ABSTRACT

Title of Dissertation: CERAMIDE METABOLISM AND TRANSPORT:
IMPLICATIONS ON THE INITIATION OF APOPTOSIS

Johnny Stiban, Doctor of Philosophy, 2006

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Apoptosis is a process by which unwanted cells are eliminated in a controlled manner. Early in apoptosis, ceramide levels rise and the mitochondrial outer membrane becomes permeable to proteins. The permeability of the outer membrane is attributed to the self-assembly of ceramide in form of channels. In the only direct structural study, to date, ceramide channels were visualized in liposomes using transmission electron microscopy. Those channels were of various sizes, averaging 10 nm in diameter. In concert, using electrophysiological techniques, the estimated diameter of ceramide channels was also around 10 nm. These channels are large enough to release all the pro-apoptotic intermembrane space proteins to initiate apoptosis.

Dihydroceramide desaturase converts the inactive precursor, dihydroceramide to ceramide. Both long and short chain dihydroceramides inhibit ceramide channel formation in mitochondria. The inhibition is strong as one tenth as much

dihydroceramide inhibited the outer membrane permeabilization by 95% (C_2) and 51% (C_{16}). Other mitochondrial components are not required for such inhibition as comparable amounts prevented the permeabilization of liposomes. Hence, the apoptogenic activity of ceramide may depend on the ceramide to dihydroceramide ratio perhaps resulting in a more abrupt transition from the normal to the apoptotic state.

The location of the desaturase is the endoplasmic reticulum (ER). Only minimal activity was measured in mitochondria. However, newly synthesized ceramide from ^{14}C-C_8-dihydroceramide or ^{3}H-sphingosine (in the ER) can transfer rapidly to mitochondria (40 % in 10 min) and permeabilize them to cytochrome *c* and adenylate kinase. The transfer of sphingolipids is bidirectional and non-specific. The transfer mechanism is consistent with direct membrane contact, since reducing the organellar concentrations by half resulted in a four-fold reduction of the transfer rate. Thus this ceramide exchange obviates the need for a complete ceramide *de novo* pathway in mitochondria in order for cells to use ceramide to activate mitochondria-mediated apoptosis.

These results demonstrate the ability of ceramide to form large channels capable of releasing proteins from mitochondria. Ceramide can rapidly reach mitochondria and there are mechanisms to control the propensity for ceramide channel formation. Clearly ceramide channels play a central role in the decision to undergo apoptosis.

CERAMIDE METABOLISM AND TRANSPORT: IMPLICATIONS ON THE INITIATION OF APOPTOSIS

by

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PREFACE

This is my thesis in which I present a synopsis of my research on the metabolism and transport of a vital lipid molecule, ceramide. I joined Dr. Marco Colombini's lab at the University of Maryland, College Park about 4 years ago and I embarked on studying ceramide channel activities in planar phospholipid membranes. For about two years, I have tried to find molecules that either enhance or destabilize ceramide's ability to form channels in a completely biophysical study. I discovered that trehalose and, to a lesser extent, sucrose were able to disrupt channel formation and they might be possible inhibitors of this process. I also developed a method to measure the channel formation activity in unilamellar liposomes. Using this method, and in collaboration with Prolexys, a chemical company in Salt Lake City, Utah, we are planning to scan a variety of compounds to see their effects on channel formation. This could potentially help future studies on apoptosis.

Although I did enjoy the electrophysiological studies, I wanted to go back to my original passion, biochemistry. Following an excellent advice from Dr. Jeffery Davis, I tested the ability of the inactive precursor, dihydroceramide, to influence the channel formation activity of ceramide. Amazingly, not only did dihydroceramide prove to be inactive, but inhibitory as well. I showed that in liposomes and in isolated mitochondria, dihydroceramide is able to significantly inhibit ceramide channel formation. This was presented in my first paper (Stiban *et al.*, 2006).

After the first paper, I realized that it is important to localize the enzyme that is responsible for the conversion of dihydroceramide to ceramide, dihydroceramide

desaturase. Studies have shown that this enzyme is a resident of the endoplasmic reticulum. I wanted to test whether it is also found in mitochondria. Indeed, I was not able to find significant enzyme activity there. However, when both substrates of this enzyme were mixed with mitochondria, permeabilization of the outer membrane to cytochrome *c* occurred, similar to the effects of adding ceramide. This was surprising. Nevertheless, I found that ceramide and dihydroceramide added to ER membranes transport rapidly to mitochondria. *In vivo*, this is important because of the close proximity of these two membranes. This work was summarized in my second first-author paper submitted for publication in *Biochimica et Biophysica Acta – Biomombranes*.

I also tried to develop a technique to visualize those channels in liposomes by electron microscopy. I wanted to use Osmium tetroxide as a fixative agent that adds to double bonds (in ceramide) and stains them. I started the work but then I focused on my previous projects. In the meantime, Ms. Sarah O'Connell continued the EM work and was able to get a formula that worked well. After Ms. O'Connell left the lab, I took over and finished the project of visualizing these interesting structures. This work will be submitted soon.

I have six chapters in this dissertation. In the first chapter, I introduce the general background of apoptosis; the sphingolipid ceramide and its metabolism. In chapter two, I detail the methods used to obtain my results. Chapters three, four and five are distinct manuscripts that summarize my research experience in Dr. Colombini's lab. In chapter three, I address the issue of dihydroceramide inhibition of ceramide channels. The next chapter characterizes the metabolism and transport of ceramide between neighboring

membranes; and chapter five introduces the imaging of ceramide channels. In the final chapter, I summarize and discuss my results shedding some light on future research directions.

After the good experience and unconditional support from Dr. Colombini who never ceased to amaze me not only with scientific prowess but with his creativity and craftsmanship, I am glad that my work has finally come to fruition.

DEDICATION

I would like to dedicate this thesis to my parents, Pierre and Lucy Stiban, as well as my grandmother Georgette and my late grandfather Anton for their understanding and full support. I would also like to thank my extended family in Palestine and the U.S. as well as my high school chemistry and biology teacher, Mr. Elias Katan, for getting me into science. All the hard work invested in this thesis came after a long and steady backing from my brothers, Suheil and Fadi; my cousins Samer, Daleen, Mira and Dima; my best friends Hamzeh Yassin, Rami Bahbah, Baha Sharaf and especially my beloved fiancée, Razan. Thank you all, I love you dearly.

ACKNOWLEDGMENTS

After 3 beautiful years as an undergraduate chemistry student at Birzeit University in Palestine, I decided to transfer to the University of Maryland to get my undergraduate and graduate degrees in biochemistry and biology, respectively. I was privileged to be mentored by Dr. Marco Colombini all these years. In his lab, not only did I find the environment friendly to work effectively, but also I made friends for life. Dr. Colombini aided me in every aspect that he was capable of to make me realize the beauty of science and the significance of research. This thesis could not have been possible without his generous encouragement and unrestricted assistance. Becoming a budding scientist with a hopefully rewarding career, I like to thank the father figure and great friend in Dr. Marco Colombini.

I also thank Drs. Elizabeth Quinlan, Hey-Kyoung Lee, Jeffery Davis and Sergei Sukharev for serving as my committee members. Their fruitful discussions and suggestions given to me impacted my research immensely and guided me in the right direction.

The members and ex-members of my lab, particularly Alexander Komarov, Wenzhi Tan and Suzanne Hudak, were true friends and outstanding coworkers. I thank them for that. I also thank Dr. Leah Siskind for all the help and support given to me during my tenure.

Last but not least, I would like to thank all the undergraduate students who worked with me on my projects, Ms. Laura Caputo, Mr. Daniel Fistere Jr., and Ms. Selam Wubu. I also extend my sincere gratitude to Lois Reid for her fantastic logistic support. I thank NIH fellowship (NS42025) for financial support.

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CHAPTER 1

GENERAL INTRODUCTION

Programmed Cell Death (Apoptosis)

Programmed cell death, or apoptosis, is the mechanism by which cells repackage and feed themselves to neighboring cells when they receive death signals. There are two pathways that trigger apoptosis, extrinsic and intrinsic. Apoptosis can be initiated from signals coming from outside the cell, for instance, a killer lymphocyte which has Fas ligand will bind to Fas receptors on the target cell. This generates a set of intracellular signal transduction events involving the activation of caspase-8 that eventually leads to the repackaging of the cell. In addition, the removal of growth factors induces DNA damage, mitochondrial permeabilization and ultimately apoptosis. Alternatively, the signal can originate within the cell. The permeablization of the mitochondrial outer membrane releases cytochrome *c* and other intermembrane space proteins to the cytosol. Such a release causes a series of events involving the activation of caspase-9 and the initiation of the caspase cascade that leads to the death of the cell. Consequently, solute and protein efflux from the intermembrane space of mitochondria is significant for apoptosis. Even though there are various pathways proposed for this release, this dissertation will focus on the evidence for a ceramide-based large mitochondrial outer membrane channel.

