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METHODS

A Three-Step Assay for Ceramide Synthase Activity Using a Fluorescent Substrate and HPLC

Timothy A. Couttas · Xin Y. Lim · Anthony S. Don

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Abstract Ceramides are a family of signalling lipids with diverse physiological functions that include pro-differentiative and pro-apoptotic signalling. Ceramides and their derivatives are major constituents of myelin, maintaining neuronal conductivity. Ceramides are synthesized by ceramide synthases, of which there are six isoforms in mammals (CERS1–6). These enzymes catalyse the transfer of a variable length fatty acid to a sphingoid base, typically sphingosine or dihydrosphingosine. We previously reported a fluorescent thin-layer chromatography assay for ceramide synthase activity. In this paper we describe an improved fluorescent assay, using HPLC to achieve clear resolution of closely related ceramide species and to facilitate easy quantification of both product and substrate. Our HPLC assay protocol eliminates the need for a chloroform extraction step. Instead a simple three-step procedure is used: (1) reactions are run; (2) reactions are terminated with addition of methanol and centrifuged; (3) products are quantified with HPLC. HPLC resolution enables assays in which multiple fatty acid substrates are used in the same reaction. Using this approach, we show that CERS2 demonstrates a preference for the monounsaturated C24:1 fatty acid substrate compared to the saturated C24:0 substrate, potentially explaining why myelin is enriched in ceramides containing the monounsaturated form of very long chain fatty acids.

Keywords Ceramide · Ceramide synthase · CERS · Dihydrosphingosine · Sphingolipid · Fluorescent assay · Method · HPLC

T. A. Couttas · X. Y. Lim · A. S. Don (✉)
Prince of Wales Clinical School, University of New South Wales, Level 2, C25 Lowy Building, Sydney, NSW 2052, Australia
e-mail: anthonyd@unsw.edu.au

Abbreviations

CERS	Ceramide synthase
CoA	Co-enzyme A
HPLC	High-performance liquid chromatography
TLC	Thin-layer chromatography

Introduction

Sphingolipids are a diverse class of membrane lipids that modulate the biophysical properties of cell membranes, are involved in cell–cell recognition and/or act as secondary messengers in signal transduction. The central metabolite in the sphingolipid biochemical pathway is the group of lipids called ceramides [1]. Ceramides are involved in a broad range of cellular and physiological functions, including cell differentiation [2], apoptosis [3, 4], autophagy [5], induction of cellular senescence [6] and as major functional constituents of myelin [7, 8] and the epidermal water barrier [9]. Increased ceramide levels have been strongly implicated in the pathogenesis of diabetes [10] and neurodegenerative conditions [11], whilst decreased levels are suggested to fuel cancer cell resistance to apoptosis [12]. Ceramides are precursors for more complex sphingolipids via the attachment of functional groups, including phospho-choline, phosphate, glucose and galactose [1].

In mammals, ceramides are usually comprised of an 18-carbon sphingosine or dihydrosphingosine base, linked via an amide bond to a fatty-acyl chain that can vary in length (between 14 and 26 carbons) and degree of unsaturation [1]. The most common fatty acids found in ceramide are palmitic (C16:0), stearic (C18:0), tetracosanoyl (C24:0) and nervonoyl (C24:1), although there are more than 200 structurally distinct forms of ceramide [13].

De novo ceramide synthesis occurs in the endoplasmic reticulum (ER), catalysed by ceramide synthases, which are found throughout the eukaryotic kingdoms. In humans and rodents a family of six ceramide synthases [CERS1–6; also referred to as longevity assurance (LASS) genes] catalyse the transfer of the variable length fatty acid to dihydrosphingosine (a.k.a. sphinganine), forming dihydroceramide [14]. Dihydroceramides are rapidly desaturated by specific desaturases (DEGS1 and 2), forming ceramides. Alternatively, ceramides may be formed in the sphingolipid "salvage" pathway, whereby degradation of complex sphingolipids in the lysosomes forms sphingosine that is then recycled to the ER for new ceramide synthesis. Although all CERS members can acylate dihydrosphingosine or sphingosine, the different isoforms of CERS preferentially attach different length fatty acids. For example, CERS1 preferentially catalyses the addition of 18-carbon (C18:0) fatty acids to dihydrosphingosine, whereas CERS2 preferentially adds very long chain fatty acids (C22:0–C26:0) and CERS6 has high specificity for C14:0 or C16:0 fatty acids [15–17]. The various fatty acid compositions may define the specific role of ceramide species in cell physiology. For instance, C18 ceramide synthesized by CERS1 is implicated in apoptosis and lethal autophagy [4, 5], very long chain ceramides synthesized by CERS2 are essential constituents of myelin [7], and even longer chain ceramides (longer than C24) synthesized by CERS3 are critically important for skin barrier function [9].

