acyl chain lengths after labeling with [U-^{13}C]palmitate.

Fatty acid[a]	16:0	18:0	20:0	22:0	24:1	24:0	26:1	26:0
Unlabeled								
MRM *m/z* [b]	700.7 264.4	728.7 264.4	756.7 264.4	784.8 264.4	810.9 264.4	812.9 264.4	838.9 264.4	840.9 264.4
r [c]	0.6309	0.6169	0.6035	0.5907	0.5780	0.5777	0.5653	0.5650
^{13}CFA								
MRM *m/z* [b]	716.7 264.4	744.7 264.4	772.7 264.4	800.8 264.4	826.9 264.4	828.9 264.4	854.9 264.4	856.9 264.4
r [c]	0.7496	0.7326	0.7168	0.7018	0.6859	0.6859	0.6720	0.6716
Dual ^{13}C								
MRM *m/z* [b]	732.7 280.4	760.7 280.4	788.7 280.4	816.8 280.4	842.9 280.4	844.9 280.4	870.9 280.4	872.9 280.4
r [c]	0.8897	0.8711	0.8518	0.8340	0.8157	0.8150	0.7974	0.7974
^{13}C Base								
MRM *m/z* [b]	716.7 280.4	744.7 280.4	772.7 280.4	800.8 280.4	826.9 280.4	828.9 280.4	854.9 280.4	856.9 280.4
r [c]	0.7496	0.7326	0.7168	0.7018	0.6859	0.6859	0.6720	0.6716

a The x:y abbreviation denotes the number of carbon atoms (x) and double bonds (y) in the N-acyl chain.
b Precursor-Product ion *m/z*
c Fraction of total quantity that is measured by the shown MRM pair; for the 12:0 internal standard this value was 0.6588.

Table III. Sphingomyelin precursor and product ion *m/z* values for isotopologues with different N-acyl chain lengths after labeling with [U-^{13}C]palmitate.

Fatty acid[a]	16:0	18:0	20:0	22:0	24:1	24:0	26:1	26:0
Unlabeled								
MRM *m/z*[b]	703.8 184.4	731.8 184.4	759.8 184.4	787.9 184.4	813.9 184.4	815.9 184.4	841.9 184.4	843.9 184.4
r[c]	0.6382	0.6242	0.6109	0.5974	0.5848	0.5848	0.5718	0.5718
Single ^{13}C								
MRM *m/z*[b]	719.8 184.4	747.8 184.4	775.8 184.4	803.9 184.4	829.9 184.4	831.9 184.4	857.9 184.4	859.9 184.4
r[c]	0.7576	0.7413	0.7252	0.7097	0.6944	0.6940	0.6789	0.6789
Dual ^{13}C								
MRM *m/z*[b]	735.8 184.4	763.8 184.4	791.8 184.4	819.9 184.4	845.9 184.4	847.9 184.4	873.9 184.4	875.9 184.4
r[c]	0.9009	0.8811	0.8621	0.8432	0.8244	0.8244	0.8071	0.8071

a The x:y abbreviation denotes the number of carbon atoms (x) and double bonds (y) in the N-acyl chain.
b Precursor-Product ion *m/z*
c Fraction of total quantity that is measured by the shown MRM pair; for the 12:0 internal standard this value was 0.6662.

Table IV. Ceramide-1-phosphate precursor and product ion *m/z* values for isotopologues and isotopomers with different N-acyl chain lengths after labeling with [U-^{13}C]palmitate.

Fatty acid[a]	16:0	18:0	20:0	22:0	24:1	24:0	26:1	26:0
Unlabeled								
MRM *m/z*[b]	618.7 264.4	646.7 264.4	674.7 264.4	702.8 264.4	728.9 264.4	730.9 264.4	756.9 264.4	758.9 264.4
r[c]	0.6761	0.6623	0.6477	0.6333	0.6200	0.6200	0.6068	0.6064
^{13}CFA								
MRM *m/z*[b]	634.7 264.4	662.7 264.4	690.7 264.4	718.8 264.4	744.9 264.4	746.9 264.4	772.9 264.4	774.9 264.4
r[c]	0.8039	0.7868	0.7692	0.7524	0.7364	0.7364	0.7205	0.7205
Dual ^{13}C								
MRM *m/z*[b]	650.7 280.4	678.7 280.4	706.7 280.4	734.8 280.4	760.9 280.4	762.9 280.4	788.9 280.4	790.9 280.4
r[c]	0.8039	0.7868	0.7692	0.7524	0.7364	0.7364	0.7205	0.7205
^{13}C Base								
MRM *m/z*[b]	634.7 280.4	662.7 280.4	690.7 280.4	718.8 280.4	744.9 280.4	746.9 280.4	772.9 280.4	774.9 280.4
r[c]	0.9551	0.9337	0.9141	0.8945	0.8741	0.8741	0.8554	0.8554

a The x:y abbreviation denotes the number of carbon atoms (x) and double bonds (y) in the N-acyl chain.
b Precursor-Product ion *m/z*
c Fraction of total quantity that is measured by the shown MRM pair; for the 12:0 internal standard this value was 0.7067.