Originally, morphological analysis was used to determine whether the cell is dying from apoptosis or from necrosis. Necrotic cells release proteins and osmolytes to the environment. This causes inflammatory reactions (Kerr *et al.*, 1972). During apoptosis, such reactions do not take place (Gulbins *et al.*, 2000). The modern characterization of apoptosis can be summarized as follows: The chromatin of an

apoptotic cell condenses, the nucleus fragments and phosphatidylserine flips from the inner to the outer leaflet of the bilayer (Jacobson *et al.*, 1993; Jacobson *et al.*, 1994). The cell loses its attachment to other cells (Wyllie *et al.*, 1980; Ruoslahti and Reed, 1994), the plasma membrane becomes disorganized and blebbed and the cell shrinks (Jacobson *et al.*, 1993; Jacobson *et al.*, 1994). Metabolic energy is needed to synchronize and coordinate these events. The apoptotic cell is transformed into membrane-bound entities called the apoptotic bodies (Kerr *et al.*, 1972), which are identified and endocytosed by phagocytes (Hart *et al.*, 1996) avoiding any inflammation that would be caused by the release of certain proteins and metabolites to the environment.

Causes of Apoptosis

As aforementioned, various factors are known to affect the initiation of apoptosis. DNA damage, for instance, induces apoptosis. Stress and disruption of calcium homeostasis also cause programmed cell death. The inhibition of metabolite exchange between the cytosol and mitochondria, by VDAC (voltage-dependent anion channel) closure as a result of treatment with phosphorothioate nucleotides is also linked to inducing apoptosis (Lai *et al.*, 2006; Tan *et al.*, 2006). Cell proliferation and cell death are tightly regulated by the cell cycle enzymes as well as by Bcl-2 family proteins, NAIP (neuronal apoptosis inhibitor protein), TNF (tumor necrosis factor), etc. The mechanisms by which each protein is involved in preventing or inducing apoptosis are diverse and not very well characterized; however, it seems that the interactions of such proteins with mitochondria lead to the release of mitochondrial intermembrane space proteins such as, among others, cytochrome *c*, SMAC/Diablo and apoptosis-inducing factor (AIF).

Once cytochrome *c* is released from the intermembrane space into the cytosol, it can bind to the apoptotic protease-activating factor 1 (APAF-1). The immediate effect of this binding is the activation of caspase-9 by cleaving procaspase-9. Caspase-9 then cleaves and hence activates caspase-3, which is the main effector of the caspase cascade in the intrinsic pathway (Liu *et al.*, 1996; Yang *et al.*, 1997). Through the caspase cascade events, other enzymes, such as endonucleases, transglutaminases and DNases get activated and start degrading the cell, each within its capacity. Moreover, other caspases are activated by the cascade and these are important in activating other cytosolic proteins that eventually facilitate the blebbing of the plasma membrane and the rest of the morphological changes associated with apoptosis. Even though studies have showed that the cell that is committed to die has to pass through the irreversible step of cytochrome *c* release from mitochondria (Susin *et al.*, 2000), cells must have a functional APAF-1 in order to die when cytochrome *c* is released (Sanchis *et al.*, 2003). Fig. 1.1 demonstrates the many pathways that lead to apoptosis.

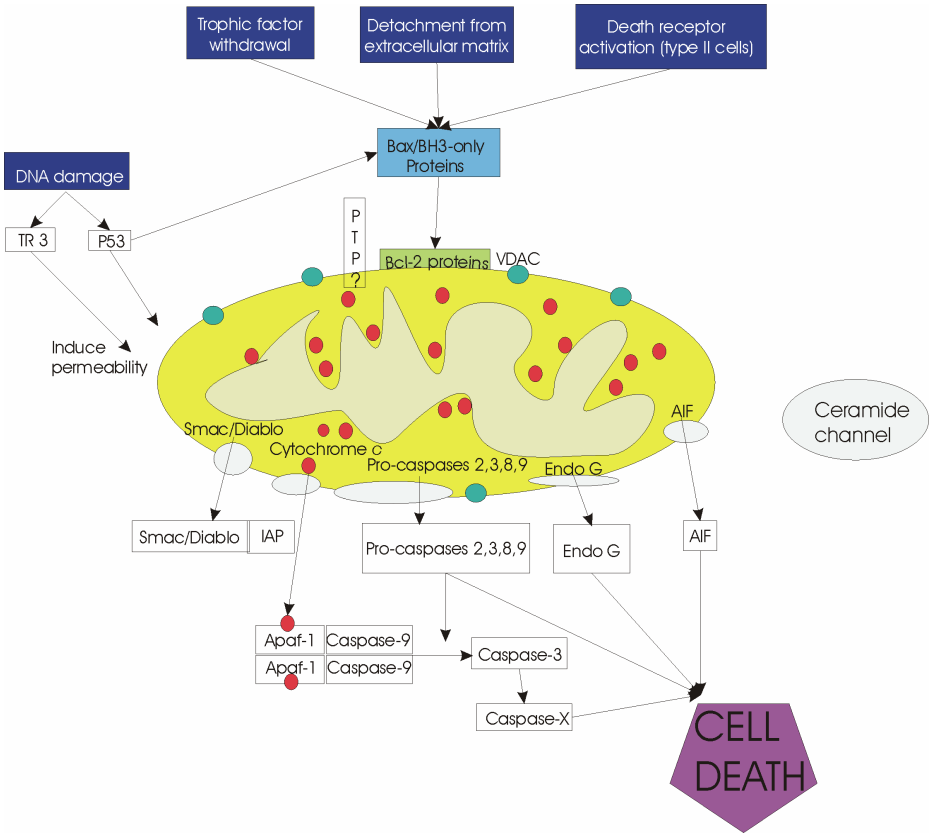


FIG. 1.1. General apoptosis pathways (information reproduced and modified from Parone *et al.*, 2002).

The Release of Pro-apoptotic Proteins

The execution phase of intrinsic apoptosis begins with the egress of intermembrane space proteins into the cytosol. The mechanism by which mitochondria release cytochrome *c* and other pro-apoptotic proteins is still under investigation and a variety of processes have been proposed. This thesis investigated the hypothesis that ceramide-based water-filled pathways, or channels, in the mitochondrial outer membrane mediate the release of intermembrane space proteins and initiate apoptosis.

An aqueous pathway through the membrane is both necessary and sufficient to allow the release of cytochrome *c* from mitochondria. Even though cytochrome *c* is a component of the mitochondrial electron transport chain, it is not an integral part of an

enzyme complex residing in the mitochondrial inner membrane. Due to its solubility, it transports electrons from the ubiquinone:cytochrome *c* oxidoreductase (complex III) to cytochrome oxidase (complex IV), which reduces molecular oxygen to water (Tyler, 1992; Nelson and Cox, 2000). Cytochrome *c* is confined between the mitochondrial outer and inner membranes (the intermembrane lumen). Mostly, it is soluble but some is attached to the outer surface of the inner membrane (Tyler, 1992; Nelson and Cox, 2000). As a highly charged (+7) small (13 kDa) globular protein, cytochrome *c* should not be able to cross membranes spontaneously. The two pathways across the outer membrane, VDAC and TOM, are either too small or too highly regulated to allow cytochrome *c* release. The mechanism by which cytochrome *c* leaks out of the mitochondrial intermembrane space is still unclear. Nevertheless, there are many theories in the field today to explain this phenomenon.

A variety of mechanisms have been proposed for the release of cytochrome *c* (reviewed in Siskind, 2005): the opening of the permeability transition pore (PTP) (Crompton, 1999), the forming of lipidic pores induced by Bax (Basañez *et al.*, 1999), the oligomerization of Bax monomers to achieve channel activity (Antonsson *et al.*, 2000, 2001; Saito *et al.*, 2000), the opening of the mitochondrial apoptosis-induced channels (MAC) (Pavlov *et al.*, 2001), the interactions of BH3/Bax/cardiolipin (Kuwana *et al.*, 2002), the interactions between Bax and ceramide (Lee *et al.*, 2002; Belaud-Rotureau *et al.*, 2000; Pastorino *et al.*, 1999), and ceramide channels (Siskind and Colombini, 2000; Siskind *et al.*, 2002; 2003). There are many conflicting reports that the PTP complex includes VDAC, cyclophilin D and ANT (adenine nucleotide translocator), yet knockout experiments using mice lacking these proteins are also capable of undergoing apoptosis (Krauskopf *et al.*, 2006; Kokoszka *et al.*, 2004; Halestrap, 2004; Forte and Bernardi,

2005). Hence, the PTP is not a well characterized channel as it was once thought to be. Moreover, cardiolipin is a molecule known to destabilize membranes; therefore any induction of apoptosis by its action on mitochondria can be simply by disintegrating the outer membrane in a detergent-like manner. Furthermore, MAC channels were reported to be involved in later stages of apoptosis rather than initiating the process (Guihard *et al.*, 2004) thus cannot be the pathway for protein release to start the execution phase.

No direct structural evidence for any of the aforementioned pathways has been presented thus far. However, results presented in this thesis (detailed in chapter 5) show the structure of ceramide channels, negatively stained in liposomes. The size of these channels is consistent with a pore that allows large (around 115 kDa) proteins to pass through. Hence, to date, ceramide channels provide the only ultrastructural characterization for a pore capable of initiating apoptosis.

Ceramide

More than a century ago, in 1884, German physician-biochemist Johann Thudichum discovered a class of lipids whose properties were so enigmatic that he named them sphingolipids, after the mysterious Sphinx (Thudichum, 1884). When they were discovered, the role of sphingolipids was not understood, hence the name. Nonetheless, in this past decade, sphingolipid research has prospered and many roles have been assigned to these molecules.

