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Very long chain ceramides interfere with C_{16}-ceramide-induced channel formation: A plausible mechanism for regulating the initiation of intrinsic apoptosis

Johnny Stiban [a,*], Meenu Perera [b,1]

[a] Department of Biology and Biochemistry, Birzeit University, P.O. Box 14, West Bank 627, Palestine
[b] Department of Biology, University of Maryland, College Park, MD 20742, USA

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ABSTRACT

Mitochondria mediate both cell survival and death. The intrinsic apoptotic pathway is initiated by the permeabilization of the mitochondrial outer membrane to pro-apoptotic inter-membrane space (IMS) proteins. Many pathways cause the egress of IMS proteins. Of particular interest is the ability of ceramide to self-assemble into dynamic water-filled channels. The formation of ceramide channels is regulated extensively by Bcl-2 family proteins and dihydroceramide. Here, we show that the chain length of biologically active ceramides serves as an important regulatory factor. Ceramides are synthesized by a family of six mammalian ceramide synthases (CerS) each of which produces a subset of ceramides that differ in their fatty acyl chain length. Various ceramides permeabilize mitochondria differentially. Interestingly, the presence of very long chain ceramides reduces the potency of C_{16}-mediated mitochondrial permeabilization indicating that the intercalation of the lipids in the dynamic channel has a destabilizing effect, reminiscent of dihydroceramide inhibition of ceramide channel formation (Stiban et al., 2006). Moreover, mitochondria isolated from cells overexpressing the ceramide synthase responsible for the production of C_{16}-ceramide (CerS5) are permeabilized faster upon the exogenous addition of C_{16}-ceramide whereas they are resistant to permeabilization with added C_{24}-ceramide. On the other hand mitochondria isolated from CerS2-overexpressing cells show the opposite pattern, indicating that the product of CerS2 inhibits C_{16}-channel formation ex vivo and vice versa. This interplay between different ceramide metabolic enzymes and their products adds a new dimension to the complexity of mitochondrial-mediated apoptosis, and emphasizes its role as a key regulatory step that commits cells to life or death.

1. Introduction

Ceramides are a family of lipid molecules whose functions exceed merely being part of the structure of the membranes as was initially presumed [2]. Ceramides are sphingolipids, composed of a sphingosine backbone *N*-acylated with different fatty acyl-CoA. This *N*-acylation reaction is performed by one of 6 mammalian ceramide synthases (CerSs). While CerS can use sphingosine as a substrate, in the cell, CerS utilizes sphinganine (dihydrosphingosine) to produce dihydroceramide since de novo ceramide synthesis pathway produces sphinganine abundantly [3,4].

Interestingly, different CerSs produce different species of ceramides, varying by the length of their hydrophobic tail. CerS2, for instance, preferentially acylates sphinganine with very long chain fatty acyl-CoA whereas CerS5 uses C_{16}-fatty acyl-CoA as its substrate [4,5]. Ceramides were shown by biochemical and biophysical methods to possess a unique ability to perforate lipid bilayers, especially the mitochondrial outer membrane, by forming water-filled channels [1,6–10]. This evidence was later validated by transmission electron microscopy visualization of these channels in liposomes [11]. The current model of the ceramide channel consists of layers of ceramide molecules stacked in an anti-parallel fashion on top of one another to form ceramide columns. The amide bond stacks in a manner similar to peptide bonds in an alpha-helix stabilizing the channel. An ice-lattice-like structure of hydrogen bonding between the hydroxyls further stabilizes the channel, holding adjacent ceramide columns together to form the lumen of the channel [12] (reviewed in [6,13,14]). The ability of ceramides to perforate mitochondrial outer membranes was shown for ceramides with

Abbreviations: C16-ceramide, N-palmitoyl-D-erythrosphingosine; C22-ceramide, N-behenoyl-D-erythrosphingosine; C24-ceramide, N-lignoceroyl-D-erythro-sphingosine; IMS, intermembrane space; DPX, *p*-xylene-bis-pyridinium bromide; CerS, ceramide synthase; CF, 5/6-carboxyfluorescein; TBST, Tris buffered saline tween 20.

* Corresponding author at: Department of Biology and Biochemistry, Birzeit University, P.O. Box 14, Ramallah, West Bank, Palestine. Tel.: +97 2 298 2162, 97 2 54 392 1144; fax: +970 2 298 2084.
E-mail addresses: jstiban@birzeit.edu (J. Stiban), meenunp@gmail.com (M. Perera).
[1] Present address: Intralytix, Inc. 701 East Pratt Street, Baltimore, MD 21202.

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different fatty acyl chain lengths (C_2-, C_8-, C_{16}- and C_{24}-ceramides [1,9,15]).

Ceramide has been implicated to play a key role in apoptosis, a form of programmed cell death. During the initiation phase of apoptosis, mitochondrial ceramide levels increase [16] and the mitochondrial outer membrane starts to lose its integrity. When the mitochondrial outer membrane becomes permeabilized, leading to the egress of cytochrome *c* and other pro-apoptotic proteins from the IMS into the cytosol, the cell is irreversibly committed to death [17]. Ceramide can form channels in the mitochondrial outer membrane which are large enough to allow the egress of all proteins that are known to be released during apoptosis [9]. Ceramide channels are also regulated by the Bcl-2 family of proteins, which regulate apoptosis [18–20].

