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Dihydroceramide hinders ceramide channel formation: Implications on apoptosis

J. Stiban, D. Fistere and M.Colombini

Department of Biology, University of Maryland, College Park, Maryland 20742

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Early in apoptosis, ceramide levels rise and the mitochondrial outer membrane becomes permeable to small proteins. The self-assembly of ceramide to form channels could be the means by which intermembrane space proteins are released to induce apoptosis. Dihydroceramide desaturase converts dihydroceramide to ceramide. This conversion may be removing an inhibitor as well as generating a pro-apoptotic agent. We report that both long and short chain dihydroceramides inhibit ceramide channel formation in mitochondria. One tenth as much dihydroceramide was sufficient to inhibit the permeabilization of the outer membrane by about 95% (C_2) and 51% (C_{16}). Similar quantities inhibited the release of carboxyfluorescein from liposomes indicating that other mitochondrial components are not necessary for the inhibition. The apoptogenic activity of ceramide may thus depend on the ceramide to dihydroceramide ratio resulting in a more abrupt transition from the normal to the apoptotic state when the *de novo* pathway is used in mitochondria.

Keywords: ceramide channels; ceramide-induced apoptosis; cytochrome *c* release; *de novo* synthesis; dihydroceramide; liposomes; mitochondria.

Abbreviations: CF: carboxyfluorescein; C_2-ceramide: *N*-acetyl-D-*erythro*-sphingosine; C_{16}-ceramide: *N*-palmitoyl-D-*erythro*-sphingosine; DHC_2: *N*-acetyl-D-*erythro*-sphinganine or C_2-dihydroceramide; DHC_{16}: *N*-palmitoyl-*erythro*-sphinganine or C_{16}-dihydroceramide; DPX: *p*-xylene-bis-pyridinium bromide.

Introduction

Ceramides are sphingosine-based lipids that normally exist in cell membranes at a steady state level. This level is increased in cells undergoing apoptosis (for review, see[1–3]) and this increase is thought to be an important step in the apoptotic process.

Ceramide's role in apoptosis is quite extensive and diverse. It has been proposed that ceramide induces apoptosis via several routes. For instance, by clustering into ceramide-enriched domains in the plasma membrane, ceramide is able to recruit or cluster Fas receptors to induce Fas-mediated apoptosis.[4–7] Ceramide is also believed to be involved in reorganizing small membrane rafts into signaling platforms in response to stress. The control seems to arise from ceramide locally generated within rafts (for review, see[8]). Ceramide administration changed the phosphorylation states of some MAP kinases[9–13] and since this coincides with the release of cytochrome *c*, AIF, SMAC, and Omi from mitochondria this change in phosphorylation state is believed to be causal.[13] In differentiating embryonic stem cells, ceramide is reported to mediate the binding of protein kinase C zeta (PKC ζ) to its inhibitor PAR-4 (prostate apoptosis response-4) and this is proposed to be responsible for the induction of apoptosis in these cells.[14]

Ceramide could also initiate apoptosis by permeabilizing the mitochondrial outer membrane to pro-apoptotic proteins found in the intermembrane space. The release of these proteins into the cytosol has been shown to be a critical step in committing a cell to irreversible apoptosis. This permeabilization can be performed by ceramide directly because both short chain (C_2) and long chain (C_{16}) ceramides form channels in planar phospholipid membranes[15] and in liposomes (this study). The addition of ceramide to whole cell cultures induces the release of cytochrome *c* into the cytosol.[16–19] Ceramide also induces the release of cytochrome *c* in isolated mitochondria.[20–22] The permeability of the mitochondrial outer membrane to proteins can be enhanced in a dose- and time-dependent fashion by incubation with ceramide.[23] This permeability showed a molecular weight cutoff of 60 kDa.[23] This is an appropriate size for releasing the known pro-apoptotic proteins from the mitochondrial intermembrane space.

Dihydroceramide is almost identical to ceramide except that it lacks a critical *trans* double bond at the 4, 5 position. It is an intermediate in the *de novo* synthesis pathway and is believed to be biologically inactive in that it cannot cause cells to undergo apoptosis[24,25] nor can it induce the release of cytochrome *c* when added to isolated mitochondria[23] or cell cultures. Dihydroceramide also does not form channels in planar phospholipid membranes[15] or in liposomes (this study). However, rather than simply being inactive, we provide evidence that it can be inhibitory. Indeed we find that both C_2- and C_{16}-dihydroceramide interfere with ceramide

Correspondence to: M.Colombini, Department of Biology, University of Maryland College Park, MD 20742. Tel.: (301) 405-6925; Fax: (301) 314-9358; e-mail: colombini@umd.edu

channel formation both in mitochondria and in liposomes. Generation of ceramide by the *de novo* pathway involves converting dihydroceramide to ceramide. Our results indicate that both the process of depleting dihydroceramide and augmenting ceramide levels are important in the generation of ceramide channels and the induction of apoptosis. This combined effect would sharpen the transition from impermeability to permeabilization of the outer membrane.