There are many members of the sphingolipid family. The main sphingolipid, ceramide, (Fig. 1.2) is a molecule involved in intracellular signaling. C_{16}-ceramide, or *N*-palmitoyl-$_D$-erythro-sphingosine, is one of the forms of naturally occurring ceramides. Ceramides differ in their fatty acid groups. They can have either long or short tails.

Naturally occurring ceramides have long tails. In this thesis, short-chained C_2-ceramide, or *N*-acetyl-$_D$-erythro-sphingosine, medium-chained C_8-ceramide, or *N*-octanoyl-$_D$-erythro-sphingosine, and long-chained C_{16}-ceramide, or *N*-palmitoyl-$_D$-erythro-sphingosine were used.

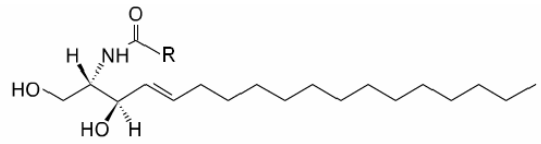


FIG. 1.2. The structure of ceramide.
R = $–(CH_2)_{14}CH_3$ → *N*-Palmitoyl-D-*erythro*-sphingosine (C_{16}-ceramide)

Ceramide is also considered a second messenger that gets activated by extracellular messages. For instance, cannabinoids, the active ingredients in marijuana, bind to a $G_{I/o}$-protein coupled receptor inducing the inhibition of adenylyl cyclase, modulation of ion channels, activation of extracellular signal-regulated kinases as well as the synthesis of ceramide. Ceramide then mediates cannabinoid-induced apoptosis (Guzman *et al.*, 2001). The method by which it does that is still unclear, but the increase in the ceramide concentration as a result of *de novo* synthesis or sphingomyelin hydrolysis might indicate that it favors apoptosis via channel formation.

Ceramide Metabolism

The formation of ceramide channels depends on the steady-state level of ceramide in the membrane. Thus ceramide metabolism is involved in influencing ceramide channel formation. Ceramides are sphingosine-based lipids that have two pathways by which they can be synthesized. A *de novo* synthesis from sphingosine and acyl-CoA is one pathway (Fig. 1.3). The amino acid serine adds on a fatty acyl CoA in a condensation reaction by the serine palmitoyl transferase (SPT). This enzyme is a heterodimer (53 and 63 kDa

subunits) that is bound to the ER membrane (Hanada, 2003). The product (3-ketosphinganine) is later reduced by the NAD(P)H ketosphinganine reductase in a rapid reaction resulting in sphinganine. Dihydroceramide synthase (an ER enzyme) acylates sphinganine into dihydroceramide (Lahiri and Futerman, 2005). The latter is desaturated by dihydroceramide desaturase into ceramide.

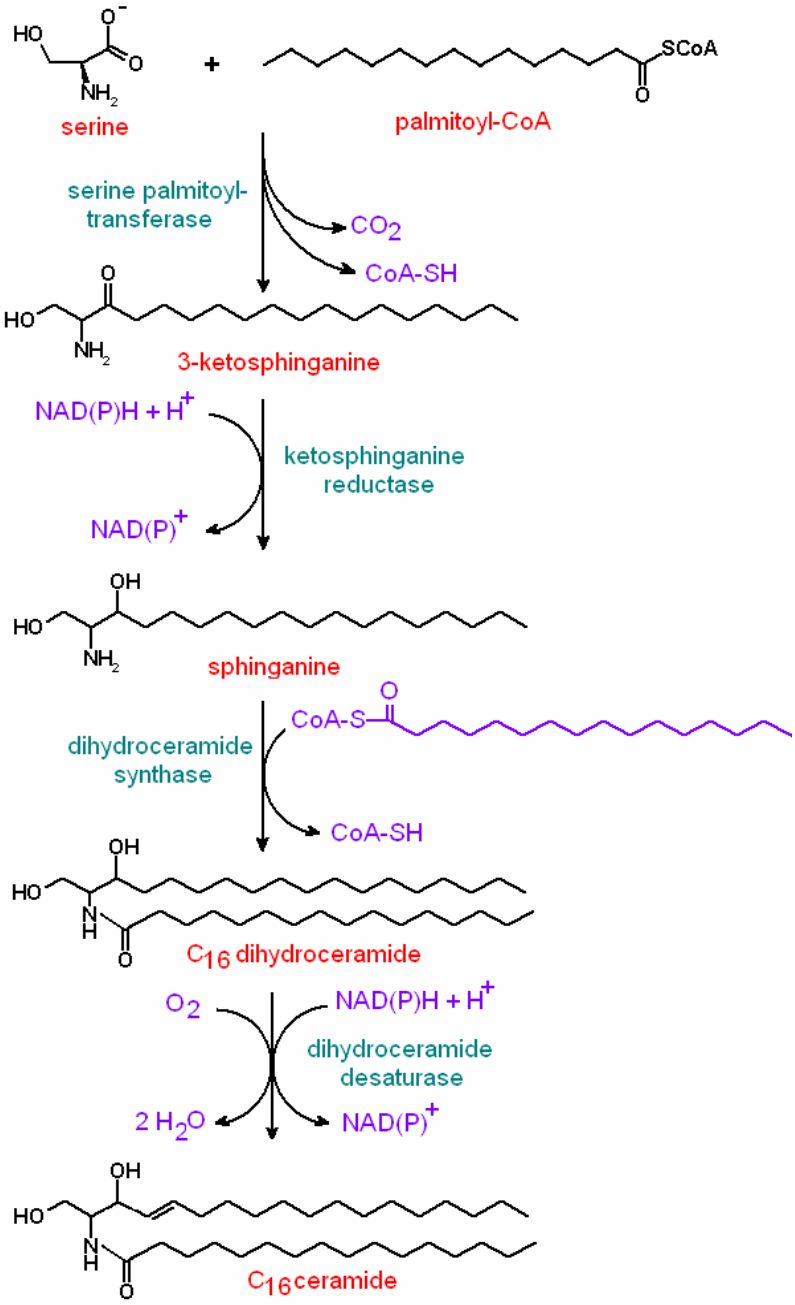


FIG. 1.3. The *de novo* ceramide synthesis pathway.

Another way of producing ceramide in the cell uses sphingomylinase (SMase) to hydrolyze sphingomyelin (Fig. 1.4). There are five different isoforms of SMase. Three of them are acidic (requiring an acid environment to function) and the other two are neutral (Marchesini and Hannun, 2004). Neutral sphingomyelinases are cloned and localized to the endoplasmic reticulum (ER), Golgi apparatus or plasma membrane (Clarke *et al.*, 2006). The localization of ceramide metabolism enzymes has been shown to be primarily in the ER and Golgi even though some reports find some enzyme isoforms in mitochondria (El Bawab *et al.*, 2000; Bionda *et al.*, 2004).

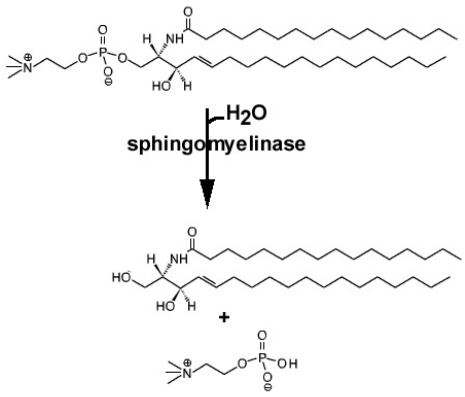


FIG. 1.4. Sphingomyelin hydrolysis pathway.

Although mitochondrial ceramide machinery might be less efficient than that in the ER, ceramide levels increase in mitochondria prior to apoptosis. Thus, the trafficking of ceramide from its production site to its functional site is important. The intercellular trafficking of ceramide is discussed in details in chapter 4.

Lipid Transport

In the cell, lipids have to move from one compartment to another. Most of the cell's lipids are made in the ER (Bishop and Bell, 1988) before being transported to the

Golgi complex where they are sorted and distributed to various destinations. There are three main mechanisms of lipid movement. First, lipids can be transported via vesicular trafficking. Vesicles containing the desired lipid form from the donor membrane, with the help of necessary proteins, then they bud off and move to the acceptor membrane where they dock and fuse releasing the lipid in that membrane. Second, since lipids are hydrophobic, they can be transported through the aqueous environment of the cell using protein shuttles. A cytosolic, soluble protein extracts the needed lipid molecule from the donor membrane and transports it to the acceptor membrane. The third mechanism of lipid trafficking occurs by direct membrane contact. Some lipids can flip from one membrane to a neighboring membrane quickly. Because of the close proximity of the ER to mitochondria, a unique system of interorganellar lipid transport system is found for the transport of some phospholipids.

The close apposition between ER and mitochondria allows for membrane contact and lipid exchange. For instance, even though most of the enzymes responsible for phospholipid synthesis are found in the ER, mitochondria also carry some enzymes such as phosphatidylserine decarboxylase (Dennis and Kennedy, 1972). This enzyme catalyzes the reaction of decarboxylating phosphatidylserine (PS) to make phosphatidylethanolamine (PE) (Dennis and Kennedy, 1972; Voelker, 1989; Vance, 1991). In order to make PE, PS (formed in the ER) needs to rapidly move to mitochondria, get decarboxylated and move back to the ER as PE (Vance, 1990; Vance, 1991; Shiao *et al.*, 1995; Gaigg *et al.*, 1995; Vance and Shiao, 1996). The transport of PS and PE to and from the ER and mitochondria provides the evidence of membrane contact trafficking (Daum and Vance, 1997; Voelker, 2005) rather than vesicular transport (Voelker, 1989; Vance *et al.*, 1991).

