Phytoceramide in Vertebrate Tissues: One Step Chromatography Separation for Molecular Characterization of Ceramide Species

Somsankar Dasgupta, Jina Kong, Erhard Bieberich*

Program in Developmental Neurobiology, Institute of Molecular Medicine and Genetics, Georgia Regents University, Augusta, Georgia, United States of America

Abstract

Ceramide is a precursor for complex sphingolipids in vertebrates, while plants contain phytoceramide. By using a novel chromatography purification method we show that phytoceramide comprises a significant proportion of animal sphingolipids. Total ceramide including phytoceramide from mouse tissue (brain, heart, liver) lipid extracts and cell culture (mouse primary astrocytes, human oligodendroglioma cells) was eluted as a single homogenous fraction, and then analyzed by thin layer chromatography, and further characterized by gas chromatography-mass spectrometry (GC-MS). We detected a unique band that migrated between non-hydroxy fatty acyl ceramide and hydroxy fatty acyl ceramide, and identified it as phytoceramide. Using RT-PCR, we confirmed that mouse tissues expressed desaturase 2, an enzyme that has been reported to generate phytoceramide from dihydroceramide. Previously, only trace amounts of phytoceramide were reported in vertebrate intestine, kidney, and skin. While its function is still elusive, this is the first report of phytoceramide characterization in glial cells and vertebrate brain, heart, and liver.

Citation: Dasgupta S, Kong J, Bieberich E (2013) Phytoceramide in Vertebrate Tissues: One Step Chromatography Separation for Molecular Characterization of Ceramide Species. PLoS ONE 8(11): e80841. doi:10.1371/journal.pone.0080841

Editor: Maurizio Del Poeta, Stony Brook University, United States of America

Received August 9, 2013; Accepted October 15, 2013; Published November 29, 2013

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Funding: This work was supported by National Institutes of Health grant R01AG034389 and an NSF grant NSF1121579 to EB. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ebieberich@gru.edu

Introduction

Ceramide (Cer) participates in a wide variety of biological processes, for example modulating protein phosphorylation and activity of protein kinase C (PKC), regulating cell development and differentiation and signal transduction leading to apoptosis [1,2,3]. Besides de novo synthesis, Cer can be generated by hydrolysis of sphingomyelin [3], and by metabolic degradation of glycosphingolipids or GSLs by glycosidases [4]. The cell or tissue-specific biological function of sphingolipids is pertinent to their concentration and hence, their precise characterization and quantification is critically important. A variety of analytical methods such as high performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), mass spectrometry-mass spectrometry (MS-MS), and lipidomics have been developed to determine the sphingolipid compositions and concentrations [5].

However, these methodologies may not be easily available to many laboratories and are very sensitive to proper extraction and separation methods, in particular when analyzing sphingolipids with intermediate polarity such as phytoceramide. We have developed a novel one-step chromatography method for the separation and identification of Cer and other neutral lipids such as cholesterol and glycerides by introducing a small silicic acid column. Using a variable solvent composition we separated bases, and GSLs remained bound [6]. Cer purified by this method can be visualized and quantified using thin-layer chromatography (TLC) followed by densitometry scanning. Its structure can be delineated using gas chromatography-mass spectrometry (GC-MS), tandem mass spectrometry, or by any other conventional method. While achieving high purification of Cer from primary astrocyte culture and human oligodendroglioma cells, we also found that there is a significant amount of phytoceramide (PHCer) in brain, heart, and liver, which apparently has been unrecognized in previous studies. Therefore, our study not only introduces a simplified method for Cer characterization, but also led us to characterize PHCer in a variety of animal tissues and cells, thereby paving the way for its functional analysis.

Materials and Methods

Silicic acid (200 mesh) was purchased from Sigma Chemical Co. (St. Louis, MO). High performance TLC plates (E. Merck, Darmstadt, Germany), oligonucleotide primers for RT-PCR, and solvents or other chemicals of analytical quality were purchased from Fisher Scientific (Pittsburg, PA). Standard ceramide (Cer) and phytoceramide (PHCer) were purchased from Avanti Polar Lipids (Alabaster, AL). Methanolic HCl and Sylon BTz were purchased from Supelco (now Supelco-Sigma-Aldrich, St. Louis, MO).