In order to study the initiation of apoptosis, the regulation of ceramide channel formation is probed in this work. We hypothesize that there are at least 6 mammalian CerS isozymes because the products of each have different roles in the cell. We propose that the products of each CerS isozyme can serve as inducers or blockers of ceramide channel formation depending on their relative amounts. Overexpression of different CerSs resulted in a differential outcome of radiation-induced apoptosis in HeLa cells. CerS5 overexpression led to more cells undergoing apoptosis whereas CerS2 overexpression protected cells from death [21]. In breast and colon cancer cells, overexpression of CerS4 and CerS6 inhibited cellular proliferation whereas overexpression of CerS2 promoted proliferation [22]. In a previous work, dihydroceramide hindered ceramide channel formation by virtue of intercalating in the structured ceramide pore and destabilizing it [1]. Here, we investigated the possibility that other ceramide structures, namely very long chain ceramides could have similar effects on C_{16}-ceramide channels, possibly by intercalating into the channel and altering channel stability. Indeed we find that a mixture of ceramides has the ability to interfere with the ability of each species to permeabilize membranes. In addition, we find that C_{16}-ceramide has the ability to form both large and small channels whereas C_{22}-ceramide was unable to form larger structures under our experimental setup.

2. Materials and methods

2.1. Materials

Sprague Dawley white laboratory rats were bred in the animal unit facility at Birzeit University and were fed standard rodent diet. The animals were sacrificed by cervical dislocation and decapitation in accordance to animal treatment regulations at the institution.

Reagents used in this research were procured from Sigma-Aldrich. Lipid species were obtained from Avanti Polar Lipids (Alabaster, AL).

Human Embryonic Kidney cells (HEK 293T), and pCMV plasmids carrying CerS2 and CerS5 genes as well as the control plasmid were kindly gifted by Prof. Anthony H. Futerman, at the Weizmann Institute of Science, Rehovot, Israel.

2.2. Isolation of rat liver mitochondria

Mitochondria were isolated from overnight-starved male rats (100–150 g) according to published protocols [1,10,23]. Isolated mitochondria were suspended in H-buffer (280 mM mannitol, 2 mM HEPES, 0.1 mM EGTA, pH 7.4) and kept on ice for the duration of experiment. The concentration of mitochondrial protein was determined spectroscopically [1].

2.3. Cytochrome c permeability assay

The permeability of the outer membrane of isolated mitochondria was measured indirectly as the rate of oxidation of exogenously-added reduced cytochrome *c* [1,10]. In short, 11 mg of cytochrome *c* was fully reduced with 4 mg of ascorbic acid in buffer Q (180 mM NaCl, 20 mM HEPES, 10 mM EGTA, pH 7.5) for 5 min at room temperature. Excess ascorbic acid was removed by gel filtration of the mixture over Sephadex G-10 beads equilibrated with buffer Q. The bright red eluent (reduced cytochrome *c*) was kept on ice for the duration of the experiments.

When the outer membrane of isolated mitochondria is intact, respiratory chain complex IV (cytochrome *c* oxidase), which is an integral protein of the mitochondrial inner membrane, has no access to any added cytochrome *c*, and hence the rate of oxidation of exogenous cytochrome *c* should be minimal. If mitochondria are hypotonically shocked in water, the outer membrane disintegrates and the rate of oxidation of cytochrome *c* by the complex would be maximal (designated 100%). The permeability of mitochondria under any given circumstances can hence be measured as the rate of oxidation of cytochrome *c* compared to the maximal rate of oxidation.

For intact mitochondria, concentrated mitochondrial suspension was diluted to 0.2 mg/mL in H-buffer supplemented with 5 μM antimycin A and 5 mM DNP (HAD-buffer). For hypotonically-lysed mitochondria, mitochondria were diluted to 0.4 mg/mL in double distilled water for 10 min on ice then the osmolarity was restored by the addition of an equal volume of 2× HAD-buffer (making the final concentration of hypotonically-shocked mitochondria 0.2 mg/mL).

In all experiments, 25 μg of mitochondria (50 μL of 0.2 mg/mL) was dispersed in 650 μL HAD and after 5 min incubation, the indicated amount of C_{16}-, C_{22}-, C_{24}-ceramide, or a mixture of ceramides was added in a volume of 30 μL while vortexing. All lipids were added from 2 mg/mL stocks in isopropanol. The volume of added isopropanol never exceeded 8% of the total assay volume. Controls with similar volumes of isopropanol were assayed in parallel. The mitochondrial suspension was then incubated for 15 min at room temperature before the assay was performed. Reduced cytochrome *c* (~25 μM, final concentration) was added and the rate of oxidation was measured immediately at 550 nm. Volume of solvent is kept constant in all samples and when a premixed combination of ceramides was added.