Whereas the import of phospholipids from the ER into mitochondria has been studied in details (reviewed in Daum and Vance, 1997) the exchange of sphingolipids between these two organelles has not. Sphingolipids use vesicular trafficking to move from ER to Golgi (van Meer and Lisman, 2002; Futerman and Riezman, 2005). The movement of sphingolipids between ER and mitochondria has yet to be investigated thoroughly, although some mechanisms were proposed (Futerman, 2006). As mentioned above, chapter 4 describes that ceramide transport into mitochondria occurs via direct membrane contact.

Ceramide Channels

Once formed in the mitochondria, ceramide channels induce the execution phase of apoptosis. The addition of ceramide to whole cell cultures induces the release of cytochrome *c* (Zamzami *et al.*, 1995; Castedo *et al.*, 1996; Susin *et al.*, 1997; De Maria *et al.*, 1997). It also stimulates the release in isolated mitochondria (Arora *et al.*, 1997; Di Paola *et al.*, 2000; Ghafourifar *et al.*, 1999). The permeability of the mitochondrial outer membrane to proteins can be enhanced by the incubation with ceramide in a dose- and time-dependent fashion (Siskind *et al.*, 2002). Previous experiments showed the molecular weight cutoff of ceramide channels to be 60 kDa (Siskind *et al.*, 2002). This molecular weight cutoff of the ceramide channel is large enough to allow the passage cytochrome *c* and other small pro-apoptotic intermembrane space proteins during apoptosis. In chapter 5, the cutoff size for protein release from mitochondria (using a different vehicle) was measured to be higher (around 115 kDa), indicating the delivery of ceramide can be important for its ability to permeabilize the outer membrane of mitochondria. Ceramide channels (Fig. 1.5) are proposed to form from columns

composed of six ceramide molecules. Many columns, arranged in an anti-parallel fashion, self-assemble to form cylindrical channels of varying diameters. The insertions of more columns increase the diameter of the channel. The lumen of the channel is bounded by an ice-lattice like structure of hydrogen bonding between the ceramide hydroxyls.

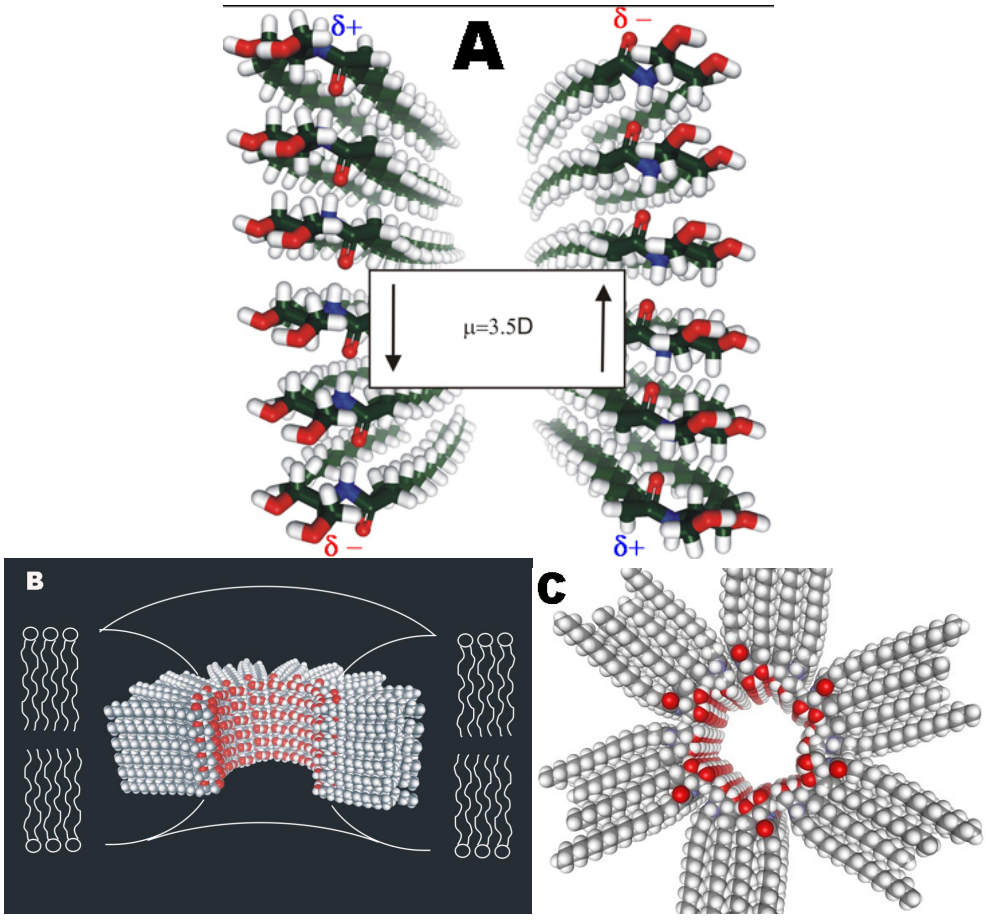


FIG. 1.5. Proposed structures of ceramide channels. A column of about 6 ceramide molecules can be generated by stacking these molecules on top of one another so as to span the hydrophobic part of the membrane. (**A**) A pair of columns is attracted to each other by a dipole moment of 3.5 D. A group of these columns can self-assemble and form a pore with the lumen lined by the hydrogen bonded network of ceramide hydroxyls forming an ice-lattice like structure. The phospholipids tend to bend (**B**) to minimize the contact of the ceramide tails with the aqueous environment. (**C**) A depiction of a ceramide channel. The lumen of the pore is hydrophilic and the lipid tails are arranged in such a way that each pair of columns have the opposite dipole.
(Courtesy of Dr. Marco Colombini)

Even though ceramide was shown to form channels in the mitochondrial outer membrane and in planar phospholipid membranes (Fig. 1.6), it does not form channels in every type of membranes. Ceramide does not induce the permeabilization of the inner membrane of the mitochondria as there were no detectable traces of fumerase activity (Siskind *et al.*, 2002). Moreover, studies on red blood cells showed that the addition of ceramide results in no channel formation in this plasma membrane (Siskind *et al.*, 2006). The specificity of channel formation by ceramide could be due to differences in the lipid compositions of different membranes.

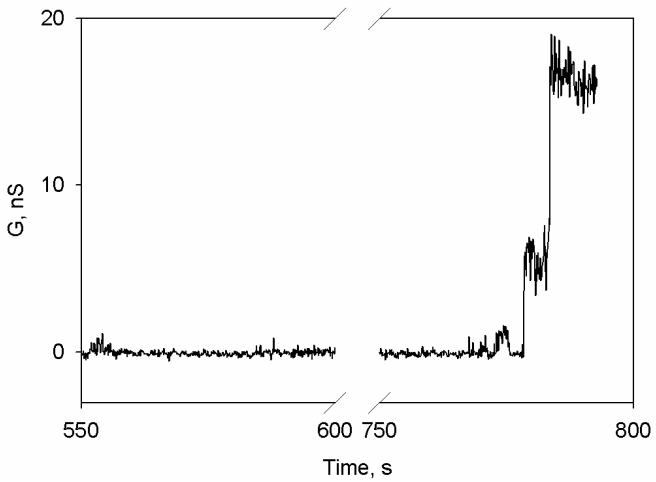


FIG. 1.6. Ceramide channel activity in planar membranes. Ceramide was added at time = 0 seconds.

Significance

Apoptosis is a factor in a wide variety of diseases. Cancer, for example, occurs when cells grow uncontrollably and continue to proliferate. These cells can become resistant to chemotherapy or radiation therapy because their apoptotic mechanism is suppressed. Following an ischemic episode, reperfusion often results in widespread inappropriate apoptosis. This often occurs in heart attacks and strokes. Characterizing and

outlining the initiation mechanism of apoptosis is essential in order to design drugs that might kill infected cells or inhibit killing of necessary cells. This approach may help in the treatment of common diseases such as cancer, stroke, heart disease, neurodegenerative diseases, autoimmune disorders, and viral diseases such as AIDS.

In the past few years, our lab has shown that ceramide is able to form organized channels in a variety of membrane systems. This unique ability of ceramide to self-assemble and permeabilize membranes, especially mitochondrial outer membrane, in an ordered fashion is believed to be critical for the initiation of the process of apoptosis. Hence, depending on the metabolic levels of ceramides in the cell, the cell can stay viable or undergo apoptosis.