Mouse brains (BALBc/6J) were collected in the lab immediately after anesthesia and decapitation as approved by Laboratory Animal Services, Georgia Regents University and the National Institutes of Health. Human oligodendroglioma (HOG) cells and
Figure 1. Thin-layer chromatography of silicic acid purified Cer fractions from astrocytes primary culture and from mouse brain lipid extract. A. The Cer fractions (using previous method) from astrocytes were applied to the HPTLC plate and developed using chloroform:methanol:acetic acid (95:5:0.5, v/v/v). Ceramide bands were visualized after iodine absorption. Lanes 1–3: Std. NFACer; Lane 4–7: Ceramide fractions isolated from astrocytes; Lane 8: Cholesterol; Lane 9: Std. HFACer. Note: The appearance of contaminants (cholesterol) in all preparations. B. Pooled Cer fractions from astrocytes (1A) were applied on a silicic acid column and eluted with solvent chloroform;acetone:acetic acid (24:10.01, v/v/v, solvent 1). After eluting the column with 10 ml of the solvent 1, the column was eluted using chloroform:acetone:acetic acid (18:2:0.01, v/v/v, solvent 2). Approximately 1 ml fractions were collected and 10 μl was applied on the TLC plate and developed using chloroform:methanol:acetic acid (95:5:0.5, v/v/v). The bands were visualized using char spray [6]. Lanes 1–2: Fractions collected using solvent 1 (approximately 5 ml each); Lanes 3–5, 7–12 fractions (1 ml each) collected using solvent 2; Lane 6: Std. NFACer. C. The purified Cer fractions (F2) from mouse brain were dissolved in Phytoceramide in Vertebrates.
primary astrocytes were cultured in the lab and preserved at −80°C until processed for lipid extraction.

**Lipid extraction**

Lipids were extracted from cells or tissues using a chloroform:methanol mixture. The cells (one 100 mm dish) or tissues (30–50 mg wet weight) were homogenized with 2 ml of methanol. Chloroform was added to make the chloroform:methanol ratio 1:2 (v/v). The volume was adjusted to 5 ml and the solution stirred on a magnetic stirrer for 1 h. The extract was centrifuged at 10,000 x g and the supernatant was collected. The lipid was re-extracted from the pellet two more times using chloroform:methanol 1:1 (v/v) and then chloroform:methanol 2:1 (v/v). All three extracts were pooled and dried. A portion of the cells/tissues was preserved for protein determination using the bicinchoninic acid (BCA) method (Pierce chemical Co/Thermo Fisher).

**Purification of Cer from astrocytes, mouse brain and HOG cells**

Ceramide fractions purified from astrocyte cultures employing our previously published method [6] showed cholesterol along with two putative Cer bands, one co-migrating with NFA-Cer and the other with a higher TLC-Rf. To remove cholesterol and enrich for the Cer fractions lipid extracts were further purified using column chromatography and stepwise elution with two solvent systems. Briefly, the dried sample was applied on a silicic acid column chromatography and stepwise elution with two solvent compositions and to test the efficacy of Cer purification using cell culture. The lipid extract was applied on a silicic acid column (0.7 g) in 1 ml of chloroform:methanol:acetic acid 95:5:0.5 (v/v/v, solvent 1), the column was washed with 25 ml of the same solvent, and collected as fraction 1 or F1. Ceramide was eluted as a pure fraction using chloroform:methanol:acetic acid 19:2:0.01 (v/v/v; 15 ml solvent 2, fraction 2 or F2). The two fractions (F1 and F2) collected separately were analyzed by TLC for their lipid composition. Sphingolipidomics (HPLC-MS) analysis was performed at the lipidomics core facility (directorship of Dr. Jacek Bielawski) of the Medical University of South Carolina (MUSC), Charleston, SC.