Materials and methods

Materials—Asolectin (polar extract of soybean lipids), C_2-ceramide, C_2-dihydroceramide, C_{16}-ceramide and C_{16}-dihydroceramide were supplied by Avanti Polar Lipids (Alabaster, AL). Analytical-grade DMSO (from Fisher) was used to dissolve C_2-ceramide and C_2-dihydroceramide whereas isopropanol (from Acros Organics) was used to dissolve C_{16}-ceramide and C_{16}-dihydroceramide. 4-Carboxyfluorescein (CF) and *p*-xylene-bis-pyridinium bromide (DPX) were obtained from Molecular Probes (Eugene, OR). Cytochrome *c* and bovine serum albumin (BSA, fatty acid depleted) and cholesterol were from Sigma (St. Louis, MO). Sodium ascorbate was bought from Acros Organics.

Mitochondria—Mitochondria were isolated from male Sprague-Dawley rat liver as described by Parsons *et al.*[26] as modified.[23] In brief, young rats (generally 200 to 300 g), fasted overnight with water *ad labitum,* were decapitated, the liver excised and cut in ice-cold isolation buffer (210 mM Mannitol, 70 mM sucrose, 10 mM HEPES, 100 μM EGTA and 0.05 mg /mL of fatty acid-free Bovine Serum Albumin, pH 7.4; i.e. HB-buffer). The liver was minced and homogenized in a motorized Potter homogenizer with loose Teflon pestle (two passes). The homogenate was centrifuged at 760 g for 10 min at 4°C. The supernatant was then spun for 10 min at 9000 g to recover mitochondria. This sequence was repeated but the second high-speed spin was performed without BSA (H-buffer). Finally the mitochondrial pellet was resuspended in the isolation buffer without BSA.

The protein concentration of the mitochondrial suspension was measured by using a spectroscopic method.[27] In short, 25 μL of the suspension was diluted in 475 μL of H-buffer without BSA. 500 μL of 100 mM Tris: H_2SO_4, 0.4% SDS pH 8.0 was added to the mitochondrial suspension and the absorbance measured at 280 nm and 310 nm. The protein concentration is $\frac{40\times(A280-A310)}{1.05}$. Reduced cytochrome *c* was prepared by mixing 11 mg of cytochrome *c* with 4 mg sodium ascorbate in 0.5 mL of buffer Q (200 mM HEPES, 10 mM EGTA, pH 7.5). The reduced cytochrome *c* was separated from the ascorbate on a Sephadex G-10 gel filtration column pre-equilibrated with buffer Q.

The mitochondria (50 μL of 0.2 mg/mL) were suspended in 0.75 mL of isotonic H-medium supplemented with 2.2 μM antimycin A and 2 mM 2, 4-dinitrophenol and treated with ceramide or dihydroceramide in different doses. The permeabilization of the mitochondria was measured by monitoring the reduction of the absorbance of added reduced cytochrome *c* at 550 nm. C_2-ceramide and dihydroceramide were added as 10 μL of a DMSO solution. The dose of ceramide therein was kept the same (2.5 μL of 4 mg/mL or a total of 29 nmoles) and it was mixed with various amounts of dihydroceramide and DMSO to achieve the concentrations of DHC_2 used in each experiment. C_{16}-ceramide and dihydroceramide were added as 50 μL of an isopropanol solution. It contained a constant 25 μL of 2 mg/mL C_{16}-ceramide (93 nmoles) diluted with 25 μL of either isopropanol or DHC_{16} in isopropanol (1 mg/mL) to achieve the desired amount of dihydroceramide. Vehicle controls were performed as appropriate.