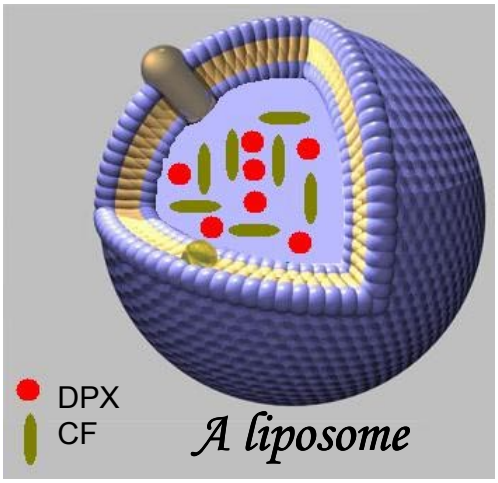
Controlling ceramide channel formation *in vivo* may help the shift from cell viability to cell death, or vice versa. If, for example, one can prevent channel formation in cardiac cells following a heart attack, those cells may not undergo apoptosis and therefore be able to rescue the patient. On the other hand, if one can figure out ways to stabilize these channels (or enhance ceramide production in mitochondria), cancerous cells can be killed. *In vivo*, results presented in chapter 3 indicate that the ratio of ceramide to dihydroceramide may be a toggle switch between cells with permeabilized mitochondria (cells destined to die) and cells with intact mitochondrial outer membrane. The dihydroceramide inhibition of ceramide function is remarkable because it gives insight into a natural process of selecting for cell death or cell viability. Indeed, other molecules, whether physiological or drugs, must be tested for their ability to stabilize or destabilize ceramide channels because of their potential therapeutic effects.

CHAPTER 2

METHODS

Liposome Assays

A method was developed to measure channel formation in liposomes. This assay uses the dequenching of the fluorophore carboxyfluorescein as it leaves the liposomes as a means of detecting its release. The liposomes (Fig. 2.1) are made so that they contain carboxyfluorescein (CF) and quencher *p*-xylene-bis-pyridinium bromide (DPX). When these molecules are entrapped in vesicles, the fluorescence signal is small (because of the quenching). When the liposomes are permeabilized, these molecules are quickly released, hence diluting them in the medium and the fluorescence signal increases.



This drawing was modified from
http://avantilipids.com/liposomes.asp

FIG. 2.1. A depiction of a liposome entrapping CF and DPX molecules.

A soybean polar lipid extract, asolectin, was used because its lipid composition is close to that of the outer membrane of mitochondria. Basically, 2.9 mg asolectin and 0.1 mg cholesterol are mixed together in chloroform. This mixture is then dried under nitrogen to obtain a thin layer of lipids on the wall of the container. Afterwards, the lipids

are desiccated overnight under house vacuum. The next morning, the lipids are hydrated in 1 mL of CDL Buffer (1.5 mM CF, 6 mM DPX, 50 mM NaCl, 10 mM HEPES, 1 mM EDTA pH 7.0). After vortexing the mixture and forming the liposomes, 4 freeze-thaw-sonication steps are performed to thoroughly disperse the lipids. In order to obtain uniform single walled liposomes that are approximately 0.2 μm in diameter, the vesicles are extruded through a 0.2 μm-polycarbonate membrane. To remove the un-trapped fluorescent molecules, gel filtration is used (Sephacryl S-200).

The liposomes are ready to be used after being eluted from the column. The liposomes (100 μL) are diluted into 2 mL of L medium (50 mM NaCl, 10 mM HEPES, 1 mM EDTA pH 7.0). The fluorescence is measured in a spectrofluorometer set at the necessary wavelengths (for CF, the excitation wavelength is 495 nm and the emission wavelength is 520 nm). The liposomes are stable and for at least 2 days, however, most of the experiments are done on the day the liposomes were formed. Fluorescence is measured as a function of time. Ceramide, in different combinations, (please see chapter 3 for details) is added and the fluorescence increase is recorded. In order to assess the total fluorescence in that batch of liposomes, towards the end of the experiment, 150 μL of 5% Triton-X 100 is added to release the CF from all the liposomes. This allows the results to be expressed as a percentage of the fluorescence released by adding ceramide.

Isolation of Rat Liver Organelles

Mitochondria are isolated from male Sprague-Dawley rat liver as described by Parsons *et al.* (1966) as modified by Siskind *et al.* (2002) and Stiban *et al.* (2006). Briefly, a rat (200 to 300 g) is fasted overnight, anesthetized using CO_2 and decapitated. Immediately the liver is removed, rinsed and chopped in ice-cold HB-isolation buffer

containing 210 mM Mannitol, 70 mM sucrose, 10 mM HEPES, 0.1 mM EGTA and 0.05 mg/mL of fatty acid-free Bovine Serum Albumin, pH 7.4. Small pieces of the cut liver are homogenized in HB-buffer using a motorized Teflon-glass Potter homogenizer. The homogenized tissue is then centrifuged at 760 ×g for 10 minutes at 4 ^{o}C in order to pellet cell debris and nuclei. The supernatant, consisting of mitochondria, microsomes and peroxisomes, among other things, is collected and re-spun for 10 minutes at 9000 ×g so that mitochondria can pellet, leaving the microsomes (including ER vesicles) in the supernatant. Multiple low and high-speed steps were used to purify the mitochondria prior to resuspending the mitochondrial pellet in HB lacking BSA. In all the resuspension steps, cut pipette tips were used to prevent sheering of mitochondria and to increase the degree of intactness.

The protein content of these isolated mitochondria can be measured using a method that takes advantage of the differential absorbance of proteins and DNA. Using this rapid spectroscopic method (Clarke, 1976), a 20-fold dilution of stock mitochondria in H-buffer is followed by a 2-fold dilution in 100mM Tris·SO_4, 0.4% SDS, pH 8.0 solution. The absorbance at 2 wavelengths (280 nm and 310 nm) is measured and the concentration of protein (in mg/mL) is given by: $\frac{40 \times (A_{280} - A_{310})}{1.05}$.

ER membranes are separated following the modified procedure of Schulze *et al.* (1999). The supernatant from the first 9000 ×g spin is centrifuged at 105,000 ×g for 1 hour at 4^{o}C. The pellet is washed 3 times prior to resuspension in H-buffer. Another 9000 ×g spin is performed for 10 minutes in order to separate some attached mitochondria in the pellet. The supernatant (ER vesicles) is collected and the protein concentration is determined using the BCA protein assay kit from Pierce (Rockford, IL).

In order to purify the mitochondria further (mainly from the mitochondria-associated ER membrane, or MAM), isolated mitochondria are layered on a 28% self-generating Percoll gradient following the procedure of Holden and Colombini (1993). The Percoll-layered mitochondria are then centrifuged at 39,000 $\times g_{max}$ for 30 minutes collecting the mitochondrial fraction near the bottom of the tube. The collected fraction is diluted in H-buffer and spun at 9000 ×g for 10 minutes to remove the Percoll.

Isolation of Rat Erythrocytes

Mammalian red blood cells (erythrocytes) are a model system for the plasma membrane. They lack intracellular organelles, and and thus are essentially sacks of hemoglobin. In order to isolate erythrocytes, blood from a decapitated rat is collected in a tube pre-filled with an anticoagulation buffer (AB) containing 150 mM NaCl, 4 mM EGTA and 5 mM HEPES, pH 7.4. Red blood cells easily sediment by centrifugation, therefore, the blood sample is spun at 600 ×g for 10 minutes, these cells were collected in the pellet. The cells are washed and resuspended in AB and pelleted 3 times. After the third spin, the hemoglobin was released from the cells by osmotic shock (in double distilled water). The membranes of these lysed cells are collected as the pellet of a 12,000 ×g spin for 10 minutes. After every spin, the membranes were resuspended in double distilled water to effectively wash out hemoglobin. This step is repeated 3 times. Finally, the membranes are resuspended in AB and their protein content is measured by the BCA assay.

Cytochrome Oxidase Accessibility Assays

The permeability of the mitochondrial outer membrane to cytochrome *c* was

measured using the method of Siskind *et al.* (2002). Reduced cytochrome *c* is made by reducing it with ascorbic acid (5.5 mg cytochrome *c* with 2 mg ascorbic acid in 250 µL of 150 mM NaCl, 10 mM HEPES, 1 mM EDTA, pH 7.2). The reduced cytochrome *c* is then separated from the ascorbic acid by running the sample on a gel filtration column. When added to mitochondria the oxidation rate can be a determined by recording the absorbance at 550 nm. This oxidation rate is a measure of the permeability of the outer membrane to cytochrome *c*.

Planar Membrane Technique

Ceramide channels were first discovered (Siskind and Colombini, 2000) using the planar membrane technique (Montal and Mueller, 1972; Schein *et al.*, 1976; Colombini, 1987). I have used this technique sparingly to look at inhibitors and enhancers of ceramide channels and to measure of the size of the channel. The apparatus consists of a Teflon chamber with two compartments (*cis* and *trans*) divided by a Saran partition with a 100 µm hole.

Various lipids can be used to generate these membranes. The lipids used in my research were either 5:5:1 diphytanoylphosphatidylcholine (DPhPC), asolectin (soybean phospholipids polar extract), cholesterol (w/w/w) or 10:1 DPhPC, cholesterol. Lipid monolayers are used to form a bilayer membrane (Fig. 2.2). The current is measured as the voltage across the membrane is clamped (Fig. 2.3). The membrane should not be conductive as monitored by the current recorded as positive and negative voltages are applied. The aqueous solution normally contained 100 mM KCl, 1 mM $MgCl_2$, 5 mM PIPES (pH 7.0). All the experiments were performed at room temperature (about 23^oC).