**TLC resolution of glycerides, cholesterol (F1) and Cer fractions (F2)**

The lipid components of dried F1 and F2 were resolved using TLC. Briefly, the fractions were dissolved in a defined volume (50 µL/mg of protein) of chloroform:methanol 19:1 (v/v) and an equal volume of each sample (3 µl for F1; 15 µl for F2) was applied on a high-performance TLC (HPTLC) plate. The bands were resolved using chloroform:hexane, 4:1 (v/v, results not shown) for F1, and chloroform:methanol:acetic acid 95:5:0.5 (v/v/v) for F2 [6]. The individual bands were initially visualized after iodine absorption and then by char spray. The bands were quantified using densitometry and ImageJ software and compared to the reference standards. The recovery of Cer through the silicic acid column was >95% as reported [6]. Besides glycerides, cholesterol, and Cer detection, our method allows for examining other lipid profiles, such as phospholipids and glycolipids which are still bound to the column, and can be eluted using suitable solvents [6].

Table 1. Fatty acyl composition of purified ceramide from astrocytes.

| Fatty acyl chain | Ceramide (%) | Phytoceramide (%) |
|-----------------|-------------|-------------------|
| C16:1           | ND*         | 1                 |
| C16:0           | 13          | 24                |
| C18:1           | 5           | 4                 |
| C18:0           | 57          | 36                |
| C20:1           | 3           | 2                 |
| C20:0           | 5           | 4                 |
| C22:1           | 7           | 11                |
| C22:0           | 3           | 4                 |
| C24:1           | 4           | 9                 |
| C24:0           | 3           | 5                 |

ND* means not detectable.

doi:10.1371/journal.pone.0080841.t001

Characterization of Cer using gas chromatography-mass spectrometry (GC-MS)

The fatty acyl composition of Cer was analyzed as methyl esters while the base composition was characterized as trimethylsilyl (TMS)-derivatives [6,7,8]. Briefly, a defined amount of Cer solution was transferred into a screw-cap tube, dried under nitrogen, and hydrolyzed with methanol-water-HCl 29:4:3 (v/v/v) [8] [the most effective of several available methods [7,9,10,11]] at 80°C for 18 h in a sealed tube. The mixture of free fatty acids and methyl esters of fatty acids (FAME) was recovered by partitioning with hexane and re-methylated using 1 N methanolic HCl for 16 h-18 h at 80°C. The base was recovered from the methanol-HCl layer and analyzed as TMS-derivative after N-acetylation [12].

Both FAME and TMS-base were analyzed by GC-MS (Hewlett Packard 5890 series II, MS 5972) using a DB-1 column. FAMEs and per-O-trimethylsilyl 2-OH FAMEs were analyzed using the GC-MS conditions described above for per-O-trimethylsilyl methyl glycosides, but with the temperature program extended to 300°C to ensure elution and detection through the 2-OH, C26 FAME derivatives [13,14]. The EI-MS detector acquired 50–500 amu for fatty acid analysis, and 50–600 amu for base character-
Figure 2. GC-MS of TMS-bases of Cer purified from astrocytes. The base composition of purified Cer was analyzed as TMS-derivative after N-acetylation as described in the text. A. TMS-Sphingosine base. B. TMS-Phytosphingosine base. Inserts indicate the primary m/z fragmentations. doi:10.1371/journal.pone.0080841.g002
Characterization of DHCer desaturase (DES) mRNA levels in vertebrate tissues

DHCer is converted to Cer or PHCer by the enzyme designated as Δ4-desaturase or DES1 and 2 [16]. While the DHCer is rapidly converted to Cer by DES1, DES2 is capable of producing PHCer along with Cer [17]. Hence, we examined the expression of DES2 mRNA by RT-PCR in brain, liver, and heart muscle. Briefly, total RNA was prepared from cells and tissues using TRIzol reagent following the manufacturer’s protocol (Invitrogen, Grand Island, NY). First-strand cDNA was synthesized using an Omniscript RT kit according to the manufacturer’s protocol (Qiagen, Valencia, CA). The amount of template from each sample was adjusted until PCR yielded equal intensities of amplification products for β-actin, which was used as a standard. Primers with the following sequences were used for RT-PCR [18].

mDES2 (sense): 5′-GATCATACCAGACTCGTGACAGA-3′
mDES2 (antisense): 5′-CGGTTTCGGGAGACACAACT-3′
β-actin (sense): 5′-CAT CGA GCA CGG CAT CGT CA-3′
β-actin (antisense): 5′-TAG CAC AGC CTG GAT AGC AAC-3′

Statistics

Average and standard deviation for quantification of Cer and PHCer were calculated in Microsoft Excel from the intensity of stained HPTLC bands following densitometry.