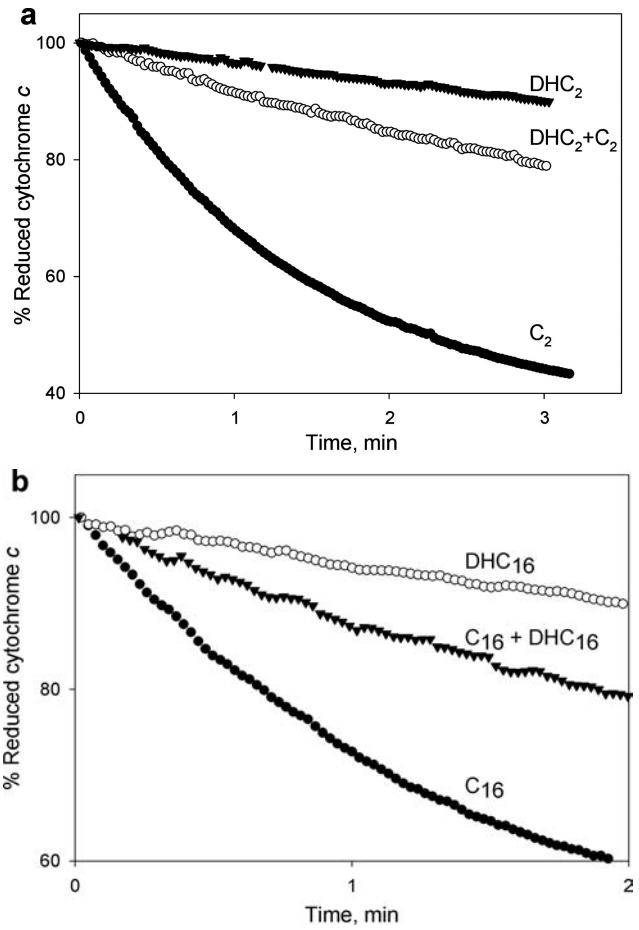
Liposomes—Liposomes were made after mixing the lipids (93% asolectin and 7% cholesterol, by weight) in chloroform and the solvent was evaporated under nitrogen and overnight in vacuum. The lipids (total of 5 mg) were hydrated in a buffer containing 1.5 mM CF 6 mM DPX 38.8 mM NaCl 10 mM HEPES and 1 mM EDTA pH 7.0. The liposomes were vortexed and subjected to 4 cycles of freeze-thaw-sonication followed by freeze-thawing and extrusion through a polycarbonate membrane to form uniform single walled vesicles (100 nm). A Sephacryl S200 gel filtration column (1.5 × 30 cm) was used to separate the liposomes from unloaded fluorophore. 100 μL of the liposome suspension (containing approximately 0.1 mg of lipid) were diluted into 2 mL of 50 mM NaCl, 10 mM HEPES, 1 mM EDTA pH 7. The liposomes were assessed for their fluorescence in a Deltascan spectrofluorometer (Photon Technology Instruments). CF was excited at 495 nm and the emitted light detected at 520 nm. The fluorescence was measured as a function of time and then different concentrations of C_2-ceramide, C_{16}-ceramide or C_2-ceramide premixed with DHC_2 were added. The increase in fluorescence was the result of the release of CF from the liposomes and its dilution from the quenching agent, DPX. The maximal increase in fluorescence was measured following the addition of 150 μL of 5% Triton-X 100.

Results

Ceramide permeabilizes mitochondria and dihydroceramide blocks this permeabilization

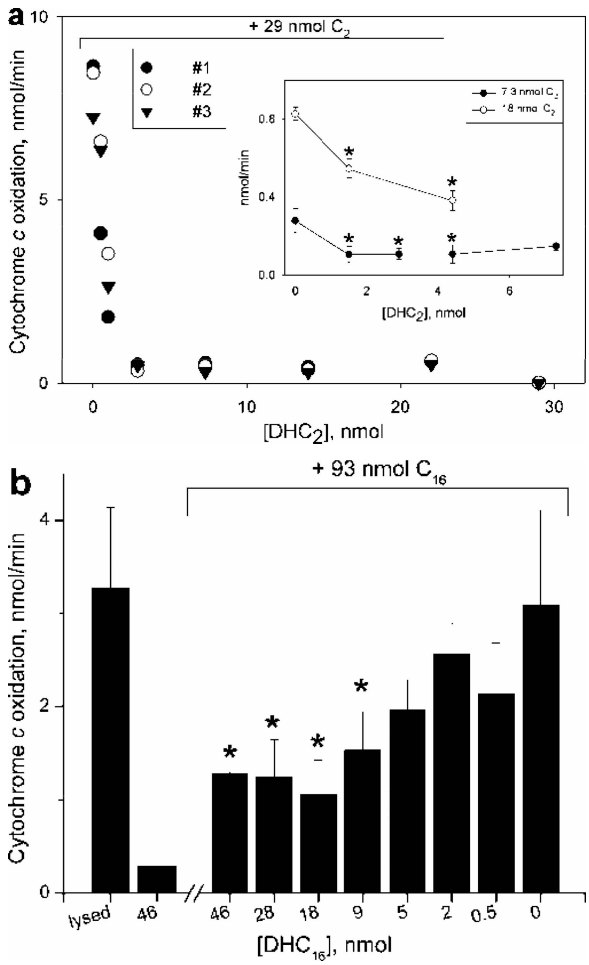
The permeabilization of the outer membrane of isolated rat liver mitochondria was achieved by measuring the oxidation of exogenously added cytochrome *c* by mitochondria. Cytochrome oxidase carries electrons from cytochrome *c* to molecular oxygen, thus oxidizing cytochrome *c*. If the outer membrane is intact, cytochrome *c* will not be able to cross it to get to the cytochrome oxidase. Permeabilization of the outer membrane to cytochrome *c* can be detected by the oxidation of cytochrome *c* measured as a reduction in absorbance at 550 nm. Incubation of mitochondria with 29 nmoles of C_2- (Figure 1a) or 93 nmoles of C_{16}-ceramide (Figure 1b) for