The side that is kept at ground (normally the far side) is defined as *trans* and the front side is the *cis*-side. The voltage is controlled in the *cis*-side. Ceramide was added to both sides, typically 25 μL of 0.1 mg/mL or 2.5 μL of 1 mg/mL. The ceramide solution was made in DMSO.

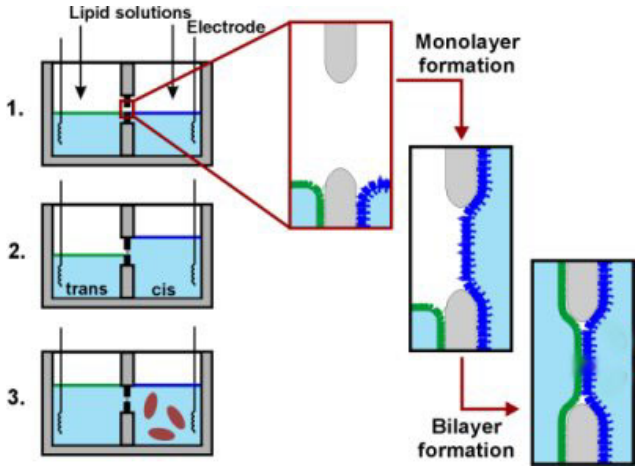


FIG. 2.2. A general scheme of membrane making.

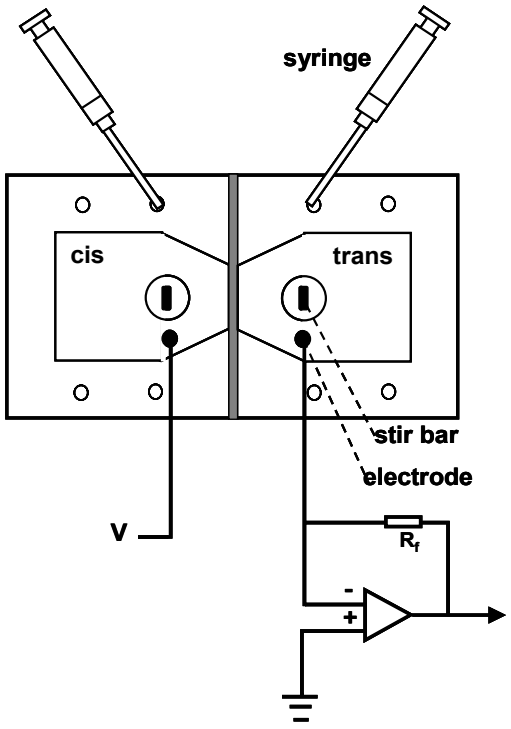


FIG. 2.3. The experimental set-up used for planar membrane measurements. A Teflon partition divides the chamber into two compartments. The lipid bilayer is formed across the hole of 100 μm diameter. The ion flux across the membrane is recorded as a current and the voltage (V) is controlled by a pair of potassium chloride electrodes connected to the ground and amplifier (►), respectively. The amplifier is set in the voltage clamp mode with feedback resistor R_f.

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

Blue Native (BN)-PAGE is a technique used to separate proteins on a gel based on their size but under native conditions (Schägger and von Jagow, 1991; Schägger *et al.*, 1994). It involves incorporating a negatively-charged dye, Coomassie Brilliant Blue G-250 (Fig. 2.4) into the cathode buffer. This dye coats all the proteins in the sample and gives them a total negative charge. The extent of binding of the dye is proportional to the size of the protein, hence, the farther the protein migrates on the gel, the smaller it is. The binding of the dye to the proteins typically does not denature them, unlike SDS-PAGE, and hence the proteins remain in native form (Schägger *et al.*, 1994). Preferably, a

gradient polyacrylamide gel is used. In my experiments, I used 4-16% polyacrylamide pre-made gels from Invitrogen. Following the manufacturers' instructions, the cathode buffer contains 50 mM BisTris, 50 mM Tricine and 0.02% Coomassie G-250, pH 6.8. The anode buffer consists of the same ingredients without the dye. Also, a 4× sample buffer is made. That consists of 50 mM BisTris, 50 mM NaCl, 10% glycerol and 0.001% bromophenol blue, pH 7.2.

The protein sample is mixed with the 4× sample buffer in a 3:1 volume ratio and then loaded on the gel. The electrophoresis was run for 2 hours at room temperature at 150 V. After the run is complete, the gel is destained in 40% CH_3OH, 10% CH_3COOH at first then overnight in 8% CH_3COOH. After the destaining, the bands are stained but further staining with GelCode Blue may be used to enhance the contrast.

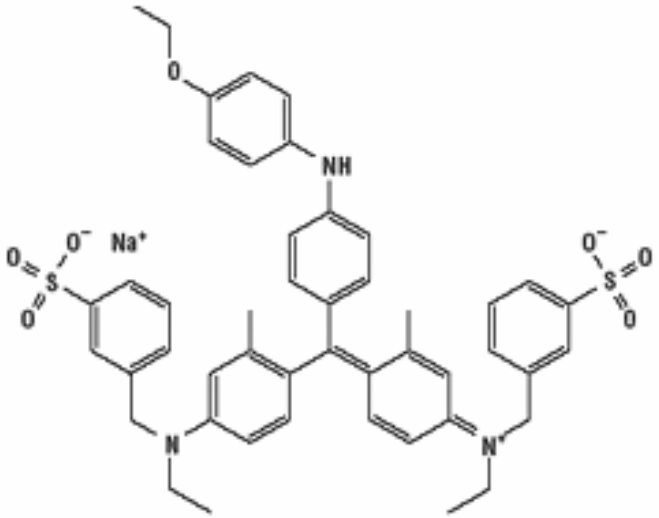


FIG. 2.4. Coomassie Brilliant Blue G-250 molecule.

N.B. Other methods were used and they are detailed in each chapter, where appropriate. In this chapter, I described, in details, methods that were essential for my research as well as methods that were not explained explicitly in their respective chapters.

CHAPTER 3

DIHYDROCERAMIDE HINDERS CERAMIDE CHANNEL FORMATION: IMPLICATIONS ON APOPTOSIS

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Running Title: ***DHC inhibits ceramide channel formation***

KEYWORDS:

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

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.

ABSTRACT

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.

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 Hannun, 1996; Kolesnick and Fuks, 2003; Reynolds *et al.*, 2004) 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 (Grassme *et al.*, 2001A; Grassme *et al.*, 2001B; Cremesti *et al.*, 2001; Miyaji *et al.*, 2005). 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 Gulbins and Kolesnick, 2003). Ceramide administration changed the phosphorylation states of some MAP kinases (Willaime *et al.*, 2001; Kitatani *et al.*, 2001; Shin *et al.*, 2002; Willaime-Morawek *et al.*, 2003; Stoica *et al.*, 2005) 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 (Stoica *et al.*, 2005). In differentiating embryonic stem cells, ceramide is reported to mediate the binding of protein kinase C zeta (PKCζ) to its inhibitor PAR-4 (prostate apoptosis response-4) and this is proposed to be responsible for the induction of apoptosis in these cells (Wang *et al.*, 2005).

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 (Siskind and Colombini, 2000) and in liposomes (this study). The addition of ceramide to whole cell cultures induces the release of cytochrome *c* into the cytosol (Zamzami *et al.*, 1995; Castedo *et al.*, 1996; Susin *et al.*, 1997; De Maria *et al.*, 1997). Ceramide also induces the release of cytochrome *c* in isolated mitochondria (Arora *et al.*, 1997; Ghafourifar *et al.*, 1999; Di Paola *et al.*, 2000). The permeability of the mitochondrial outer membrane to proteins can be enhanced in a dose- and time-dependent fashion by incubation with ceramide (Siskind *et al.*, 2002). This permeability showed a molecular weight cutoff of 60 kDa (Siskind *et al.*, 2002). 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 (Bielawska *et al.*, 1993; Sugiki *et al.*, 2000) nor can it induce the release of cytochrome *c* when added to isolated mitochondria (Siskind *et al.*, 2002) or cell cultures. Dihydroceramide also does not form channels in planar phospholipid membranes (Siskind and Colombini, 2000) 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 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.* (1966) as modified (Siskind *et al.*, 2002). 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, 1 mM TRIS, 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 minutes at 4 ^{o}C. The supernatant was then spun for 10 minutes 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 (Clarke, 1976). In short, 25 μL of the suspension was diluted in 475 μL of H-buffer without BSA. Five hundred 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 $\frac{40 \times (A_{280} - A_{310})}{1.05}$. 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 1mM 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 cm×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 (Fig. 3.1).