We recently described a fluorescent thin-layer chromatography (TLC)-based assay for CERS activity in crude extracts of cultured cells and tissues. This assay uses commercially available 7-nitro-2-1,3-benzoxadiazole (NBD)-labelled dihydrosphingosine [(2*S*,3*R*)-2-amino-18((7-nitrobenzo[*c*][1,2,5]oxadiazol-4-yl) amino)octadecane-1-3-diol] as a substrate in place of the natural dihydrosphingosine substrate [18]. A drawback with the protocol described is that TLC on silica gel plates cannot distinguish ceramide species with similar mass (C16:0 from C18:0, or C24:0 from C24:1, for example). Reversed-phase chromatography produces better resolution of different ceramides. In the present study, we describe a fluorescent high-performance liquid chromatography (HPLC) assay that effectively detects endogenous levels of CERS activity in cell and tissue extracts, and retains all the positive features of our recently published TLC method, including the absence of radioactive isotopes and relatively low cost. The primary advantages of the HPLC method are (1) it is much more effective than our published TLC assay at distinguishing individual dihydroceramide species, (2) reproducible column elution times provide greater confidence in the assignment of product peaks, (3) it is also simple to quantify the dihydrosphingosine substrate in each sample, (4) the method is more automated and less prone to human factors that affect the quality of the TLC image (e.g. uneven bands and the "smile" effect) and (5) the use of HPLC allows one to omit the chloroform extraction step, thus shortening the overall assay protocol.

Materials and Methods

Reagents and Ceramide Standards

Dihydrosphingosine-NBD (sphinganine-NBD) and d18:1-NBD/18:0 ceramide standard were purchased from Avanti Polar Lipids, USA. Dihydroceramide-NBD standards d18:0-NBD/16:0 ceramide, d18:0-NBD/18:0 ceramide, d18:0-NBD/24:0 ceramide and d18:0-NBD/24:1 ceramide were prepared as described previously [18].

Cell and Tissue Extracts

U87MG cells were from American Type Culture Collection and cultured in DMEM medium supplemented with 10 % foetal bovine serum and 2 mM L-glutamine. Human frontal cortex grey matter was obtained from the NSW Brain Bank Network, and its use in this project was approved by the UNSW Human Research Ethics Committee (approval HREC13120). Crude homogenates were prepared by resuspending approximately 10^6 cells or 20 mg tissue in 500 μL of lysis buffer containing 20 mM Hepes, pH 7.4, 10 mM KCl, 1 mM dithiothreitol, 3 mM β-glycerophosphate and complete protease inhibitor cocktail (Roche, Mannheim, Germany). Cells or tissues were homogenised by ultrasonication for 5 min in an ice-cold water bath, using a Diagenode Bioruptor (Denville, NJ, USA). Extracts were cleared by centrifugation at 800*g* for 10 min and the supernatant was stored at −80 °C. Protein concentrations were determined with the bicinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA). Extracts of CERS1- and CERS2-expressing HEK293 cells, as well as mouse liver, were prepared as described previously [18].

CERS Reaction

The reaction buffer consisted of 20 mM Hepes, pH 7.4, 25 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.1 % (w/v) fatty acid free BSA, 10 μM sphinganine-NBD and 50 μM fatty acid-CoA. Reactions (50 μL), in eppendorf tubes, were started with the addition of 5–25 μg lysate protein, and were generally run for 20 or 30 min at 37 °C. Reactions were stopped with the addition of four volumes (200 μL) of methanol, then centrifuged at 14,000 rpm to pellet insoluble material. The supernatant was then transferred to an HPLC vial for analysis.

Alternatively, in the two-phase extraction protocol, reactions were stopped with the addition of 100 μL chloroform/

methanol (2:1). The phases were separated by 15 s centrifugation at 14,000 rpm, and the lower organic phase transferred to a glass tube for drying. The aqueous phase was then re-extracted with another 100 μL chloroform/methanol (2:1), and the lower organic phase combined with the first organic extract. The organic extracts were dried in a SpeedVac SC210 (ThermoFisher Scientific), reconstituted with 50 μL HPLC mobile phase (80 % methanol/20 % water/0.2 % formic acid) and transferred to glass HPLC vials with 400 μL fused glass inserts for HPLC analysis.