Figure 1 Dihydroceramide inhibits the permeabilization of the mitochondrial outer membrane by ceramide. The percent of cytochrome *c* in the reduced state is plotted as a function of time after the addition of 29 nmol C_2-ceramide, 29 nmol dihydroceramide (DHC_2) and 22 nmol DHC_2+29 nmol C_2 (panel a), 93 nmol C_{16}, 46 nmol DHC_{16} and 46 nmol DHC_{16}+93 nmol C_{16} (panel b) to 0.75 mL of mitochondrial suspension (13 μg of mitochondrial protein per mL).



10 min induced a permeability to cytochrome *c* as evident by the fast decrease of the level of reduced cytochrome *c*. Alternatively, incubating mitochondria with a similar dose of C_2- or C_{16}-dihydroceramide (DHC_2 or DHC_{16}) caused no permeabilization of the mitochondria. The slow decrease in the reduced cytochrome *c* after dihydroceramide occurred at the same rate as untreated mitochondria or mitochondria treated with DMSO (results not shown; see also[23]). When dihydroceramide is premixed with ceramide, the ability of ceramide to permeabilize mitochondria was effectively reduced (Figure 1a and b) and this reduction occurred in a dose-dependent manner (Figure 2a and b). The amounts of C_2- and C_{16}-ceramides used were chosen to produce close to maximal effects, hence increasing the signal to noise ratio. These amounts were higher than the physiological levels of ceramide in mitochondria (about 52 pmol C_2 per nmol mitochondrial phospholipid and 168 pmol C_{16} per

Figure 2 Dihydroceramide reduces ceramide channel formation in mitochondria. (a) The initial rate of oxidation of cytochrome *c*, obtained from experiments similar to those shown in Figure 1, is shown as a function of the amount of DHC_2 added to a fixed amount of C_2-ceramide (as indicated). The points collected at 29 nmoles of DHC_2 had no added C_2-ceramide. The results were collected in three different experiments. The amount of mitochondria used in the experiments 13 μg/mL. The baseline oxidation rate (DMSO alone) was subtracted from each point. *Inset*: Low levels of ceramide and dihydroceramide were used along with a higher mitochondrial protein concentration (133 μg/mL). (b) A bar graph showing similar results for the effects of DHC_{16} on C_{16}-ceramide channel formation. Again the results were obtained from 3 independent experiments, hence the large error bars. "Lysed" refers to the rate of cytochrome *c* oxidation after hypotonic shock (20 μL of mitochondria were diluted to 1.5 mL with water). The results represent the mean of three experiments±S.D. The level of statistical significance from control (0 nmol DHC_{16}) is shown with *, $P \leq 0.05$.



nmol mitochondrial phospholipid*). However, under physiological conditions, C_2-ceramide channel formation (Figure 2a *inset*) was inhibited by DHC_2 and C_{16}-ceramide was also inhibited by DHC_{16} (data not shown). Moreover, the

*The calculation is based on the percent insertion of added ceramide into the mitochondrial membranes.[28] For 40 μM C_2, about 2 percent inserts in the membranes. In mitochondria, total phospholipid in the membranes is about 680 μg phospholipid per mg protein.[32]

estimated physiological levels of ceramide needed to initiate apoptosis are average levels, whereas the local concentration of ceramide (produced by dihydroceramide desaturase) must be much higher. Thus the reported influence of dihydroceramide on channel formation should be physiologically relevant.

Mitochondria were incubated with each of the mixtures for 10 min and the reaction was started by the addition of reduced cytochrome *c*. Pre-incubation with ceramide alone induced the greatest permeabilization. Premixing ceramide with dihydroceramide greatly reduced the ceramide-induced permeabilization. The effect was dose-dependent and even a small ratio of dihydroceramide to ceramide reduced the ability of ceramide to permeabilize the outer membrane. This indicates that dihydroceramide is able to block the formation of ceramide channels in mitochondria.