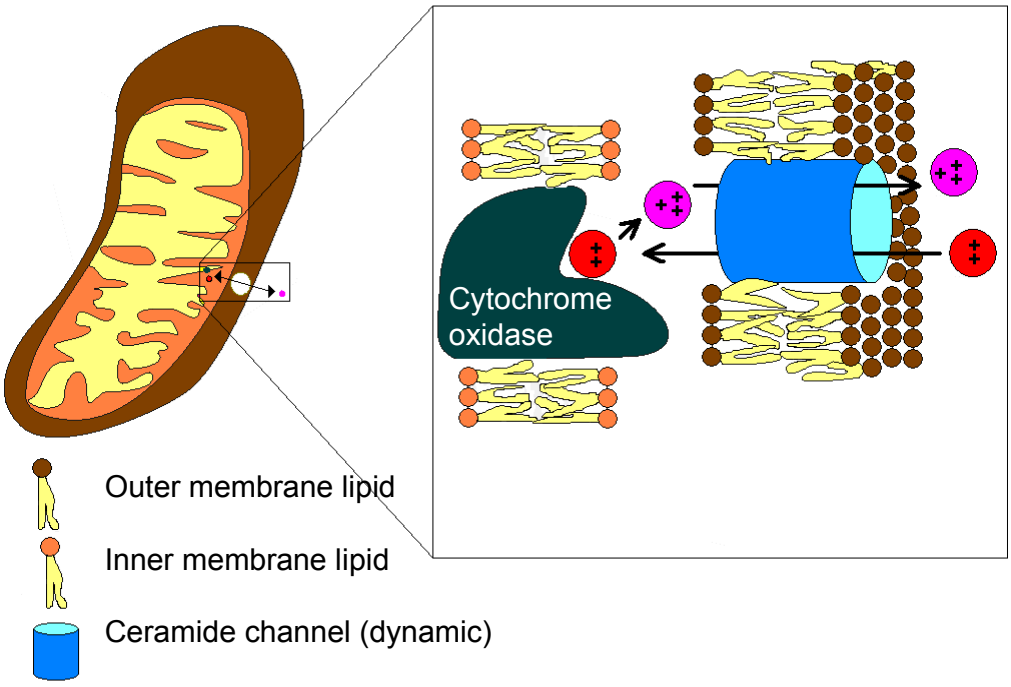

FIG. 3.1. Cytochrome oxidase accessibility assay. Measuring MOM permeabiliy. A permeabilized outer membrane allows for the rapid oxidation of exogenously added reduced (+2) cytochrome *c* using the inner membrane integral enzyme, cytochrome oxidase.

Incubation of mitochondria with 29 nmoles of C_2- (Fig. 3.2**a**) or 93 nmoles of C_{16}-ceramide (Fig. 3.2**b**) for 10 minutes 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 Siskind *et al.*, 2002). When dihydroceramide is premixed with ceramide, the ability of ceramide to permeabilize mitochondria was effectively reduced (Fig. 3.2**a** and **b**) and this reduction occurred in a dose-dependent manner (Fig. 3.3**a** and **b**).

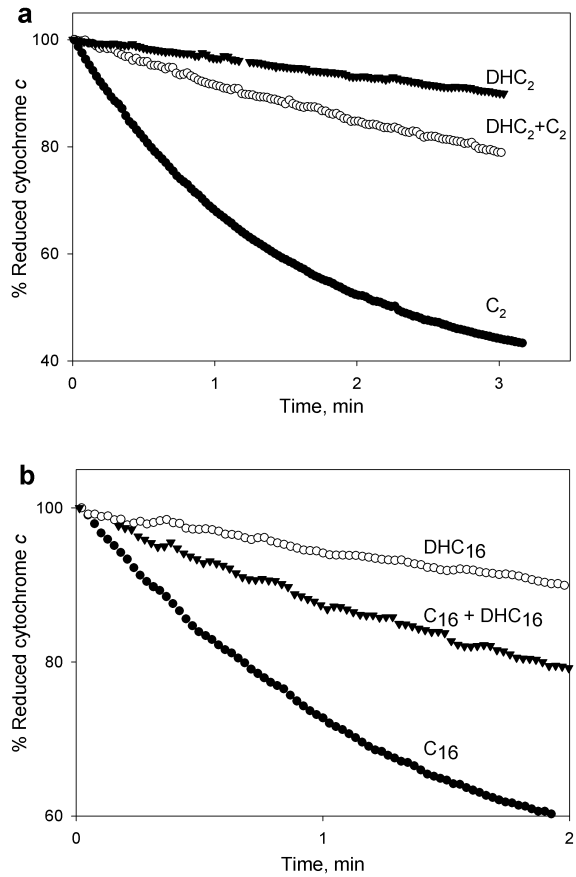

FIG. 3.2. Dihydroceramide inhibits the permeabilization of the mitochondrial outer membrane by ceramide. The percent of cytochrome *c* in the reduced state is plotted as a function of time after the addition of 29 nmol C_2-ceramide, 29 nmol dihydroceramide (DHC_2) and 22 nmol DHC_2 + 29 nmol C_2 (panel **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).

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 nmol mitochondrial phospholipid[♦]) However, under physiological conditions, C_2-ceramide channel formation (Fig. 3.3**a** *inset*) was inhibited by DHC_2 and C_{16}-ceramide was also inhibited by DHC_{16} (data not shown). Moreover, the 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.

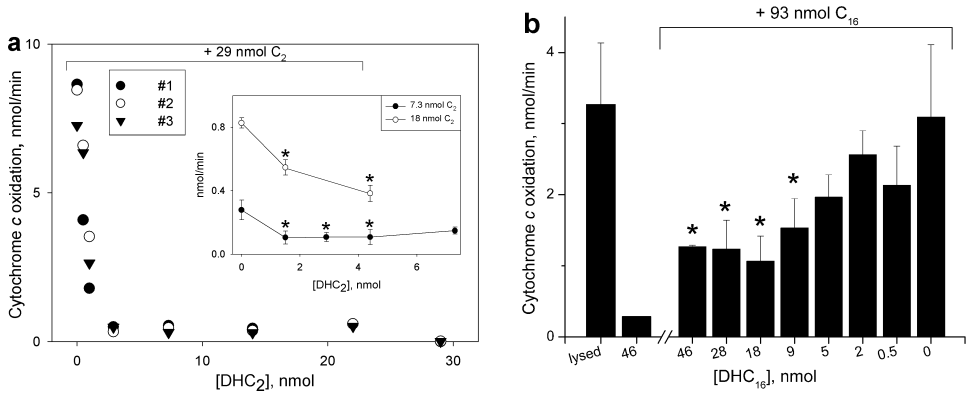

FIG. 3.3. Dihydroceramide reduces ceramide channel formation in mitochondria. **(a)** The initial rate of oxidation of cytochrome *c*, obtained from experiments similar to those shown in Fig. 1, is shown as a function of the amount of DHC_2 added to a fixed amount of C_2-ceramide (as indicated). The 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 *, $P \leq 0.05$.

♦ The calculation is based on the percent insertion of added ceramide into the mitochondrial membranes (Siskind *et al.*, 2005). 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 (Tyler, 1992).

Mitochondria were incubated with each of the mixtures for 10 minutes 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 (Fig. 3.4**a**). 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- (Fig. 3.4**b**) or C_{16}-ceramide (Fig. 3.4**c**) 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 (Siskind and Colombini, 2002; Siskind *et al.*, 2002), 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 (Siskind *et al.*, 2005).

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).

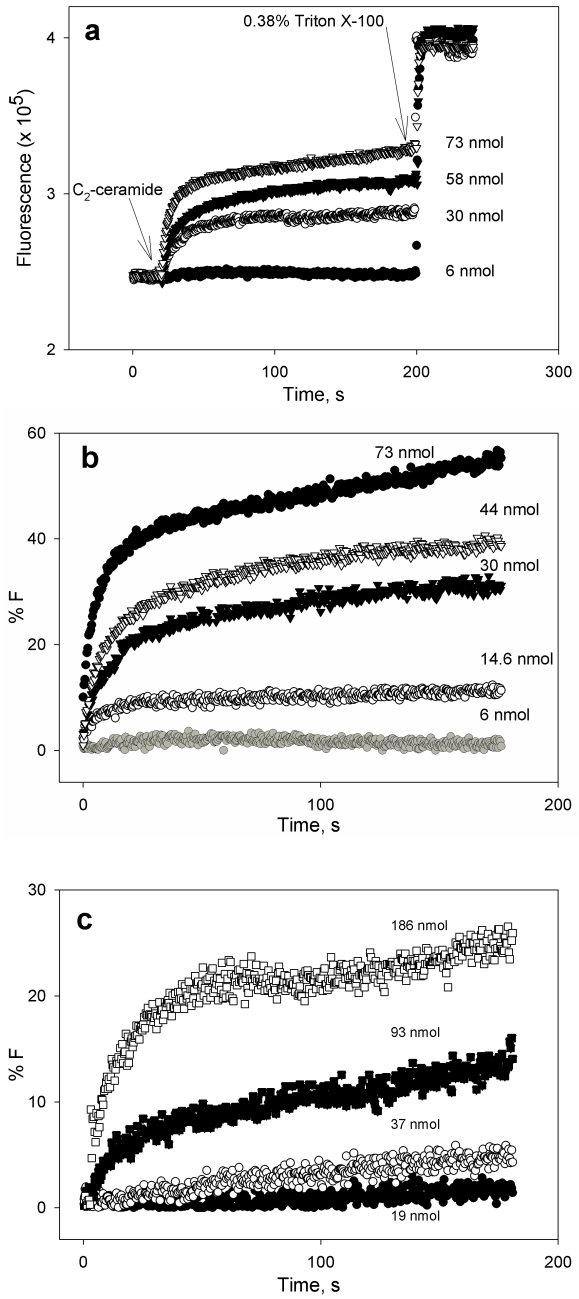

FIG. 3.4. The concentration dependence of ceramide permeabilization of liposomes. In panel **a**, the indicated amount of ceramide was added after the baseline fluorescence was recorded. The total permeabilization of liposomes was achieved with 0.38% Triton-X 100. In panel **b**, the concentration dependence 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 (Fig. 3.5**a**, 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 reduced ceramide channel formation in a dose-dependent fashion (Fig. 3.5**b**).