Results and Discussion

Most recently, the simple sphingolipid ceramide (Cer) has gained more attention due to its diversified functions in cell growth and development including the regulation of exosome formation [19] and ciliogenesis [20,21]. Precise quantification of Cer and its structural elucidation is an absolute requirement to evaluate its biological function. Recently, many methodologies have been introduced to identify and characterize Cer and other sphingolipids [22] demanding sophisticated instruments and special operating skills.

We have developed a one step purification method to quantify Cer species in cells and tissues. While examining the regulation of Cer content in cells (astrocytes) and tissues using our previous method [6], we observed that most Cer preparations contain a contaminant (Figure 1A) that was identified as cholesterol and might interfere with the structural elucidation of the Cer fraction. In addition, we observed that our Cer preparation showed an additional band with TLC-Rf between NFA-Cer and HFA-Cer (Figure 1A), which required further purification and characterization of the putative Cer fraction. Using a silicic acid column and employing a modified solvent system (solvent 1 followed by solvent 2) we were able to separate all three components and purify the two Cer bands to homogeneity (Figure 1B). The newly developed method was then applied successfully to purify Cer from the total lipid extracts of HOG cells (data not presented), and mouse brain tissues (Figure 1C), and later examining the Cer content and composition of kidney, heart, and liver.

The fatty acyl composition of the individual purified Cer bands was analyzed as FAME as described earlier (7,11). A variation of the fatty acyl composition was observed in the two purified ceramide fractions, Cer and PHCer, obtained from primary cultured mouse astrocytes. The higher TLC-Rf band (characterized as Cer) contained the C18:0 (57%) as the only major fatty acid, while the slower migrating band (characterized as PHCer) contained C16:0 (24%) and C18:0 (36%) fatty acyl groups at a ratio of 2:3 (Table 1). In addition, a small amount of C16:1 (1%) was present in the slower migrating band, which has not been identified in the band with higher TLC-Rf (Table 1). A variation of other fatty acyl groups between the two ceramide components was also recorded.

The trimethylsilyl derivative of the N-acetyl base of the higher TLC-Rf band (GC retention time 22.38 min) clearly indicated sphingosine (m/z at 470, 426, 311, 247, 174) with a trace amount (less than 5%) of dihydro sphingosine (m/z at 472, 313, 247, 174, see inserts in Fig. 2 for fragmentation products). No additional peak was detected in this Cer fraction (Figure 2A and Table 2). Interestingly, the analysis of the TMS-base of the slower migrating band (GC retention time 23.5 min) with a lesser TLC-Rf confirmed the structure as phytosphingosine (m/z at 560, 401, 311, 247, 174) (Figure 2B and Table 2). No sphingosine or dihydrosphingosine was identified in this fraction. Hence the upper band contained a mixture of predominantly Cer and a trace of dihydroCer (DHCer), while the lower band contained phytoceramide (PHCer). The potential uptake of PHCer from the medium was ruled out because astrocytes were cultured for one week in serum-free medium prior to the extraction of lipids.

Since astrocytes contained significant amounts of PHCer, we further proceeded to characterize the Cer fractions purified from mouse brain and HOG cells, a human oligodendrocyte cell line available in our laboratory. We used HOG cell culture [1] to refine the purification procedure and [2] to validate the applicability of our method to cell culture. It is worth mentioning that similar slow migrating bands were detected in both purified Cer preparations. Instead of purifying the individual bands, we have purified the Cer fraction (F2) and elucidated the structures as a mixture. To our expectations, we found Cer (49%), DHCer (15%), and PHCer (36%) in HOG cells and in brain (Cer-82%; DHCer-7%; PHCer-11%). A list of the primary ions (m/z) detected is presented in Table 2.