Liposomes are permeabilized by ceramide in a dose-dependent manner

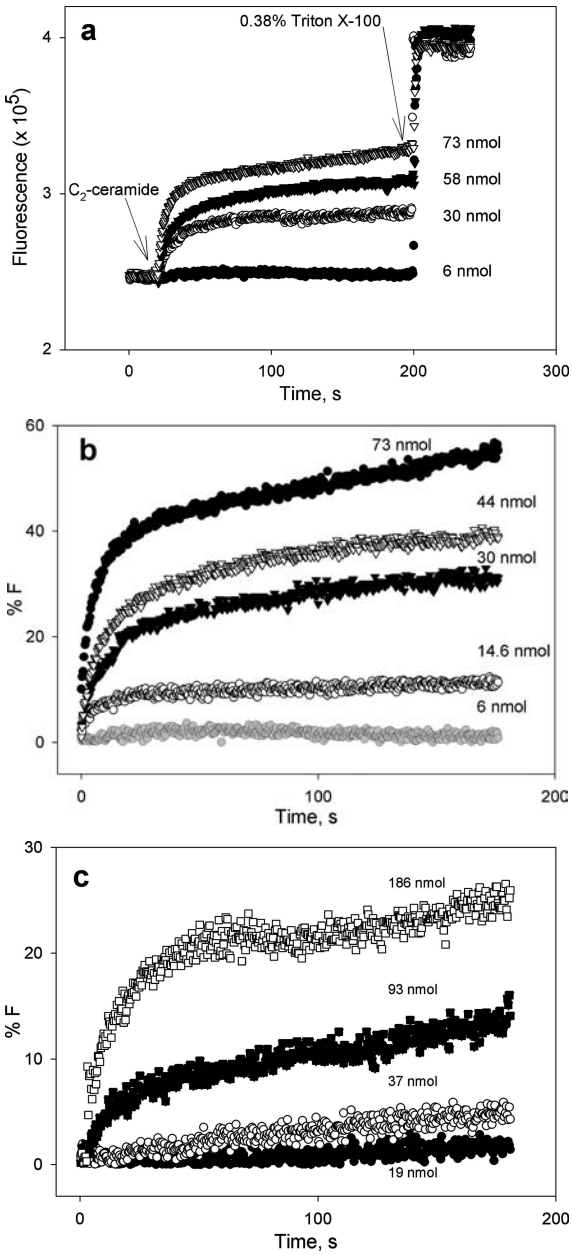
The release of trapped carboxyfluorescein (CF) from liposomes was monitored by an increase in fluorescence. The addition of ceramide to liposomes preloaded with CF and the quencher *p*-xylene-bis-pyridinium bromide (DPX) leads to an increase in fluorescence. Permeabilization of the liposomes allows the efflux and dilution of the CF and the quencher resulting in an increase in fluorescence (Figure 3a). The maximal possible increase was obtained by the addition of the detergent, Triton-X100.

The amount of CF released depends on the amount of ceramide added. The larger the amount of C_2- (Figure 3b) or C_{16}-ceramide (Figure 3c) that was added, the greater was the rate of fluorescence increase and thus the greater the chance for channels to form and release CF. Considering the small size of the liposomes and the large size of the ceramide channels,[15,23] once a channel is formed the liposome should rapidly equilibrate its content with the medium. Thus the rate of fluorescence increase reflects the rate of channel formation in liposomes.

The greater apparent potency of C_2 as compared to C_{16} is probably due to the problem of delivering ceramide to the liposome membrane. A smaller fraction of C_{16}-ceramide partitions into the membrane as compared to C_2-ceramide.[28]

Both DHC_2 and DHC_{16} did not cause permeabilization when added to liposomes even at concentrations 10 times higher than the respective ceramides (data not shown).

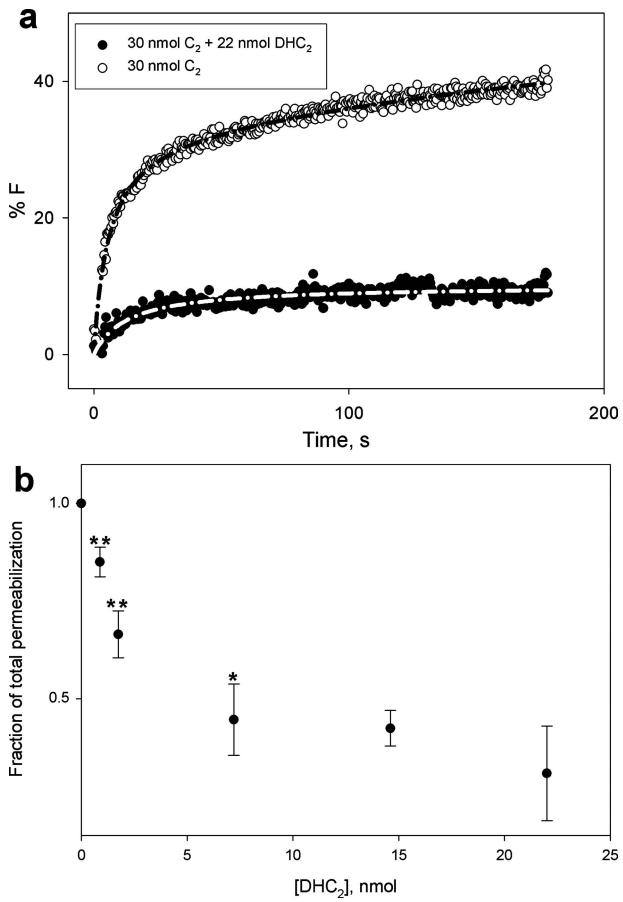
Figure 3 The concentration dependence of ceramide permeabilization of liposomes. In panel a, the indicated amount of ceramide was added after the baseline fluorescence was recorded. The total permeabilization of liposomes was achieved with 0.38% Triton-X 100. In panel b, the concentration dependence of ceramide permeabilization of liposomes is shown as the percentage of the total fluorescence increase after Triton addition. Long-chain ceramide (C_{16}) was used in the experiments illustrated in panel c. Each concentration trace was the average of at least 3 independent experiments.