HPLC

Dihydroceramide-NBD products were resolved by reversed-phase chromatography on a C8 column (3 × 150 mm Agilent XDB-C8 column; 5 μM pore size) and analysed on a Thermo Scientific Surveyor HPLC, connected to a Shimadzu RF-10AXL fluorescent detector. Sample was injected with a 20-μL injection loop. The fluorescent detector was set to a gain of 3. We recommend that each individual user optimise the fluorescent detector associated with their particular HPLC. Data was acquired over a 12-min chromatography run using a two-solvent system: solvent A, 0.2 % formic acid, 2 mM ammonium formate in water; solvent B, 0.2 % formic acid, 1 mM ammonium formate in methanol. The gradient started at 20:80 A/B, increasing to 5:95 A/B over 2 min, then to 100 % B from 2 to 8 min. The gradient is then held at 100 % B for 2 min, before re-equilibration to 20:80 A/B for 2 min. The total run time is 12 min. Peak areas were integrated using Thermo Scientific Xcalibur software, and converted to picomoles of product using a standard curve ranging from 0.2 to 20 pmol.

TLC

TLC was run using aluminium-backed silica gel 60 TLC plates (Merck), using the solvent system chloroform/methanol/water (8:1:0.1), as described previously [18]. Fluorescence was detected directly on the TLC plates using a LAS Mini 4000 imaging system, and fluorescent pixel intensity was quantified using ImageQuant software (GE Healthcare).

Results

HPLC Resolves Fluorescent Ceramides That Cannot Be Distinguished Using TLC

We first established the resolution of different NBD-labelled dihydroceramides separated on a C8 HPLC column. The 12 min protocol described in the methods section was readily able to resolve d18:0-NBD/16:0 ceramide (C16:0 dihydroceramide-NBD), d18:0-NBD/18:0 ceramide (C18:0 dihydroceramide-NBD), d18:0-NBD/24:0 ceramide (C24:0 dihydroceramide-NBD) and d18:0-NBD/24:1 ceramide (C24:1 dihydroceramide-NBD) (Fig. 1a–e). Normal-phase TLC on silica gel 60 plates is not capable of resolving C16:0 from C18:0 dihydroceramide-NBD, or C24:0 from C24:1 dihydroceramide-NBD, as shown in Fig. 1f.

Limit of Detection and Quantification

Detection of a commercially available C18:0 ceramide-NBD (i.e. d18:1-NBD/18:0 ceramide) standard was linear over the range 0.1–20 pmol (Fig. 2). Standard curves for peak areas obtained with dihydrosphingosine-NBD substrate were very similar to the C18:0 ceramide-NBD standard, although the peak areas for dihydrosphingosine-NBD were 50–60 % of those obtained with C18:0 ceramide-NBD (Fig. 2). The dihydrosphingosine-NBD elutes at 2.4 min, much earlier than the NBD-dihydroceramides, which elute between 7 and 11 min (Fig. 2). Therefore the dihydrosphingosine-NBD substrate can be used to generate a standard curve for relative quantification of product if one does not wish to purchase the ceramide-NBD standard (note that C18:0 ceramide-NBD from Avanti Lipids is currently the only commercially available ceramide carrying an NBD label on the sphingosine base). C18:0 ceramide-NBD is preferable for quantification, since our chromatography gradient is optimised to give sharp peaks for ceramides.

Reproducibility

HPLC column elution times and peak areas for C16:0, C18:0 and C24:1 NBD-dihydroceramide reaction products were highly reproducible when the same reaction sample was run on subsequent days, as shown in Table 1. The average coefficient of variance (CV) for column elution time was 0.57 %; whilst the average CV for peak area was 2.3 %.

Linearity of the Reaction With Respect To Time and Lysate Concentration

C16:0, C18:0 and C24:1 dihydroceramide-NBD product formation was linear with respect to the concentration of U87MG glioblastoma cell extract used (20 min reaction) (Fig. 3a); and using 20 μg U87MG or human frontal cortex grey matter extract, product formation was linear with respect to time over 30 min (Fig. 3b, c). Lysates of human frontal cortex grey matter produced a different pattern of preferential substrate use, with C18:0 CERS activity being higher than C16:0 or C24:1 CERS activity in these