2.4. Adenylate kinase assay

The release of adenylate kinase was performed as published [23]. In short, 200 mg of isolated mitochondria was suspended in 3 mL of H-buffer supplemented with 5 mg leupeptin, 5 mg aprotinin, and 5 mg pepstatin (each from 5 mg/mL solutions, to make the final volume of suspended mitochondria 5 mL). Mitochondria were then treated with 40 μg (74 nmol) of C_{16}-ceramide, 46 μg (74 nmol) of C_{22}-ceramide, or a pre-mixed mixture of 74 nmol C_{16}- and 74 nmol of C_{22}-ceramides. At time points 0, 5, and 15 min (or 0, 10, 20 and 40 min) 1 mL was removed and centrifuged at 14,000 rpm for 2 min. The recovered supernatant (~900 μL) was removed and kept on ice. In each experiment, 300 μL of the supernatant was mixed with 700 μL of the reaction mixture (50 mM Tris, pH 7.5, 5 mM $MgSO_4$, 10 mM glucose, 1 mM ADP, and 0.2 mM $NADP^+$). After equilibrating for 1 min, 9 μL of the enzyme mixture (25 units of hexokinase and 25 units of glucose 6-phosphate dehydrogenase) was added, and the production of NADPH at 340 nm was monitored.

2.5. Liposome permeabilization assays

Liposome permeabilization assays were performed as previously published [23] following a fluorescence dequenching protocol. Unilamellar liposomes consisting of 93% asolectin and 7% cholesterol were prepared in buffer LP containing 1.5 mM carboxyfluorescein, 6 mM DPX, 40 mM NaCl, 10 mM HEPES, and 1 mM EDTA, pH 7. After 5 cycles of freeze-thawing followed by the extrusion through a polycarbonate membrane, excess unloaded fluorophore was removed by gel filtration on a Sephacryl S200 gel filtration column. In a 96-well plate 10 μL of liposome suspension was diluted in 100 μL of 50 mM NaCl, 10 mM HEPES, and 1 mM EDTA, pH 7. The liposomes were assessed for their fluorescence in a TECAN spectrofluorometer. The excitation filter was 492 nm and the emission filter was 520 nm for CF. Fluorescence

was measured as a function of time. Ceramides were added (40 nmol of C_{16}- or C_{22}-ceramide, or a mixture of both at an amount of 40 nmol each) and the fluorescence was measured kinetically. The total CF content in liposomes was assessed after the addition of 0.4% Triton X-100. The results were normalized using the values of the Triton permeabilization as 100%.

2.6. SOX assay

The release of sulfite oxidase, a mitochondrial intermembrane space protein of 120 kDa, was assayed according to Ganesan and colleagues [24].

2.7. Cytochrome c release and Western blotting

Human embryonic kidney (HEK 293T) cells were grown in DMEM supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum in a 5% CO_2 incubator at 37 °C until ~80% confluent. The cells were then transfected with the genes encoding CerS2 or CerS5 in a pCMV plasmid (20 μg of vector DNA containing CerS genes per 10 cm culture plate) using the calcium phosphate method [25,26]. Control cells were mock transfected with a pCMV only vector lacking any gene of interest. Twenty-four hours post-transfection, cells were collected by mild trypsinization. Cells were homogenized in H-buffer and mitochondria were isolated. Exogenous C_{16}-ceramide or C_{24}-ceramide (74 nmol) was added and the mixture was incubated for 20 min at room temperature. The suspensions were spun down at 14,000 rpm for 2 min. The ensuing pellets and supernatants were collected, and protein content was measured by Bradford assay. The pellets and supernatants were then mixed with 5× sample buffer and flash frozen in $N_{2(l)}$ for SDS-PAGE analysis. Gel electrophoresis was performed on 17% polyacrylamide gels. In each well, 100 μg of each protein was loaded. Electrophoresis was carried out at constant current (25 mA per gel) for an hour. The gels were subsequently transferred onto nitrocellulose membranes and blocked with TBST and 5% fat-free milk. Anti-cytochrome *c* primary antibody was used to detect the released cytochrome *c* (1:5000 in TBST). The secondary antibody was goat-anti-mouse conjugated to HRP (1:2000). The bands were visualized by EZ-ECL detection kit. Anti-VDAC primary antibody (1:1000 in TBST) was used on the same membrane as a control without the need to strip the membrane since VDAC and cytochrome *c* have different molecular weights and hence no cross-reactivity can be seen.

2.7.1. A note of the exogenous ceramide additions

We note that the amounts of ceramides exogenously added to mitochondria or liposomes are higher than physiological ceramide concentrations, however, only a very small percentage of the ceramide added to solution inserts into mitochondrial membranes [10] and the local concentration of ceramide in a particular membrane can be much higher than the overall measured concentration.