Dihydroceramide inhibits ceramide channel formation in liposomes

To test whether mitochondrial proteins might be required to mediate the dihydroceramide inhibition of ceramide channel formation, liposome studies were undertaken. Using the same technique described above, fluorescence increases were monitored following the addition of C_2-ceramide (Figure 4a, open symbols) or C_2-ceramide premixed with DHC_2 (closed symbols). The presence of DHC_2 reduced the fluorescence increase induced by C_2-ceramide addition. Thus the inclusion of dihydroceramide with ceramide effectively

Figure 4 Dihydroceramide inhibition of ceramide-induced release of CF from liposomes. (a) The premixing of dihydroceramide with ceramide (closed symbols) greatly reduced the release of CF from liposomes observed with ceramide alone (open symbols). The average traces of 3 independent experiments are shown. (b) Dihydroceramide inhibited the ceramide-induced permeabilization in liposomes in a dose-dependent manner. The fluorescence increase following the addition of 30 nmol ceramide mixed with the values of DHC_2 shown was normalized by that induced by 30 nmol ceramide alone. The results represent the mean of 3 independent experiments ± S.D. The statistical significance between 2 adjacent points is determined with *, $P \leq 0.05$ and **, $P \leq 0.01$.

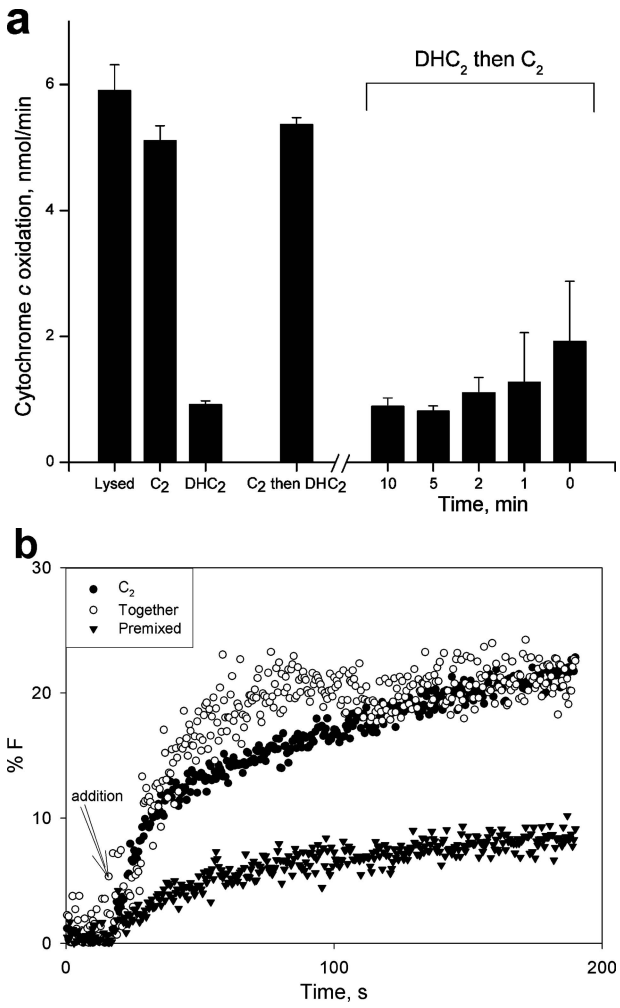


reduced ceramide channel formation in a dose-dependent fashion (Figure 4b).