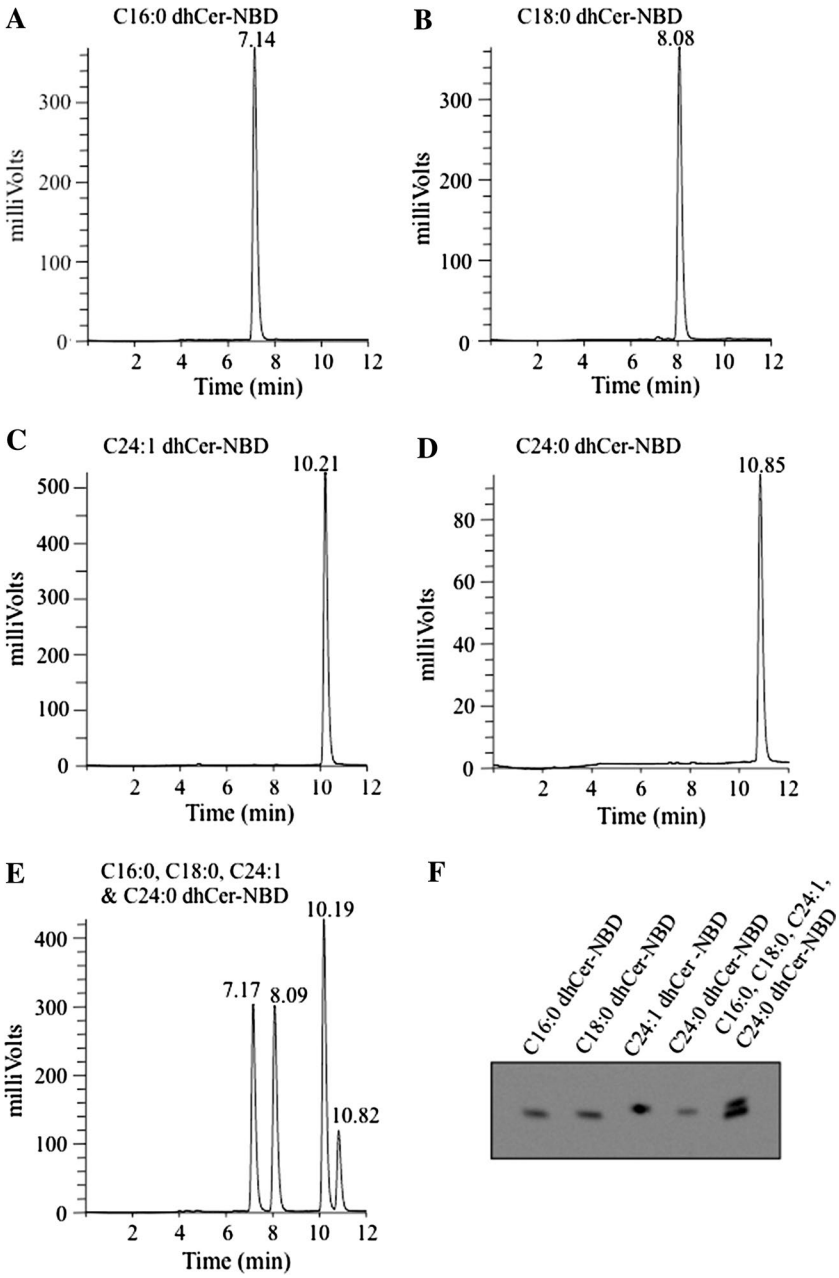

Fig. 1 Resolution of closely related ceramides using HPLC. Chromatograms show peaks for C16:0 (**a**), C18:0 (**b**), C24:1 (**c**) and C24:0 (**d**) dihydroceramide-NBD by HPLC. **e** Shows the peaks obtained with a mixture of all four dihydroceramide-NBD species, and (**f**) the corresponding TLC image illustrates the inability of TLC to separate C16:0 and C18:0, or C24:0 and C24:1, dihydroceramide-NBD species

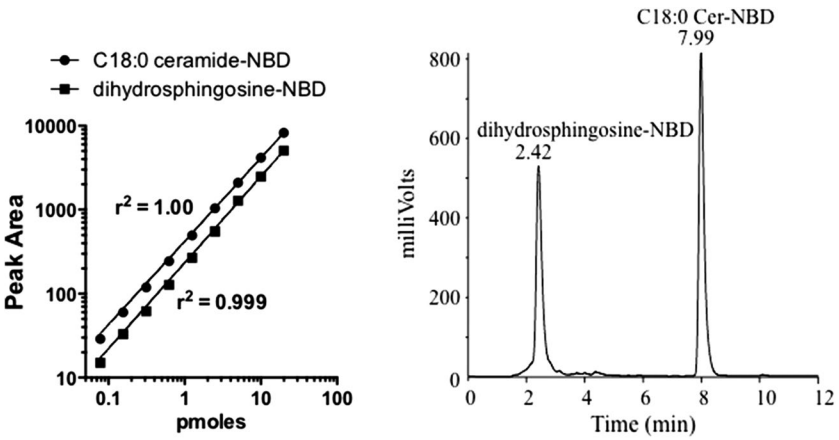

Fig. 2 Dynamic range of the HPLC method. Standard curve illustrating linearity of the fluorescent peak area for C18:0 ceramide-NBD and dihydrosphingosine-NBD over the range 0.1–20 pmol on column (loaded in a 20-μL volume). An example chromatogram for dihydrosphingosine-NBD and C18:0 ceramide-NBD is shown