To test the applicability of this newly developed method for Cer analysis from tissues and to examine their tissue specific distribution of Cer and PHCer, we also purified the Cer fractions...
from mouse brain, liver, kidney, and heart. It is worth mentioning
that we have identified the PHCer bands in all tissues examined
(Figure 3A). The lower band co-migrated with standard PHCer
below the standard NFA-Cer and above the HFA-Cer. The
density scanning of the Cer/PHCer concentration ratios in tissues
indicated the following order: liver>brain>heart. The concen-
tration of ceramide and phytoceramide (μg/mg of protein) in each
tissue has been quantified as 8.85+/−0.41 and 0.97+/−0.18
(brain), 9.88+/−0.05 and 0.44+/−0.05 (liver), and 3.17+/−0.52
and 2.82+/−0.50 (heart) respectively (see Suppl. Figure S1B for a
comparison in pmole Cer or PHCer/mg of protein). Heart tissues
appeared to contain a significantly higher proportion of PHCer and
we are currently investigating the source of PHCer generation
relevant to its function.

To test the validity of our results and compare the consistency of
our method with that of other analytical methods we also
characterized the Cer and PHCer content using HPLC-MS
(sphingolipidomics) analysis. Protocols for sphingolipidomics anal-
yses use specific extraction reagents for Cer and PHCer with one
reagent applied to animal tissues for Cer and the other one to
plant tissues or yeast for PHCer extraction. Figure S1A shows that
these extraction methods are very consistent for Cer analysis, but
not for PHCer analysis when applied to the same sample of
different animal tissues. The sphingolipidomics analysis confirms
the presence of Cer and PHCer and the quantities determined by
HPLC-MS (Figure S1A) are in consistent with those obtained with
pre-purification and HPTLC (Figure S1B). However, the variations
obtained for PHCer using HPLC-MS analysis are larger than those
obtained with lipid analysis by pre-purification followed by
HPTLC. Currently, it is not known why fluctuations are observed
when PHCer from animal tissues is analyzed. Since our method
pre-purifies the Cer and PHCer fractions by removing cholesterol
and glyceride it is possible that these lipid “contaminants”
interfere with extraction of PHCer for sphingolipidomics analysis.
In plant tissues, however, the cholesterol content is much lower
(about 100-times) and may not pose a potential obstacle for
sphingolipidomics analysis of PHCer from plants. Our method
yields robust and consistent results, suggesting that one may need
to consider pre-purification of Cer fractions if analysis of PHCer
from animal tissues is desired (Suppl. Figure S1B).

In vertebrates, Cer synthesis is initiated by the enzyme serine
palmitoyltransferase (SPT) through the condensation of serine and
palmitoyl CoA to produce 3-ketodihydro-sphingosine [23] which
is then converted rapidly to dihydrosphingosine by 3-ketohydro-
sphingosine reductase [24] in an NADPH-dependent way.
Dihydrosphingosine is then converted to DHCer by the addition
of a fatty acyl group catalyzed by Cer synthase [25,26,27,28].
Ceramide is produced from DHCer by DHCer Δ4-desaturase or
DES1 and 2 [16]. Phytoceramide or 4-hydroxyceramide is an
intermediate product during the conversion of DH-Cer to Cer and

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**Figure 3. Thin-layer chromatography of purified Cer fractions and expression of DES2 in mouse tissues.**

A. A defined amount of the purified Cer fraction (F2) from vertebrate tissues was spotted and resolved by HPTLC, as described in the text. Samples were presented in duplicate. The plate was developed using chloroform:methanol:acetic acid 95:5:0.5 (v/v/v) and Cer bands were visualized after iodine absorption and char spray. Lane 1: Std. HFA-Cer; Lane 2–3: Brain; Lane 4–5: Liver; Lane 6–7: Kidney; Lane 8–9: Heart; Lane 10: Std. PHCer; Lane 11: Std. NFA-Cer. B. mRNA was prepared from mouse tissues and expression of DES2 determined by RT-PCR. Lanes 1 and 2: Brain; Lanes 3 and 4: Liver; Lanes 5 and 6: Heart. doi:10.1371/journal.pone.0080841.g003
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References