The sequence of addition of ceramide and dihydroceramide is important for the inhibition

When mitochondria were pretreated with 15 nmoles of ceramide alone to form ceramide channels and then exposed to 15 nmoles of dihydroceramide, the latter did not reduce the rate of oxidation of exogenous cytochrome *c*. Therefore, during the time allowed, dihydroceramide was not able to disassemble the channel once it was formed (Figure 5a). However pre-incubation of mitochondria with 15 nmoles of dihydroceramide for the indicated times followed by addition of 15 nmoles of ceramide did inhibit ceramide

Figure 5 Dihydroceramide inhibits the formation of ceramide channels and does not induce channel disassembly. (a) The initial rate of cytochrome *c* oxidation by mitochondria following various treatments: hypotonic shock (lysed); 15 nmol C_2 for 15 min. (C_2); 15 nmol DHC_2 for 15 min. (DHC_2); preincubation with 15 nmoles C_2 for 10 min followed by 15 nmoles DHC_2 for 5 min (C_2 then DHC_2); preincubation with 15 nmoles DHC_2 for the indicated time followed by 15 nmoles C_2 for the remainder of 15 min (DHC_2 then C_2). (b) The premixing of 29 nmol C_2 with 22 nmol DHC_2 reduced the permeabilization of liposomes by ceramide. However, the addition of 29 nmol C_2 and 29 nmol DHC_2 separately but simultaneously (together) had almost no effect compared to the same amount of C_2 alone. Both traces are the average of 3 experiments each.



channel formation (Figure 5a). This indicates that dihydroceramide interferes with the initiation or nucleation of ceramide channels rather than disassembling them. Figure 5b shows that premixing 29 nmoles of ceramide and 22 nmoles of dihydroceramide and adding them together to the liposomes inhibits the release of CF, whereas if they are added simultaneously but separately there is almost no effect. This is consistent with the notion that sparingly soluble lipophilic molecules form aggregates that are fairly stable.[29] Adding ceramide premixed with dihydroceramide leads to aggregates containing both molecules. When these aggregates

fuse with liposomes, the propensity of ceramide to form channels is undermined by the presence of dihydroceramide. When ceramide and dihydroceramide are added at the same time but using separate pipettes, the tendency of forming mixed aggregates is now much less and the aggregates of ceramide that fuse with membranes can form channels. The path dependence of the observations clearly demonstrates the relatively slow rates of interaction between these components. It also rules out the possibility that dihydroceramide micelles might interfere with channel formation by adsorbing ceramide molecules and thus depleting the available ceramide. Indeed pre-incubating liposomes with dihydroceramide for up to 5 min prior to ceramide addition did not change the rate of ceramide channel formation (data not shown). The dihydroceramide micelles are there but ceramide channels form anyway. These results indicate that the dihydroceramide inhibition phenomenon is probably not due to its ability to form micelles that might interfere non-specifically but rather due to direct interference with ceramide nucleation and channel formation.

Discussion

Apoptosis occurs normally via two distinct pathways, extrinsic and intrinsic. Apoptosis can be initiated from signals coming from outside the cell or, alternatively, the signal can originate within the cell. The permeablization of the mitochondrial outer membrane is believed to be the first committed step in apoptosis since cytochrome *c* and other intermembrane space proteins are released into the cytosol. The starting phase of the intrinsic pathway of apoptosis is well characterized; however, the cause of the permeability of the outer membrane is still debated. The mechanism by which mitochondria release cytochrome *c* is still under investigation and a variety of processes have been proposed.[30] In our model, ceramide-based water-filled pathways, or channels, in the mitochondrial outer membrane mediate the release of intermembrane space proteins and initiate apoptosis.[23,31]

An aqueous pathway through the membrane is both necessary and sufficient to allow the release of cytochrome *c* from mitochondria. Cytochrome *c* is confined between the mitochondrial outer and inner membranes (the intermembrane lumen). Some is in solution and the rest is attached to the outer surface of the inner membrane.[32,33] As a highly charged (+7) small (13 kDa) globular protein, cytochrome *c* is not able to cross membranes spontaneously. The two pathways across the outer membrane, VDAC and TOM, are either too small or too highly regulated to facilitate the translocation of cytochrome *c*. Ceramides (both long chain and short chain) have been shown to form large stable channels in planar membranes.[31] C_{16}-ceramide (and other similar synthetic molecules) were shown to form chloride-permeant pores in liposomes.[34] Moreover, Montes *et al.*[35] showed that ceramide induced permeability in vesicles regardless of the method of delivery (*in situ* production by the hydrolysis of sphingomyelin by sphingomyelinase or by the addition of ceramide from an organic solvent). In addition, molecular dynamics simulations of ceramide channels provide some evidence that such channels can be legitimate, stable biological structures (Anishkin *et al.*, personal communication).