3. Results

3.1. C_{22}-ceramide reduces the propensity of C_{16}-ceramide channel formation in isolated rat liver mitochondria and in liposomes

In order to study the effects of a mixture of exogenously added ceramides on the permeabilization of isolated mitochondria, intact mitochondria were incubated with C_{16}-ceramide, C_{22}-ceramide or a mixture of C_{16} and C_{22}-ceramides for 15 min at room temperature. Mitochondrial permeability was measured thereafter using the cytochrome *c* accessibility assay. Whereas C_{16}-ceramide or C_{22}-ceramide treatment alone consistently induced more than 50% outer membrane permeabilization, premixing both lipids together markedly reduced the effects of each ceramide (Fig. 1A). The effect was confirmed by measuring permeabilization in real-time by monitoring the release of adenylate kinase. Mixing both ceramides together prior to addition to mitochondria significantly lowered their efficacy to induce permeability to adenylate kinase (Fig. 1B). Furthermore, this effect was also tested in artificial liposomal membranes composed purely of lipids. Indeed, C_{22}-ceramide inhibited C_{16}-channel formation in liposomes as evidenced by the lower propensity to release carboxyfluorescein from the liposomes (Fig. 1C). In addition, the effects of increasing the amount of C_{22}-ceramide compared to C_{16}-ceramide were tested in liposomes. Interestingly, the effect was biphasic. As the amount of C_{22}-ceramide became larger, inhibition of C_{16}-ceramide channel formation was evident until the concentration of C_{22}-ceramide became greater than that of C_{16}-ceramide as a reversal of the effect was observed (Fig. 1D).

3.2. Mixing different ceramides interferes with the effects of each ceramide on membrane permeability in a dose-dependent manner

In order to show that the inhibition was also present with a different very long chain ceramide, similar experiments were performed using C_{24}-ceramide as a competing species with C_{16}-ceramide channels. Moreover, the biphasic effect was further studied in isolated mitochondria using the cytochrome *c* accessibility assay. Fig. 2A shows that the effect of C_{16}-ceramide on C_{24}-ceramide channel formation and vice versa is biphasic. The effects of each ceramide on the other's ability to form channels in the outer membrane were shown to be significantly different from the addition or the average of the effects of each ceramide species alone (Fig. 2B) indicating the competitive nature of the inhibition.

3.3. Different ceramide species form different channels in mitochondria

Previous results indicated that different species of ceramide are causing membrane permeability independently from each other. In order to deduce the mechanisms by which ceramide species are perforating mitochondria, the release of two intermembrane space proteins was compared. Adenylate kinase (AK) is a small (26 kDa) protein whereas sulfite oxidase (SOX) is a large (120 kDa) enzyme of the intermembrane space. Isolated mitochondria were mixed with C_{16}-ceramide (Fig. 3, *top*), C_{22}-ceramide (Fig. 3, *middle*), or a mixture of C_{16}- and C_{22}-ceramide (Fig. 3, *bottom*) prior to assaying AK and SOX enzymes. Whereas C_{16}-ceramide caused the release of SOX (Fig. 3, *top*), C_{22}-ceramide (Fig. 3, *middle*) showed no significant release of this enzyme indicating that the channels formed by C_{22}-ceramide are much smaller than those formed by C_{16}-ceramide. Mixing both ceramide species led to a release of SOX together with AK (Fig. 3, *bottom*). Since C_{22}-ceramide channels were unable to release SOX, this release was probably only due to the formation of C_{16}-ceramide channels, or due to the formation of a C_{16}–C_{22}-hybrid channel. In all, these results are consistent with different ceramide species forming channels of different sizes, and possibly in different numbers, in isolated mitochondria.

3.4. Cells with endogenously high levels of very long chain fatty acid ceramides release less cytochrome c upon treatment with exogenous C_{16}-ceramide and vice versa

The overexpression of different CerSs in HEK cells leads to the enrichment of specific species of ceramides in these cells. For instance, the overexpression of CerS2 enriches cells with very long chain ceramides (C_{22}-, C_{24}- and $C_{24:1}$-ceramides [4]) whereas CerS5 overexpression leads to the accumulation of C_{16}-ceramide. In order to confirm the effects of exogenously added ceramide on the permeability of mitochondrial membranes enriched with different species of ceramide, the release of cytochrome *c* was measured by Western blotting. Exogenous C_{16}-ceramide was added to mitochondria isolated from HEK cells that were overexpressing CerS2 (very long chain ceramides) or CerS5 (C_{16}-ceramide). The addition of C_{16}-ceramide to cells already enriched with C_{16}-ceramide (CerS5-transfected) potentiates cytochrome *c* release