Table 1 Reproducibility of the HPLC method

	Column RT			Peak area		
	Mean	Std dev	CV (%)	Mean	Std dev	CV (%)
C16:0-CoA test 1	7.18	0.012	0.16	2,679	58.6	2.2
C16:0-CoA test 2	7.18	0.025	0.35	2,831	37.9	1.3
C18:0-CoA test 1	8.19	0.136	1.66	846	28.9	3.4
C18:0-CoA test 2	8.15	0.046	0.57	718	11.7	1.6
C24:1-CoA test 1	10.25	0.026	0.26	3,977	114	2.9
C24:1-CoA test 2	10.24	0.040	0.40	3,786	91.8	2.4

Two reactions were run for each of the three fatty acid-CoA substrates shown, using 20 μg U87MG cell extract. Reaction products were extracted with chloroform/methanol and subjected to HPLC on three separate days. Results shown are mean, Std dev and % CV for each sample over the three HPLC runs

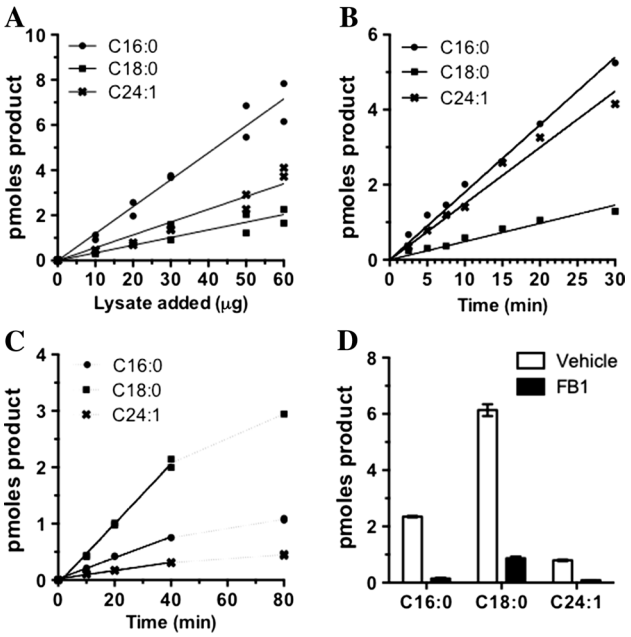

Fig. 3 Linearity of the reaction with respect to lysate concentration and time. **a** Reactions containing sphinganine-NBD and C16:0 (*circles*), C18:0 (*squares*) or C24:1 (*crosses*) dihydroceramide-NBD were started with addition of U87MG cell extract as indicated, and run for 25 min. **b** Reactions were started with addition of 20 μg U87MG extract, and run for indicated times. **c** Product formation with respect to time for extracts of human frontal cortex grey matter (15 μg/reaction). The reaction is linear out to 40 min. The data were fitted to straight lines using GraphPad PRISM software (*solid lines*). **d** Product formed in triplicate 30-min reactions using 15 μg (C16:0 and C18:0-CoA) or 30 μg (C24:1-CoA) human frontal cortex grey matter tissue, in the presence or absence of 6 μM Fumonisin B1. Mean and standard error are shown

samples. This is in agreement with the observation that C18:0 ceramides are far more abundant in human brain tissue than glioblastoma cells [19]. The well-characterised CERS inhibitor fumonisin B1 [18, 20] strongly inhibited product formation with all three fatty acid-CoA substrates, confirming the requirement for CERS activity (Fig. 3d).

Chloroform/Methanol Extraction Can Be Omitted When Using HPLC

We sought to determine if the protocol could be shortened by eliminating the chloroform/methanol extraction step. This would, in theory, improve accuracy and reproducibility by reducing the number of steps in the reaction protocol. Reactions (50 μL) were stopped with the addition of four volumes of methanol, centrifuged at 14,000 rpm to clear insoluble material, then analysed directly on the HPLC system. The shortened protocol produced a modest improvement in the calculated reaction product when compared to the protocol including chloroform extraction (Fig. 4), indicating that elimination of the chloroform extraction may afford a slight improvement in product recovery.