1. Hannun YA, Bell RM (1989) Functions of sphingolipids and sphingolipid breakdown products in cellular regulation. Science 243: 500–507.
2. Hannun YA (1994) The sphingomyelin cycle and the second messenger function of ceramide. J Biol Chem 269: 3125–3129.
3. Mathias S, Pena LA, Kolesnick RN (1998) Signal transduction of stress via ceramide. Biochem J 335 (Pt 3): 465–480.
4. Hakomori S (1990) Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. J Biol Chem 265: 18713–18716.
5. Masood MA, Yuan C, Acharya JK, Veenstra TD, Blonder J (2010) Quantitation of ceramide phosphorylethanolamines containing saturated and unsaturated hydroxy fatty acids in complex mixtures of fatty acid methyl esters by Mass chromatography. Biomed Mass Spectrom 1: 10–14.
6. Pritchard DG, Todd CW (1977) Gas chromatography of methyl glycosides as transmethylation products of glycosyl ceramides in human blood. J Lipid Res 8: 621–630.
7. Siddiqui B, Whisteadt JS, Kim YS (1978) Glycosphingolipids in human colonic adenocarcinoma. J Biol Chem 253: 2168–2175.
8. Laine RA, Young ND, Gerber JN, Sweeley CC (1974) Identification of 2-hydroxy fatty acids in complex mixtures of fatty acid methyl esters by Mass chromatography. Biomed Mass Spectrom 1: 10–14.
9. Mononen I, Karkkainen J (1975) Quantitative determination of neurodegenerative diseases [38]. Although Cer has received extensive scientific attention, the role of PHCer in cell function has gained very little or no scientific interest, mostly because of lack of proper characterization due to its paucity in vertebrate tissues. Our discovery of PHCer in CNS will open a novel direction for examining the regulation and biological functions of PHCer in CNS development and in neurodegenerative diseases. In summary, by using a novel method for Cer analysis, we have for the first time shown that many animal tissues including brain and heart contain significant amounts of PHCer which will now allows us to pursue its functional characterization.

Supporting Information

Figure S1 HPLC-MS and HPTLC analysis of ceramide and phytoceramide. A. Ceramide (Cer) and phytoceramide (PHCer) analysis by HPLC-MS shows consistent results for Cer, while the quantitative analysis of PHCer is hampered by fluctuations leading to large standard errors (indicated as bars on top of the means shown in the figure). N = 2. The figure shows pmoles of total ceramide/mg cellular protein. B. Ceramide (Cer) and phytoceramide (PHCer) analysis by quantitative HPTLC shows consistent results for Cer and PHCer. The absolute amounts are comparable to that of HPLC-MS analysis (A), but the variations between samples are smaller (N = 6 (brain), 2 (liver), 2 (heart)). The figure shows pmoles of total ceramide/mg cellular protein.

PDF

Acknowledgments

We thank the lipidiomics core facility (under supervision by Dr. Jacek Bielawski) for help with the sphingolipidomics (HPLC-MS) analyses. We also thank the Institute of Molecular Medicine and Genetics (Director Dr. Lin Mei), Georgia Regents University, GA, for institutional support.

Author Contributions

Conceived and designed the experiments: SD EB. Performed the experiments: SD JK. Analyzed the data: SD JK EB. Contributed reagents/materials/analysis tools: SD JK. Wrote the paper: SD JK EB.
21. He Q, Wang G, Dasgupta S, Dinkins M, Zhu G, et al. (2012) Characterization of an apical ceramide-enriched compartment regulating ciliogenesis. Mol Biol Cell 23: 3156–3166.

22. Gauld CR, Obeid LM, Hannun YA (2010) An overview of sphingolipid metabolism: from synthesis to breakdown. Adv Exp Med Biol 688: 1–23.