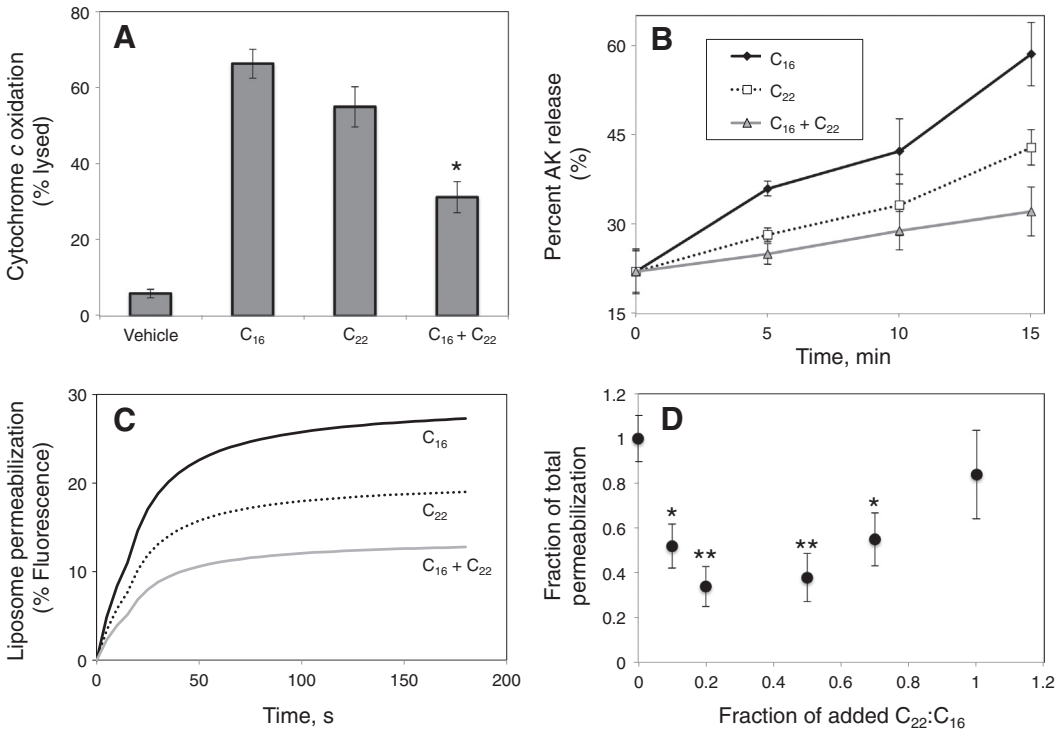

Fig. 1. C_{22}-ceramide interferes with the channel forming ability of C_{16}-ceramide. (A) The permeability of isolated mitochondria was measured by the cytochrome accessibility assay. Hypotonically-shocked mitochondria were used as a measure of 100% permeability. Isolated mitochondria were incubated with 74 nmol C_{16}-ceramide, C_{22}-ceramide or a mixture of C_{16} and C_{22}-ceramides for 15 min at room temperature prior to assaying permeability. Vehicle treatment (30 μL of isopropanol) was used as a negative control. The results represent the average and standard deviation of four replicates. (B) The release of adenylate kinase was a measure of the real-time permeabilization of mitochondrial outer membrane. Isolated mitochondria were incubated with 74 nmol C_{16}-ceramide, C_{22}-ceramide or a mixture of C_{16}- and C_{22}-ceramides and the release of adenylate kinase was monitored in a time-dependent manner. Each point represents the average and standard deviation of three replicates. (C) The permeability of liposomes was measured by a fluorescence dequenching assay. Prepared unilamellar liposomes containing DPX and CF (see the Materials and methods section) were monitored to record the kinetics of the fluorescence changes upon addition of 40 nmol C_{16}-ceramide, C_{22}-ceramide or a mixture of C_{16} and C_{22} ceramides and fluorescence intensity was recorded. The total fluorescence in liposomes was measured by lysing the vesicles with 0.4% Triton X-100. (D) Dose-dependence of C_{22}-ceramide of C_{16}-ceramide channel formation was performed in liposomes. Increasing amounts of C_{22}-ceramide were premixed with decreasing amounts of C_{16}-ceramide in the ratios given in the figure before addition into liposomes. The data represent the average and standard deviation of four replicates. $^{*}p < 0.01$; $^{**}p < 0.005$.

whereas this release is inhibited in CerS2-transfected cells (Fig. 4A). Alternatively, the addition of exogenous C_{24}-ceramide to mitochondria from CerS2-transfected cells enhanced the cytochrome *c* release and inhibited the release in mitochondria from CerS5-transfected cells (Fig. 4B) indicating that the competition is observed ex vivo as well.

4. Discussion

Apoptosis is a form of regulated cell death by which cells die without harming neighboring cells. The signals that dictate cells to undergo apoptosis can be intrinsic or extrinsic. Mitochondria play a key role in

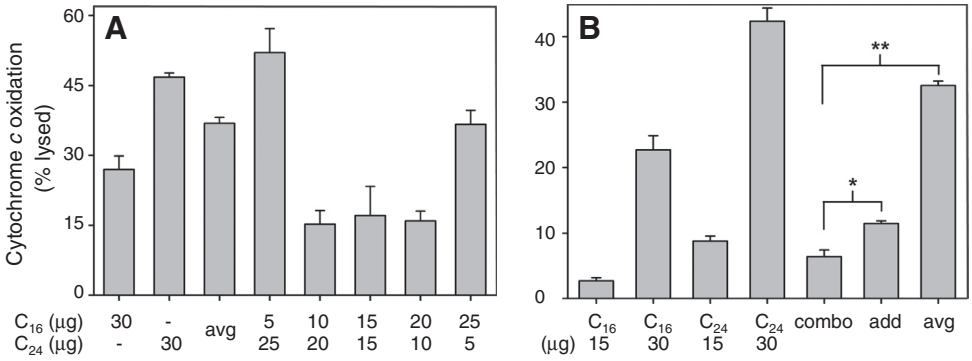

Fig. 2. Competition between C_{16}-ceramide and C_{24}-ceramide for channel formation is biphasic. Mitochondrial outer membrane permeabilization was detected by the cytochrome *c* accessibility assay. (A) Mitochondrial outer membrane permeabilization by C_{16}-ceramide or C_{24}-ceramide channel formation was monitored using 30 μg of each lipid separately or different amounts of premixed ceramides. (B) The degree of MOM permeabilization was measured following the treatments of different concentrations of lipids (15 and 30 μg). The effect of combination (15 μg of C_{16}- premixed with 15 μg of C_{24}-ceramides, combo) on mitochondrial permeabilization is compared to the added values (add) of the permeabilization by each ceramide species separately (15 μg of each) and to the average of permeabilization by each ceramide species separately (30 μg of each). Results represent the average and standard error of at least 3 different experiments. Rates are presented as a percentage of the rate measured when mitochondria are lysed by hypotonic shock. $^{*}p < 0.01$; $^{**}p < 0.005$.