Supplemental figures for

Factors to consider in using [U-^{13}C]palmitate for analysis of sphingolipid biosynthesis by liquid chromatography-tandem mass spectrometry

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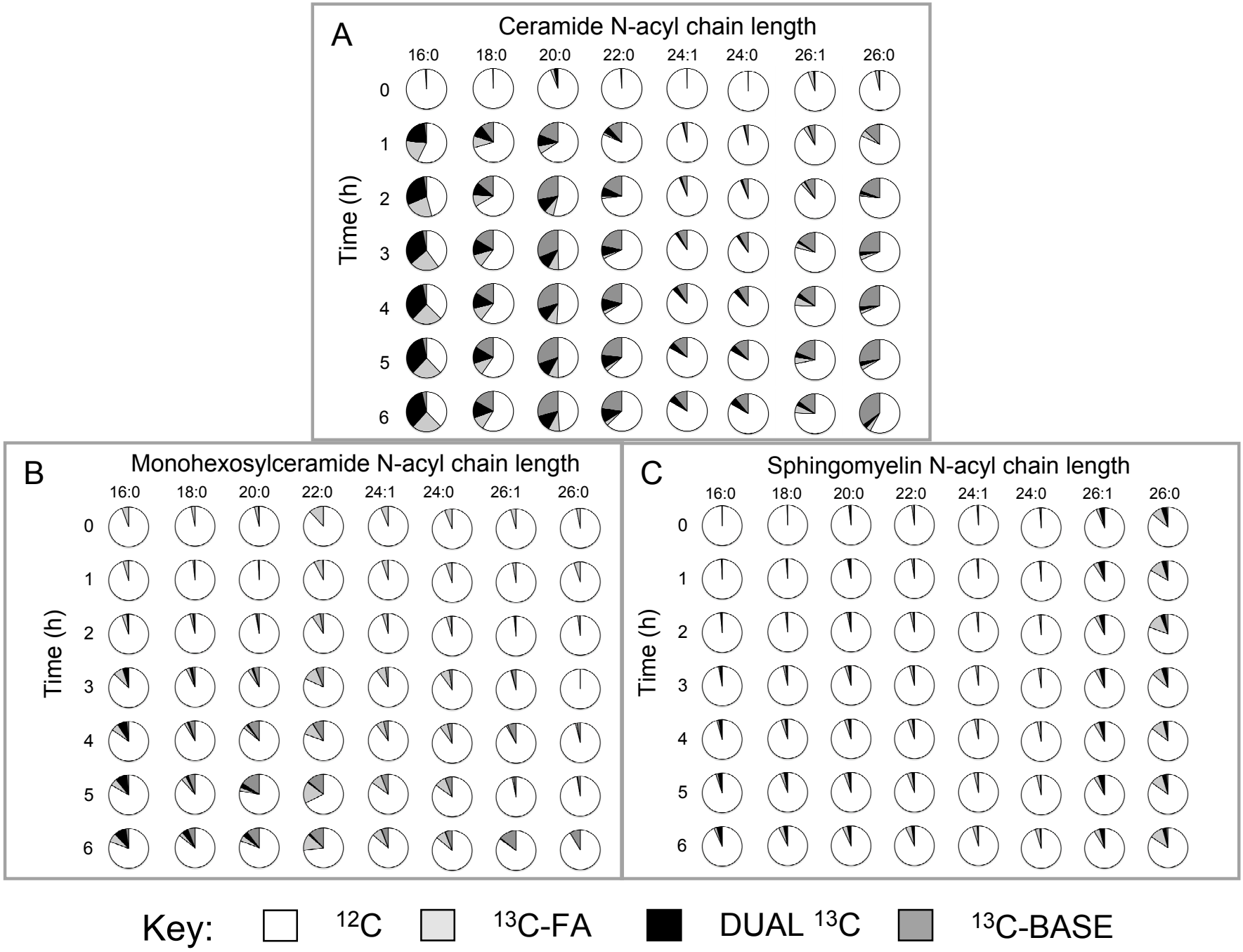
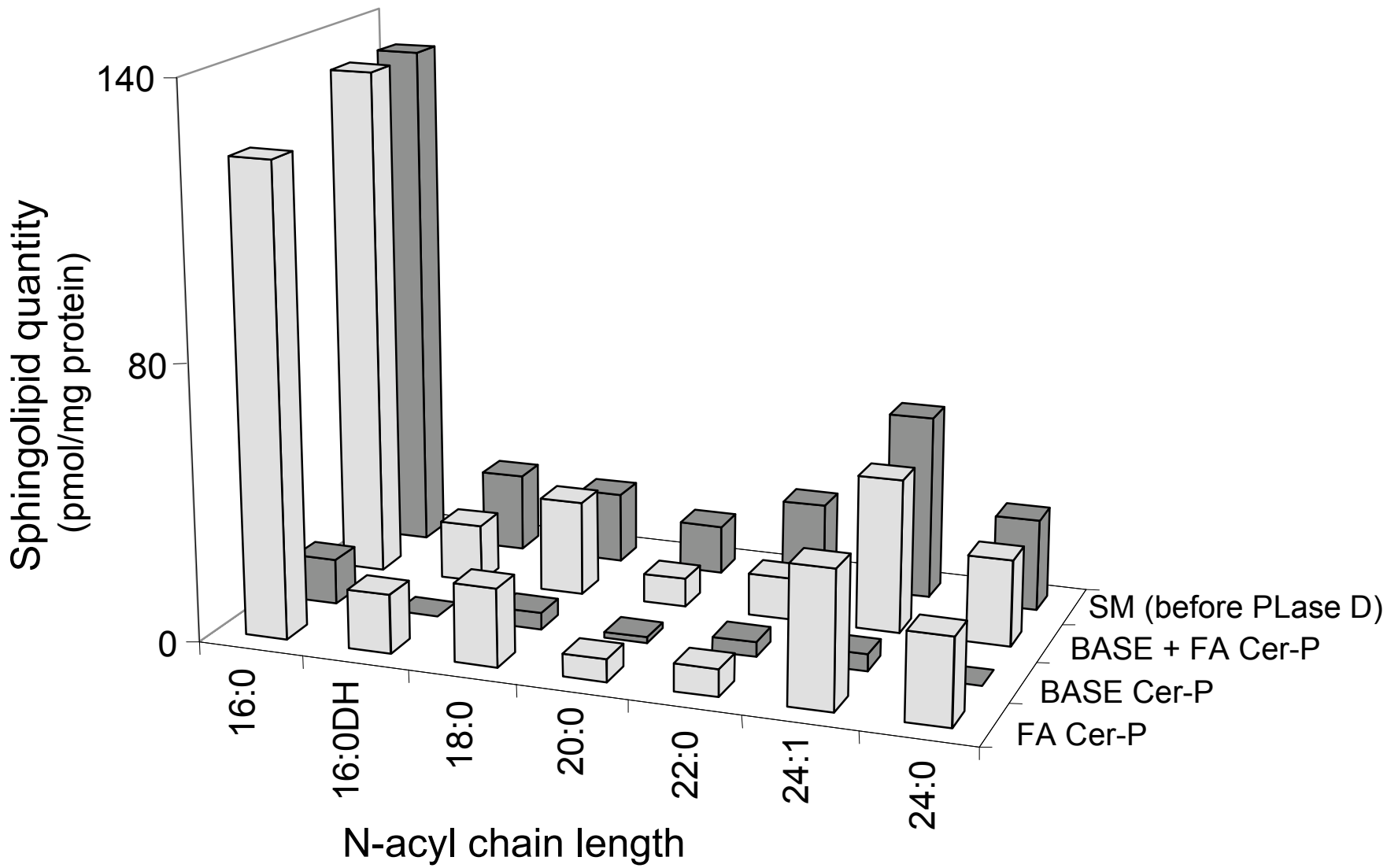
Supplementary Figure 1. Proportions of ceramide, monohexosylceramide, and sphingomyelin isotopologues and isotopomers during 6 h of incubation of Hek cells with 0.1 mM [U-^{13}C]palmitate. The ceramide N-acyl chain length is designated x:y, where x = the number of carbon atoms and y = the number of double bonds, and the shading of the pie chart segments correspond to the isotopologue / isotopomer shown in the key. Panel A is for ceramide (no headgroup); panel B, monohexosylceramide and panel C, sphingomyelin.

Supplementary Figure 2. Determination of label position in singly labeled sphingomyelin using PLaseD treatment as described in Materials and Methods.



Supplementary Figure 1



Supplementary Figure 2