23. Mandon EC, Ehres I, Rother J, van Echten G, Sandhoff K (1992) Subcellular localization and membrane topology of serine palmitoyltransferase, 3-dehydro-sphinganine reductase, and sphinganine N-acyltransferase in mouse liver. J Biol Chem 267: 11144–11148.

24. Beeler T, Bakicova D, Gable K, Hopkins L, Johnson C, et al. (1998) The Saccharomyces cerevisiae TSC10/YBR265w gene encoding 3-ketosphinganine reductase is identified in a screen for temperature-sensitive suppressors of the Ca2+-sensitive csg2Delta mutant. J Biol Chem 273: 30688–30694.

25. Laviad EL, Albee L, Pankova-Kholmyansky I, Epstein S, Park H, et al. (2008) Characterization of ceramide synthase 2: tissue distribution, substrate specificity, and inhibition by sphingosine 1-phosphate. J Biol Chem 283: 5677–5684.

26. Pewzner-Jung Y, Ben-Dor S, Futerman AH (2006) When do Lasses (longevity assurance genes) become CerS (ceramide synthases)? Insights into the regulation of ceramide synthesis. J Biol Chem 281: 25001–25005.

27. Lahiri S, Lee H, Mesicek J, Fuku Z, Haimovitz-Friedman A, et al. (2007) Kinetic characterization of mammalian ceramide synthases: determination of K(m) values towards sphingamine. FEBS Lett 581: 5289–5294.

28. Mizutani Y, Kihara A, Igarashi Y (2006) LASS3 (longevity assurance homologue 3) is a mainly testis-specific (dihydro)ceramide synthase with relatively broad substrate specificity. Biochem J 398: 531–538.

29. Cadena DL, Kurten RC, Gill GN (1997) The product of the MLD gene is a member of the membrane fatty acid desaturase family: overexpression of MLD inhibits EGF receptor biosynthesis. Biochemistry 36: 6960–6967.

30. Geeraert L, Mannazerts GP, van Velden PP (1997) Conversion of dihydroceramide into ceramide: involvement of a desaturase. Biochem J 327 (Pt 1): 125–132.

31. Carter HE, Hendrickson HS (1963) Biochemistry of the sphingolipids. XV. Structure of phytosphingosine and dehydrophytosphingosine. Biochemistry 2: 389–393.

32. Karlsson KA, Samuelsson BE, Steen GO (1973) Detailed structure of sphingomyelins and ceramides from different regions of bovine kidney with special reference to long-chain bases. Biochim Biophys Acta 316: 336–362.

33. Swamitori M, Costello C, Moser HW (1979) Analysis and quantitation of free ceramide containing nonhydroxy and 2-hydroxy fatty acids, and phytosphingosine by high-performance liquid chromatography. J Lipid Res 20: 96–96.

34. Hannun YA, Obeid LM (2006) Principles of bioactive lipid signalling: lessons from sphingolipids. Nat Rev Mol Cell Biol 9: 139–150.

35. Lee JS, Min DS, Park C, Park CS, Cho NJ (2001) Phytosphingosine and C2-phytoceramide induce cell death and inhibit carbachol-stimulated phospholipase D activation in Chinese hamster ovary cells expressing the Caenorhabditis elegans muscarinic acetylcholine receptor. FEBS Lett 499: 82–86.

36. Hwang O, Kim G, Jang YJ, Kim SW, Choi G, et al. (2001) Synthetic phytoceramides induce apoptosis with higher potency than ceramides. Mol Pharmacol 59: 1249–1253.

37. Sekiya M, Ueda K, Okazaki K, Terashima J, Katou Y, et al. (2011) A phytoceramide analog stimulates the production of chemokines through CREB activation in human endothelial cells. Int Immunopharmacol 11: 1497–1503.

38. Jung JC, Lee Y, Moon S, Ryu JH, Oh S (2011) Phytoceramide shows neuroprotection and ameliorates scopolamine-induced memory impairment. Molecules 16: 9090–9100.