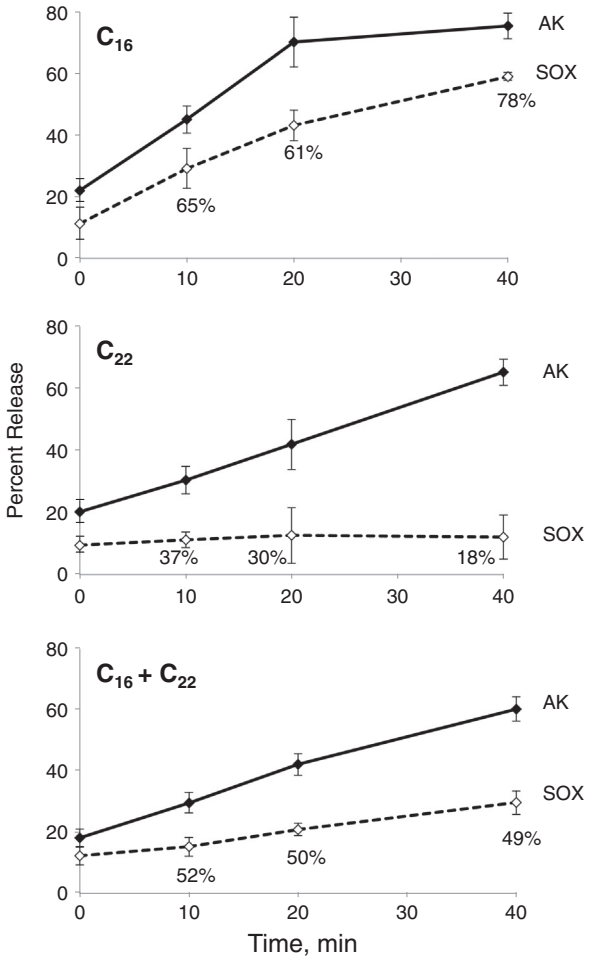

Fig. 3. C_{16}-ceramide forms channels of different sizes than C_{22}-ceramide in isolated mitochondria. Mitochondrial suspensions were incubated with 74 nmol C_{16}-ceramide (*top*), C_{22}-ceramide (*middle*) or 74 nmol C_{16}-ceramide and 74 nmol C_{22}-ceramide (*bottom*) for various time points on ice. Mitochondria were spun down and the supernatants were assayed for either AK release (solid lines) or SOX release (dashed lines). Percentage values under SOX data points indicate the percentage of AK permeable mitochondrial outer membranes that have been permeabilized to SOX. The results represent the average and standard deviation of three experiments.

intrinsic apoptosis as the loss of outer membrane integrity leads to a cascade of events that culminate in apoptotic death. Various proteins have been linked to the initiation of apoptosis by causing mitochondrial outer membrane permeabilization via the formation of different pores (reviewed in [27]), such as mitochondrial permeability transition pore (mtPTP) [28], mitochondrial apoptosis-induced channel (MAC) [29], Bax oligomerization into channels [24] and Bax/Bak hybrid channels [30]. In addition, bioactive lipids have also been implicated in channel formation in the outer membrane of mitochondria, whether in conjunction with proteins [31–33] or individually [6,14,34]. In vivo, all of the aforementioned events can take place simultaneously leading to a coordinated and regulated outcome.

Here we studied the mechanisms of ceramide-induced permeability of the mitochondrial outer membrane as one of the processes that leads to apoptotic cell death. Ceramides are produced de novo from the amino acid serine and palmitoyl-CoA in a set of reactions that end with the desaturation of the 4–5 carbon–carbon double bond to form the bioactive ceramide. The penultimate step in this pathway is a condensation reaction between a fatty acyl-CoA and the amino alcohol sphinganine. This reaction is catalyzed by a family of 6 mammalian isoforms of ceramide synthase (CerS) [35]. CerSs are localized differentially in the cell and they are specific to different subsets of fatty acids depending on the chain length [5]. Dihydroceramide desaturase, a mixed-function oxidase, finally produces the characteristic 4,5 double bond creating ceramide. Ceramides are generated in response to a plethora of stressors to initiate the apoptotic pathway. For instance, C_{16}-ceramide was shown to be increased in human colon carcinoma cells following treatment by celecoxib (a cyclooxygenase-2 selective nonsteroidal anti-inflammatory drug used to treat arthritis) thus mediating its anti-proliferative effects [36,37]. Ceramides have also been implicated in apoptotic death by facilitating the opening of mtPTP. For instance, C_6-ceramide was shown to enhance curcumin-induced cytotoxicity [38] and the efflux of AIF from mitochondria upon curcumin treatment was accelerated by a concerted action of Bax, VDAC and ceramide [39] . Other reports suggest that ceramide does not interact with the known components of mtPTP and that C_{18}-ceramide is a natural inhibitor of that channel [40]. Curcumin was recently shown to induce rapid CerS dimerization, enhancing the activity of the enzyme thus producing more ceramide [41].

