Biochimica et Biophysica Acta 1818 (2012) 1291–1301

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Ceramide channels: Influence of molecular structure on channel formation in membranes

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ARTICLE INFO

Article history:
Received 21 November 2011
Received in revised form 8 February 2012
Accepted 9 February 2012
Available online 15 February 2012

Keywords:
Ceramide
Analogs
Channels
Mitochondria
Permeabilization
Pore

ABSTRACT

The sphingolipid, ceramide, self-assembles in the mitochondrial outer membrane (MOM), forming large channels capable of translocating proteins. These channels are believed to be involved in protein release from mitochondria, a key decision-making step in cell death. Synthetic analogs of ceramide, bearing modifications in each of the major structural features of ceramide were used to probe the molecular basis for the stability of ceramide channels. Channel stability and mitochondrial permeabilization were disrupted by methylation of the C1-hydroxyl group whereas modifications of the C3 allylic hydroxyl group were well tolerated. A change in chirality at C2 that would influence the orientation of the C1-hydroxyl group resulted in a strong reduction of channel-forming ability. Similarly, methylation of the amide nitrogen is also detrimental to channel formation. Many changes in the degree, location and nature of the unsaturation of ceramide had little effect on mitochondrial permeabilization. Competition experiments between ceramide and analogs resulted in synergy with structures compatible with the ceramide channel model and antagonism with incompatible structures. The results are consistent with ceramide channels being highly organized structures, stabilized by specific inter-molecular interactions, similar to the interactions responsible for protein folding.

1. Introduction

The sphingolipid, ceramide, has been shown to be able to form channels in planar membranes [1], liposomes [2] and the mitochondrial outer membrane [3,4]. In mammalian mitochondria, channel formation occurs at physiologically relevant ceramide levels [4]; levels measured in mitochondria from cells early in the apoptotic process [5–7]. In addition, the propensity to form channels and their size is influenced by Bcl-2 family proteins [8,9]. These channels are large, stable and capable of allowing proteins to cross membranes [3,4]. The size was determined from the molecular weight of proteins released from mitochondria [3], from the conductance of single channels formed in planar membranes [10], and from visualization of the pores by electron microscopy [11]. A range of sizes was reported with a typical channel having an estimated pore diameter of 10 nm [10,11]. Thus hundreds of ceramide molecules must spontaneously self-assemble in the 2-dimensional liquid phase of the membrane. Unlike the fluid and transient toroidal pores or lipidic pores formed when lamellar lipids are disturbed by amphipathic molecules such as peptides or synthetic structures [12–14], whole proteins [15,16], or at the phase transition [17], the channels formed by ceramide seem to be highly structured and rigid. The disassembly of channels formed by short-chain ceramide shows a quantization of conductance with a strong preference for large conductance drops to be multiples of 4 nS [10]. This finding not only supports the notion that ceramide channels are highly-organized cylindrical structures, but is also consistent with a modification of the originally-proposed barrel-stave structure [1]. Each stave of the barrel is proposed to consist of a stack of ceramide molecules held together by intermolecular hydrogen-bonding through the amide and carbonyl groups of the amide linkage. These span the membrane forming a cylinder when arranged in an anti-parallel fashion (supplemental Fig. 1S) [10]. Molecular dynamic simulations indicate that this structure is stable [18].

The ability of ceramide to form channels must, at least in part, be attributed to the structural features of the ceramide molecule. The polar head group of ceramide (Fig. 1), with its amide linkage and two hydroxyl groups located in close proximity, constitutes a tridentate hydrogen-bonding donor/acceptor center and is capable of

Abbreviations: D-e-, D-*erythro*-; L-e-, L-*erythro*-; C_8-Cer, D-e-C_8-ceramide; C_{16}-Cer, D-e-C_{16}-ceramide; $C_{18:1}$-Cer, D-e-$C_{18:1}$-ceramide; DNP, 2,4-dinitrophenol; BSA, fatty acid depleted bovine serum albumin; MOM, mitochondrial outer membrane; DPX, p-xylene-bis-pyridinium bromide

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doi:10.1016/j.bbamem.2012.02.010

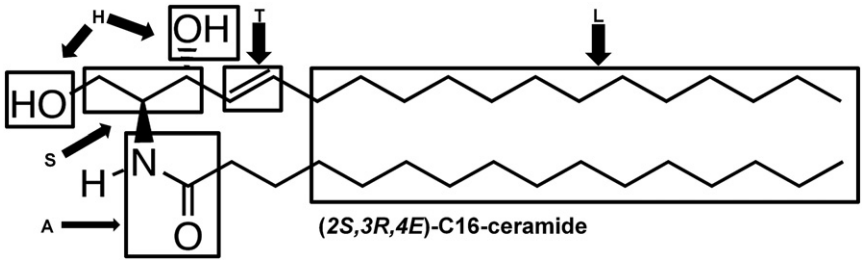

Fig. 1. Structure of *N*-palmitoyl-D-*erythro*-sphingosine (D-e-C_{16}-ceramide or C_{16}-Cer) showing the major features of the molecule and the codes used for the synthetic analogs: **H** for changes to the hydroxyl groups, **S** for changes in stereochemistry, **A** for changes to the amide group, **T** for changes to the *trans* double bond and **L** for changes to the chain length.

generating an effective network of directed hydrogen bonding interactions. These structural features may partly explain its ability to form large, stable channels in membranes. However, it is not known if the location and orientation of the interacting groups are critical to achieve a stable structure. In addition, other structural features (such as the length of the hydrocarbon chains and the number and configuration of the double bonds) may be important. Therefore, we have undertaken a study of synthetic ceramide analogs (Table 1, Fig. 2) to determine (a) if naturally occurring ceramides are uniquely suited to form large aqueous pores and (b) the structural and stereochemical features in ceramide that are necessary for the formation of large channels.

