Biochimica et Biophysica Acta 1848 (2015) 561–567

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

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

ARTICLE INFO

Article history:
Received 12 September 2014
Received in revised form 13 November 2014
Accepted 17 November 2014
Available online 21 November 2014

Keywords:
Sphingolipid
Very long chain fatty acid
Ceramide channel
Mitochondrial apoptosis
Cytochrome *c* release

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.

http://dx.doi.org/10.1016/j.bbamem.2014.11.018
0005-2736/

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