Ceramides of various fatty acyl chain lengths were shown to permeabilize mitochondria and artificial membranes when exogenously added [10,15]. This permeability is due to their ability to form channels in the membrane. The formation of ceramide channels is strongly dependent on the structure of the ceramide molecule [42]. Dihydroceramide, which lacks the 4–5 *trans* double bond in ceramide, is not only inactive in causing cell death but also inhibitory of ceramide channel formation in vitro by intercalating in the ordered ceramide channel and destabilizing it leading to channel disassembly [1]. Different ceramide species affecting one another is not surprising as the production of ceramides with differing chain lengths alter the biophysical properties of membranes differentially [26,43]. Moreover, the biophysical properties of membranes change as a result of depletion of very long chain sphingolipids [44].

The lengths of the fatty acids of ceramide serve a variety of functions. For example, they play important roles in modulation of ceramide channels by the anti-apoptotic Bcl-2 family protein, Bcl-xL [15]. Bcl-xL inhibits ceramide channel formation [20]. This inhibition is lost if the ceramide has a truncated fatty acyl chain indicating a role of the fatty acid chain length in binding Bcl-xL. Bax which potentiates ceramide channel formation, on the other hand, is insensitive to ceramide acyl chain length. The presence of a hydrophobic pocket in Bcl-xL suggests that binding ceramide is dependent on the ceramide's chain length. The optimal lengths of ceramides whose channels are inhibited by Bcl-xL were shown to be 16, 18 and 20 carbons [15]. Moreover, the roles of ceramides with different fatty acid chain lengths in a variety of neurodegenerative diseases were thoroughly discussed [45]. Depending on the type of the neurological disorder, there are increases or decreases in certain subsets of ceramides, indicating that the chain length is an integral functional feature of the ceramide molecule.

In this report, the effects of varying chain lengths at the level of the ceramide molecule were investigated. Indeed, similar to our previous results, the presence of different ceramide species in the added vehicle caused a marked decrease in ceramide channel formation as both C_{22}- and C_{24}-ceramides competed with the ability of C_{16}-ceramide to form channels. The effects were not protein-dependent as similar results were obtained with protein-free unilamellar liposomes. Interestingly, studies with liposomes confirmed a biphasic nature of the inhibition. The addition of C_{16}-ceramide to liposomes induced the release of CF from liposomes. As the mole fraction of C_{16}-ceramide was decreased in favor of C_{22}-ceramide, the release of CF was reduced then increased thereafter. This indicates that different channels were being formed. At first, C_{16}-ceramide channels were favored and C_{22}-ceramide interfered with the channel formation capability of C_{16}-ceramide, however as the relative amount of C_{16}-ceramide became less, C_{22}-ceramide channel formation dominated. Similarly, in isolated mitochondria, this effect was observed in the competition assays of C_{16}- and C_{24}-ceramides confirming the hypothesis that each ceramide species produces unique and uniform channels and the presence of other species would be inhibitory.

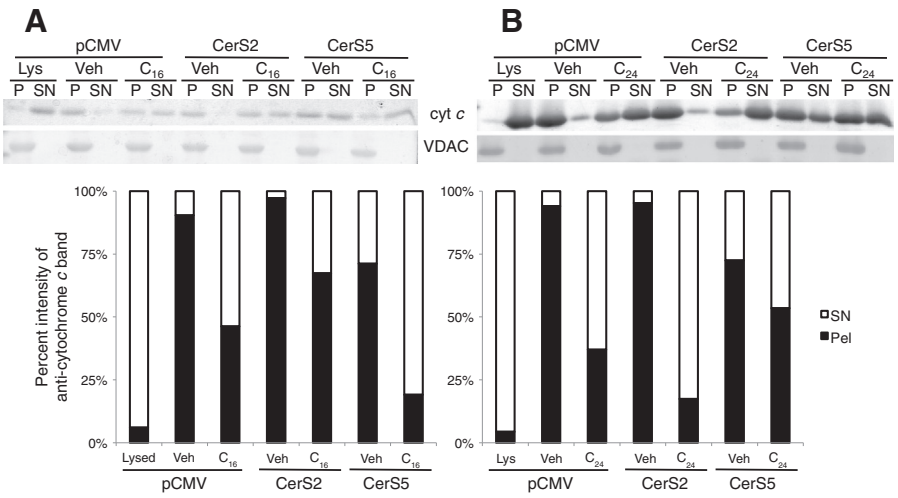

Fig. 4. Exogenous ceramide permeabilizes mitochondria isolated from different CerSs-overexpressing cells differentially. HEK cells overexpressing CerS2, CerS5 or mock transfected with plasmid only (pCMV) for 24 h were collected, homogenized and mitochondria were isolated. Mitochondria from pCMV-transfected cells were hypotonically-lysed (Lys) or treated with exogenous C_{16}-ceramide (A) or C_{24}-ceramide (B) and the mixture was incubated for 20 min at room temperature. The other batches of isolated mitochondria were treated with C_{16}-ceramide or C_{24}-ceramide in a similar manner. The suspensions were spun down and the subsequent pellets (P) and supernatants (SN) collected and flash frozen for SDS-PAGE analysis (*top*). Vehicle control was performed by the addition of isopropanol to isolated mitochondria (Veh). Western blotting using anti-cytochrome *c* primary antibody was used to detect the released cytochrome *c* upon mitochondrial permeabilization and anti-VDAC was used as a marker of mitochondrial membranes. The intensities of cytochrome *c* bands in samples from pellets and supernatants were measured by densitometry (*bottom*). The experiment was performed with three different preparations and the Western blot shown is a representation of the results.