Multiplexed CERS Assays in a Single Tube

The capacity to accurately differentiate different dihydroceramide-NBD products formed in a single reaction allows one to run multiple fatty acid-CoA substrates in a single assay. This may prove useful for determining which fatty acid substrates are preferentially utilised by particular cell or tissue types. To demonstrate the capability of the improved assay to distinguish fatty acid specificity, we examined C16:0, C18:0, C24:0 and C24:1 dihydroceramide-NBD product formation in CERS1- and CERS2-expressing HEK293 cell extracts. As expected, CERS1-expressing HEK293 cell extracts show a clear preference for utilisation of the C18:0 fatty acid substrate when presented with equimolar concentrations (25 μM) of C16:0, C18:0, C24:0 and C24:1 fatty acid substrates (Fig. 5a). CERS1 is highly expressed in neurons [8]. Accordingly, a clear preference for the C18:0 NBD-dihydroceramide synthesis was observed when extracts of human frontal cortex grey matter were incubated with NBD-dihydrosphingosine and all four fatty acid substrates (C16:0, C18:0, C24:0, C24:1) (Fig. 5b). The pattern of preferential use

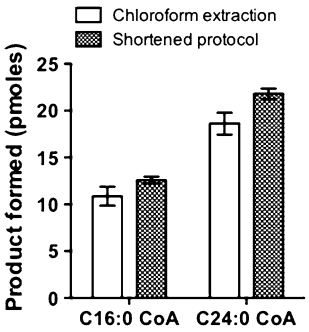

Fig. 4 HPLC method allows for omission of the chloroform extraction step. C16:0 and C24:1 CERS activity of U87MG extracts (20 μg, 30 min) was determined using chloroform/methanol extraction and drying down the samples prior to reconstitution in 50 μL 80 % methanol for HPLC; or by stopping the reactions with four volumes of methanol, centrifugation to remove insoluble material, and direct analysis of the supernatant by HPLC (shortened protocol). Graph shows mean and standard error for total product formed in triplicate reactions. Note that peak areas for the short protocol were significantly smaller than for the chloroform extraction protocol, as the final sample volume used for HPLC was 250 μL in the shortened protocol

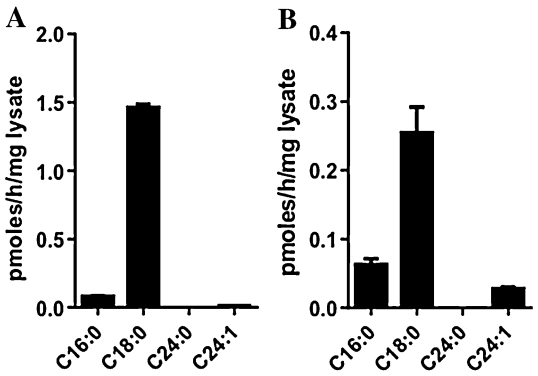

Fig. 5 Preference of CERS1 for C18:0 fatty acids in a multiplexed assay. Products formed after 20 min reaction using lysates of **a** CERS1-expressing HEK293 cells (5 μg), or **b** human frontal cortex grey matter (5 μg), with 10 μM dihydrosphingosine-NBD and a mixture of C16:0, C18:0, C24:0 and C24:1 fatty acid-CoAs, each at 25 μM. Results are mean and standard error for triplicate reactions

C18:0 > C16:0 > C24:1 is in agreement with Fig. 3c, in which the activity of frontal grey matter towards each substrate was assayed separately.

CERS2-expressing HEK293 cell lysates exhibited a strong preference towards C24:1 fatty acid substrate when equimolar concentrations of all fatty acid substrates were used (Fig. 6a). The preference of CERS2 for C24:1 over C24:0 fatty acid substrate was surprising, given that CERS2 exhibits high specificity towards 22- to 24-carbon substrates [15, 16, 21]. We therefore sought to confirm this finding using mouse liver extract, as CERS2 is highly expressed in liver [16, 21]. Results from liver tissue also demonstrated a strong preference for C24:1 fatty acid substrate (Fig. 6b). In reactions testing C24:0 and C24:1 CERS activity separately, liver extracts demonstrated a faster reaction rate with C24:1 fatty acid substrate (Fig. 6c).

Discussion

The ability to quantify CERS activity arising from the different CERS isoforms is becoming increasingly important as studies are now detailing how ceramides synthesized by one CERS isoform differ in biological activity from those synthesized by another [13, 14]. CERS activity is traditionally assayed with tritiated sphingosine or sphinganine, and the products resolved using TLC [15, 22]. The use of tritiated chemicals poses a risk to personal safety if ingested, and is increasingly difficult in countries where use of radiochemicals is heavily regulated. Tritiated compounds are also relatively difficult to quantify, generally requiring long exposure time of the TLC plate to film or a phosphoimager screen, or scraping of the bands for scintillation counting. More recently, CERS activity has been quantified using liquid chromatography tandem mass spectrometry (LC–MS/MS) [18, 23, 24]. This is a very accurate and sensitive method for quantification of CERS activity, but requires access to specialised equipment that may be associated with significant institutional access or cost recovery fees. The fluorescent approach that we described provides a radioisotope-free, cost-effective alternative, using equipment that is available in many standard biomedical research laboratories.