2. Materials and methods

2.1. Reagents

N-Palmitoyl-D-*erythro*-sphingosine (D-e-C_{16}-ceramide or C_{16}-Cer) and *N*-oleoyl-D-*erythro*-sphingosine (D-e-$C_{18:1}$-ceramide or $C_{18:1}$-Cer) were obtained from Avanti Polar Lipids (Alabaster, AL). The analogs of C_{16}-Cer and D-e-C_8-ceramide (C_8-Cer) and **{T1, T4}** (Fig. 2) were synthesized as described previously [19–26]. Antimycin A, 2,4-dinitrophenol (DNP), horse heart cytochrome c, and fatty acid depleted bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). DPX (p-xylene-bis-pyridinium bromide) was purchased from Molecular Probes (Invitrogen). 5(6)-carboxyfluorescein was purchased from Acros Organics.

2.2. Preparation of rat liver mitochondria

Rat liver mitochondria were isolated by differential centrifugation of tissue homogenates as described previously [27] as modified [3]. Briefly, male Sprague–Dawley rats were fasted overnight and sacrificed by decapitation. The liver was excised and minced in cold isolation medium, H buffer (70 mM sucrose, 210 mM mannitol, 0.1 mM EGTA, 5.0 mM HEPES, pH 7.5), supplemented with 0.5% (w/v) fatty-acid depleted BSA followed by homogenization in a motorized Potter–Thomas Teflon-glass homogenizer (two passes). The homogenate was subjected to 10-min cycles of low speed (600*g*) and high speed (8600*g*) centrifugations in the same medium (twice each) at 4 °C in a Sorvall SS-34 rotor. After the low speed spin, the supernatant was collected and spun at high speed. The pellet obtained after the high speed centrifugation was resuspended in the medium of the same composition and centrifuged. BSA was removed by centrifugation at 8600*g* in BSA-free medium. The final mitochondrial pellet was resuspended in an ice cold sucrose free isotonic buffer FH (280 mM mannitol, 0.1 mM EGTA, 2 mM HEPES, pH 7.4). The mitochondrial intactness was determined from the rate of cytochrome *c* oxidation (vide infra) compared with the rate measured after mild hypotonic shock [28].

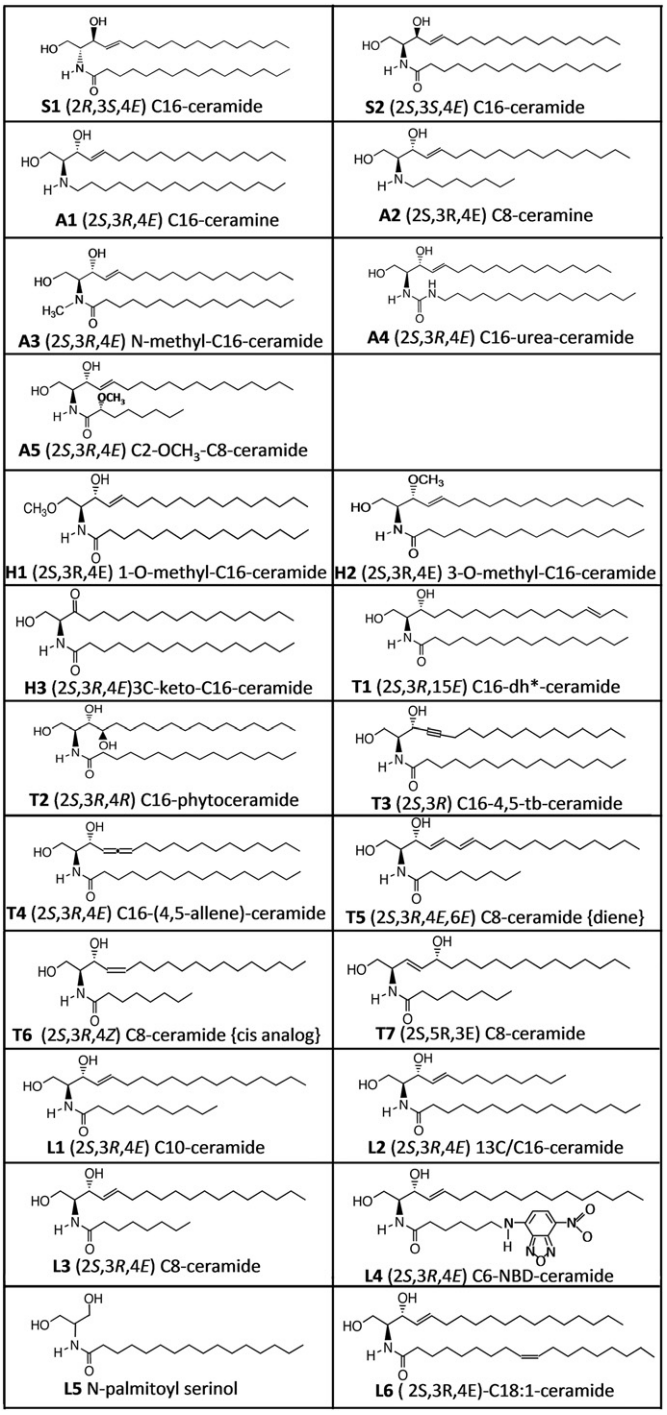

Fig. 2. Chemical structures of the analogs used.