Since there was competition between different ceramide species to form distinctive channels, we investigated the nature of each type of channel. C_{16}-ceramide channels were shown to form larger channels, as SOX (a 120 kDa protein) was released in a time-dependent manner, whereas C_{22}-ceramide was unable to release this enzyme from the IMS. Both ceramide species, however, were able to form pathways large enough to release AK (a 26 kDa protein). This indicates that C_{16}-ceramide is able to form larger channels than C_{22}-ceramide. Adding a mixture of C_{16}- and C_{22}-ceramides leads to the release of SOX presumably through the larger C_{16}-ceramide channels. Although there is a possibility that hybrid channels comprised of both C_{22}- and C_{16}-ceramides can be large enough to allow the passage of SOX outwards, it is unlikely because the presence of both species in the same channel is destabilizing.

In order to study the effects of different ceramide species ex vivo, HEK cells overexpressing different CerSs were utilized. CerS5-overexpressing HEK cells have accumulated C_{16}-ceramide in their membranes whereas HEK cells overexpressing CerS2 have high levels of very long chain fatty ceramides [4,22,46]. Mitochondria isolated from HEK cells overexpressing either enzyme or mock-transfected with vector only were subjected to C_{16}- or C_{24}-ceramides. The release of cytochrome *c*, measured by Western blotting, was an indication of channel forming activity in mitochondria. Not surprisingly, mitochondria from CerS2-transfected cells were more resistant to permeabilization by C_{16}-ceramide and the release of cytochrome *c* was potentiated when C_{24}-ceramide was exogenously added. Similar results were obtained with mitochondria isolated from CerS5-overexpressing cells, with C_{16}-ceramide potentiating cytochrome *c* release and C_{24}-ceramide inhibiting its release. These results are indicative of the presence of different mechanisms in vivo to promote or inhibit ceramide channel formation depending on the CerS that is activated and the ceramide species that are produced. It should be noted that the results presented here are limited and not based on our own analysis of ceramide contents upon overexpression of CerS2 or CerS5.

Recently, it was shown in HCT-116 human colon cancer cells as well as in MCF-7 breast cancer cells that upregulation of CerS4 and CerS6 (both of which are responsible for the production of the long chained C_{16}- and C_{18}-ceramides) induced mitochondrial damage and induction of apoptosis. The opposite effects were observed upon overexpression of CerS2 (which specifically acylates sphinganine with very long chain fatty acids) [22]. Co-overexpression of CerS2 with CerS4 or CerS6 led to a marked increase in total ceramide levels, however this increase had no effect on cell proliferation in HCT-116 cells, indicating that the equilibrium between the various ceramide species is key to instruct the cell to initiate the apoptotic machinery [47].

At the start of the apoptotic program, dying cells mainly produce C_{16} and C_{18}-ceramides [48,49] followed by the production of C_{24}-ceramides as the process reaches its final stages [48]. This selective activation of certain CerS at the expense of others is indicative of the varying roles each ceramide species plays to initiate or accelerate apoptosis. Channels made from C_{16}- and C_{18}-ceramides are inhibited by Bcl-xL which resists the permeabilization of the outer membrane until significant concentrations of these ceramides are achieved. This control of apoptosis initiation can be further exacerbated by the production of very long chain ceramides in the vicinity of mitochondria containing C_{16}- or C_{18}-ceramides. The key decision-making step of mitochondrial outer membrane permeabilization is controlled, therefore, at several levels to ensure the conditions are met before executing the cell.

5. Conclusion

In all, we present evidence of a novel mechanism of apoptotic regulation, whereby the activities of various CerS enzymes are coordinated in such a way to produce a timely response. The mode of inhibition is biophysical, and is related to the packing of different columns of ceramide on top of one another. The intercalation of a heterogeneous mixture of ceramides within the channel columns within the plane of the membrane is destabilizing the tight packing of the lipid columns and possibly affects the curvature of the channel at the interface with the mitochondrial outer membrane bilayer. This, in conjunction with dihydroceramide inhibition of ceramide channel formation [1], represents a unique process of regulation when and where ceramide is produced to cause mitochondrial permeability and apoptosis. On the other hand it can be used as a starting point in designing specific drugs that activate one type of CerS but not others and thereby controlling the initiation of apoptosis. Furthermore, provided that CerSs are regulated by homo- or hetero-dimerization, the activity of different CerSs can be modulated in a novel way in order to generate the required acyl chain composition of ceramide in vivo [41] in agreement with the results presented in this research.

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