The HPLC assay format provides vastly improved resolution of individual products compared to separation by normal-phase TLC [18]. Specific and reproducible column retention times for each dihydroceramide-NBD species provide increased accuracy and confidence in the identification and quantification of the correct product, noting that normal-phase TLC is not capable of separating out closely related dihydroceramides, such as C16:0 and C18:0, or C24:0 from C24:1 (Fig. 1) [25]. It is possible that reversed-phase TLC would achieve good separation of the different dihydroceramide-NBD reaction products (e.g. [26]), but this has not yet been tested. The improved resolution of HPLC also permits quantification of the dihydrosphingosine-NBD substrate. Further to this, TLC is technically more challenging and less automated than HPLC. Crude tissue extracts used to quantify endogenous CERS activity may contain high levels of fatty acid substrates. Although our assay does not "solve" the issue of endogenous fatty acids in tissue extracts, the improved resolution afforded by HPLC (e.g. ability to analyse C16:0 and C18:0 CERS activity independently) allows one to accurately establish the baseline value for each fatty acid chain length, in the absence of added substrate, as illustrated in Fig. 7.

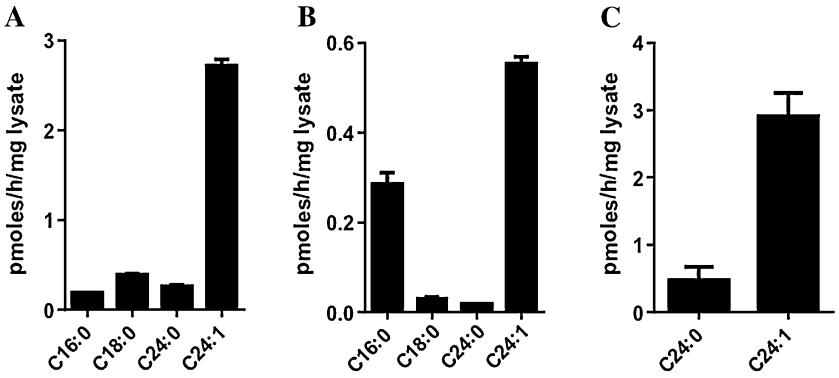

Fig. 6 Preference of CERS2 for C24:1 over C24:0 fatty acid. **a** Products formed in reactions using 5 μg extract of CERS2-expressing HEK293 cells with 10 μM dihydrosphingosine-NBD and a mixture of C16:0, C18:0, C24:0 and C24:1 fatty acid-CoAs, each at 25 μM. Products formed in reactions using 5-μg extracts of mouse liver, in the presence of 10 μM dihydrosphingosine-NBD and **b** a mixture of C16:0, C18:0, C24:0 and C24:1 fatty acid-CoAs, or (**c**) C24:0 or C24:1 fatty-acyl CoA substrate delivered in separate reactions. Results are mean and standard error for triplicate reactions