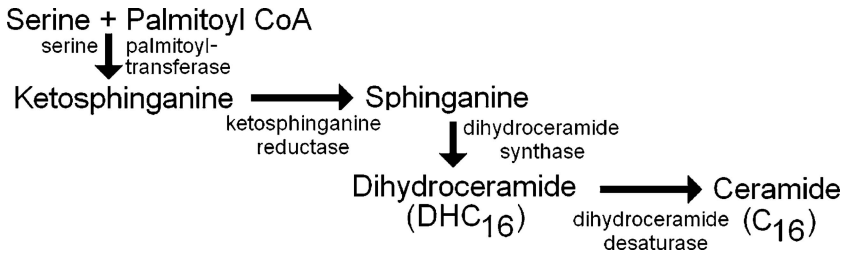
The production of reactive oxygen species (ROS) by ceramide is well documented;[16,36,37] however, under our circumstances such a production should not take place. The mitochondria are uncoupled by 2,4-dinitrophenol and hence the protonmotive force is dissipated. Moreover, the mitochondria were not supplied with substrates for the respiratory complexes and there is no glutathione in the medium. Under these conditions, ROS production should be minimal if present. In the cell ceramide can produce ROS and this may influence the results reported here. However, the results reported here do not depend on ROS production because we observed corresponding effects in liposomes. It is evident that ceramide has multiple roles in the cell and a number of these contribute to the proapoptotic action of ceramide.

Ceramide metabolism is highly regulated[1] in a cell and ceramide and its metabolites are kept at a steady state level. When the death signal arrives, ceramide production is increased by *de novo* synthesis and/or by sphingomyelin hydrolysis (for review, see[38]). One of the products of the *de novo* synthesis is dihydroceramide which has been shown to be inactive both biologically (does not cause apoptosis[24,25]) and physically (does not form channels in planar membranes,[15] mitochondria[23] or liposomes).

During *de novo* ceramide synthesis,[39] serine condenses with palmitoyl CoA to form ketosphinganine which is reduced to sphinganine. Dihydroceramide synthase then produces dihydroceramide from sphinganine and dihydroceramide desaturase eventually produces ceramide from dihydroceramide (Figure 6).

Dihydroceramide inhibits ceramide channel formation both in mitochondria and in liposomes. This attribute of dihydroceramide may be essential in regulating the effects of ceramide in cells. In order to permeabilize mitochondria to start the apoptotic pathway, the synthesized ceramide has to overcome the inhibition of channel formation by dihydroceramide. A threshold ratio of ceramide to dihydroceramide may be a plausible way to think of the initiation of ceramide-induced apoptosis. Presumably, the formation of ceramide from *de novo* synthesis leads to the decline in dihydroceramide concentration and hence enhances the ceramide effects on mitochondria. Possibly, the synthesis of ceramide by sphingomyelin is less efficient in channel formation because dihydroceramide concentration is not diminished.[40] Moreover, sphingomyelin and sphingomyelinase are mainly present in the plasma membrane. However, sphingomyelinase added to mitochondria will generate ceramide in mitochondria but this ceramide accumulation was not able to release cytochrome *c*.[41] In addition there may be a carrier protein like CERT that shuttles ceramide to mitochondria in the same way it does from ER to Golgi and plasma

Figure 6 A scheme of *de novo* ceramide synthesis pathway.



membrane.[42] Nevertheless, it is quicker and more efficient to produce ceramide directly in mitochondria by the *de novo* pathway in response to a death signal, if the intended target is to permeabilize mitochondria through ceramide channels. The *de novo* pathway has been shown to be the one by which ceramide is produced in various cell types and as a result of different stimuli (for review, see[43]). Furthermore, inhibition of the *de novo* enzymes prevented alveolar cell apoptosis.[44] When treating cells with tumor necrosis factor, isolated mitochondria showed a 2-3-fold increase in ceramide levels. This increase was not due to the hydrolysis of sphingomyelin but due to *de novo* synthesis.[36] Cannabinoids, the active ingredients of marijuana, induced an acute ceramide synthesis via sphingomyelin hydrolysis, which mediated the regulation of metabolic functions; and a sustained generation of ceramide via the *de novo* pathway. This production leads to apoptosis.[45] In addition, ceramide levels increased specifically from the *de novo* synthesis after treating cells with N-(4-hydroxyphenyl) retinamide, an event that lead to apoptosis.[46] It is clear that the *de novo* pathway participates more in ceramide production that induces apoptosis. The dihydroceramide inhibition of ceramide channel formation may be another indication of the importance of the *de novo* pathway in the regulation of apoptosis.

Conclusion

Ceramide has been shown before to form channels in planar phospholipid membranes[15] and in the outer membrane of mitochondria.[23] These channels allow proteins to cross membranes and are probably large enough to allow the known pro-apoptotic proteins to be released from mitochondria. Here, we demonstrate that ceramides (both long chain and short chain) are able to form channels in liposomes in a dose-dependent manner. Furthermore, we discovered that dihydroceramide, generally reported to be an inactive precursor, inhibits the formation of ceramide channels at a molar ratio indicative of its interfering with ceramide nucleation of channel formation. Thus dihydroceramide is probably acting as an inhibitor rather than just being unable to form channels[23] or to cause apoptosis.[24,25] We believe that we have uncovered a regulatory system, not based on enzymatic activity, that could have a strong influence on the initiation of the apoptotic process.

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