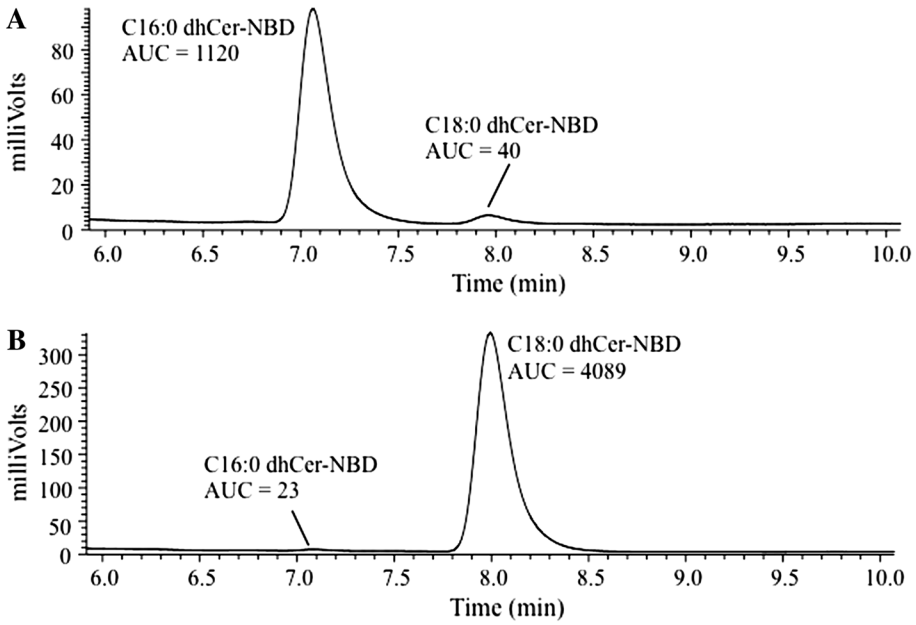

Fig. 7 Baseline dihydroceramide-NBD synthesis in the absence of fatty acid substrate. **a** Chromatogram illustrates a very low level of C18:0 dihydroceramide-NBD produced in a reaction with C16:0-CoA, but not C18:0-CoA, substrate. Reactions were run with lysates of HEK293 cells overexpressing CERS1 (5 μg). **b** The C18:0 dihydroceramide peak produced when C18:0-CoA is used as the fatty acid substrate is 100-fold larger, in terms of the area under the curve (AUC). Chromatography profiles from 6 to 10 min are shown to permit visualisation of the baseline peaks

Another key advantage with the HPLC-based assay is that it circumvents the need for two-phase extraction of the reaction mixtures with chloroform/methanol. The reaction may be stopped using four volumes of methanol, then cleared and used directly for HPLC. Although omission of the chloroform extraction step afforded a slight improvement in product recovery (Fig. 4), we note that the sample drying step that follows chloroform extraction allows for the reaction products to be reconstituted in a smaller sample volume (50 μL, compared to 250 μL for the shorter protocol with no chloroform extraction). Thus, with standard 50- or 100-μL reaction volumes, the limit of detection for the assay is better when the chloroform/methanol extraction and drying steps are included.

An additional advantage with the HPLC method is that multiple closely related fatty acid substrates (e.g. C24:0 and C24:1; or C16:0 and C18:0) can be included in one reaction. Using this approach we demonstrate the strong preference of exogenously expressed CERS1 for C18:0 fatty acid as the substrate, whilst exogenous CERS2 exhibits a preference for C24:1 as the substrate. The strong preference of CERS1 for C18:0 fatty acids has been described previously [15, 17], although not by allowing multiple fatty acid substrates to compete for the enzyme active site in a single assay. The degree of preference of CERS2 for the monounsaturated form of the 24-carbon fatty acid (Fig. 6a) was surprising and has not been demonstrated before, but is in keeping with the observation that human and mouse

brain tissues contain much greater concentrations of C24:1 than C24:0 ceramide [7, 27, 28]. These very long chain forms of ceramide are enriched in myelin lipids, and CERS2 is the enzyme that synthesizes these myelin lipids [29]. Although we observed the same preference for C24:1 over C24:0 CERS activity in mouse liver extract (Fig. 6b), an overabundance of C24:1 relative to C24:0 ceramide was not reported for mouse liver [21]. One caveat with assaying multiple fatty acid substrates in a single reaction is that the amount of product formed is reduced compared to when different fatty acid substrates are assayed in separate reactions, as illustrated in Fig. 6b, c. We speculate that this reduction in CERS activity may be a consequence of substrate inhibition, caused by the presence of excessive total levels of fatty acyl-CoA (100 μM total). Hirschberg et al. previously demonstrated reduced ceramide synthase activity when the molar ratio of fatty acyl-CoA to BSA carrier is greater than 4 [30]. Alternatively, reduced product formation could be the result of competitive inhibition between the fatty acid substrates for the CERS active site, with less favoured substrates effectively blocking the active site and slowing down the overall product turnover rate. Despite the reduced product formation we note that the relative preference for fatty acid substrates remains the same whether the reactions are performed individually or as a mixture, allowing the user to still determine the most biologically active CERS isoform in a given sample.

An issue with fluorescent assays is that exposure to fluorescent laboratory lights causes loss of the NBD signal due to photobleaching. Provided that all samples are handled equally with reduced lighting, this will not increase intra- or inter-assay variability. However, when using the NBD-ceramide external standard for quantification, one must be aware that different fluorescent yields and handling of this standard compared to the experimental samples may affect absolute quantification.

In summary, we present a new method for a simple and quantitative assay of CERS activity in cell and tissue extracts. This accessible assay will be of great benefit to studies investigating the crucial role played by ceramide synthesis in physiology and diseases such as diabetes, neurodegenerative conditions and cancer.

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