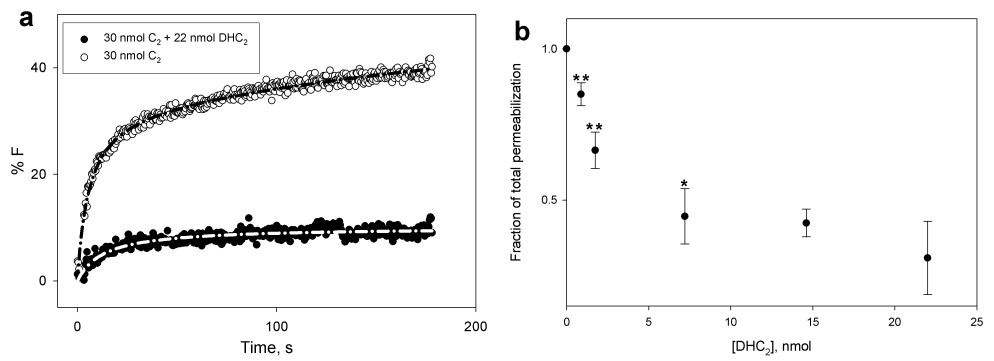

FIG. 3.5. Dihydroceramide inhibition of ceramide-induced release of CF from liposomes. **(a)** The premixing of dihydroceramide with ceramide (closed symbols) greatly reduced the release of CF from liposomes observed with ceramide alone (open symbols). The average traces of 3 independent experiments 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 **, $P \leq 0.01$.

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 (Fig. 3.6**a**). 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 channel formation (Fig. 3.6**a**). This indicates that dihydroceramide interferes with the initiation or nucleation of ceramide channels rather than disassembling them. Figure 3.6**b** 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 (Gruy *et al.*, 2005). 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 minutes 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.

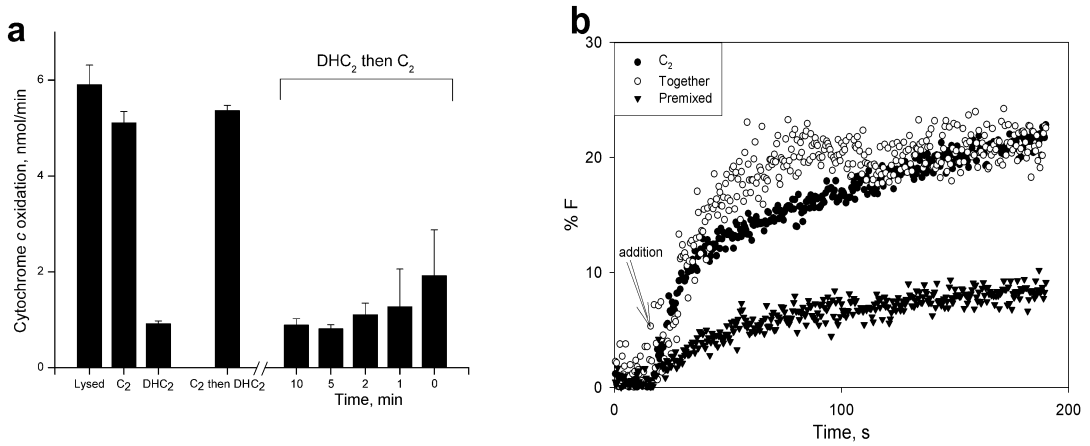

FIG. 3.6. Dihydroceramide inhibits the formation of ceramide channels and does not induce channel disassembly. **(a)** The initial rate of cytochrome *c* oxidation by mitochondria following various treatments: hypotonic shock (lysed); 15 nmol C_2 for 15 min. (C_2); 15 nmol DHC_2 for 15 min. (DHC_2); 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.

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 (Siskind, 2005). 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 (Siskind *et al.*, 2002; Siskind *et al*., 2003).

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 (Tyler, 1992; Nelson and Cox, 2000). 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 (Siskind *et al.*, 2003). C_{16}-ceramide (and other similar synthetic molecules) were shown to form chloride-permeant pores in liposomes (Pajewski *et al.*, 2005). Moreover, Montes *et al.*

(2002) 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.*, 2006). The production of reactive oxygen species (ROS) by ceramide is well documented (Zamzami *et al.*, 1995; García-Ruiz *et al*., 1997; Corda *et al.*, 2001); 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 the endogenous substrates should be low in a diluted mitochondrial suspension. Under these conditions, ROS production should be minimal if present. Indeed, ceramide can produce ROS and also can directly permeabilize membranes. In liposomes, ceramide is able to directly release CF without the help of any ROS production. It is evident that ceramide has multiple roles in the cell.

Ceramide metabolism is highly regulated (Hannun, 1996) 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 Pettus *et al.*, 2002). One of the products of the *de novo* synthesis is dihydroceramide which has been shown to be inactive both biologically (does not cause apoptosis (Bielawska *et al.*, 1993; Sugiki *et al.*, 2000) and physically (does not form channels in planar membranes (Siskind and Colombini, 2000), mitochondria (Siskind *et al.*, 2002) or liposomes).

During *de novo* ceramide synthesis (Mandon *et al.*, 1992), 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 (Fig. 3.7).

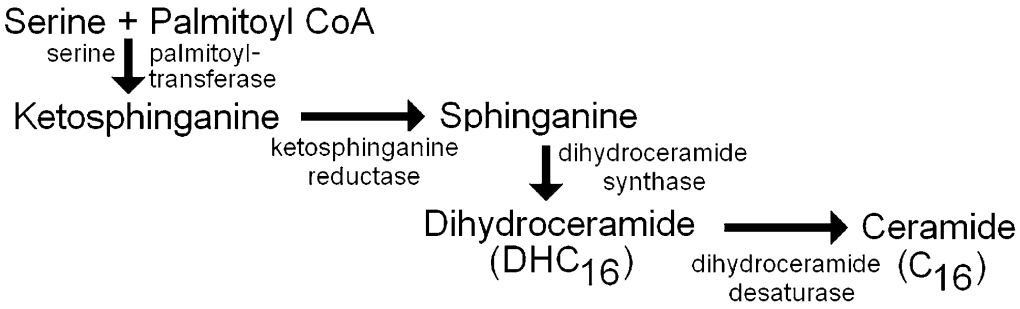

FIG. 3.7. A scheme of *de novo* ceramide synthesis pathway.

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 (Kroesen *et al.*, 2001). 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* (Birbes *et al.*, 2001). 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 membrane (Perry and Ridgway, 2005). 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 Perry, 2002). Furthermore, inhibition of the *de novo* enzymes prevented alveolar cell apoptosis (Petrache *et al.*, 2005).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 (García-Ruiz *et al*., 1997). 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 (Velasco *et al.*, 2005). 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 (Darwiche *et al.*, 2005). 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 (Siskind and Colombini, 2000) and in the outer membrane of mitochondria (Siskind *et al.*, 2002). 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 (Siskind *et al.*, 2002) or to cause apoptosis (Bielawska *et al.*, 1993; Sugiki *et al.*, 2000). 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.

ACKNOWLEDGEMENTS

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CHAPTER 4

CERAMIDE SYNTHESIS IN THE ENDOPLASMIC RETICULUM PERMEABILIZES MITOCHONDRIA TO PRO-APOPTOTIC PROTEINS

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Running Title: ***Ceramide import into mitochondria***

KEYWORDS:

Apoptosis, ceramide synthesis, ER, lipid transfer, mitochondria

ABBREVIATIONS:

ER: endoplasmic reticulum; DDase: Dihydroceramide desaturase; DHC_{16}: *N*-palmitoyl-$_D$-*erythro*-sphinganine; 1-^{14}C-DHC_8: *N*-[1-^{14}C]-octanoyl-$_D$-*erythro*-sphinganine; 1-^{14}C-C_8: *N*-[1-^{14}C]-octanoyl-$_D$-*erythro*-sphingosine; 1-^{14}C-C_{16}: *N*-[1-^{14}C]-palmitoyl-$_D$-*erythro*-sphingosine; C_8-ceramide: *N*-octanoyl-$_D$-*erythro*-sphingosine; C_{16}-ceramide: *N*-palmitoyl-$_D$-*erythro*-sphingosine.

ABSTRACT

The enzyme, dihydroceramide desaturase, converts the inhibitory precursor, dihydroceramide, into the apoptogenic sphingolipid, ceramide. Ceramide can initiate apoptosis by permeabilizing the mitochondrial outer membrane to apoptosis-inducing proteins. However, the desaturase is known to be located in the endoplasmic reticulum (ER) and here we report only minimal activity in isolated rat liver mitochondria. Nevertheless, mitochondria are one of the targets of ceramide-induced apoptosis. Here, we show that ceramide synthesized in isolated mammalian ER vesicles from either C_8-dihydroceramide or sphingosine to produce long-chain ceramide, can transfer to isolated mitochondria and permeabilize these to proteins. As expected, the transfer of the long-chain ceramide is slower. However, sufficient ceramide is transferred to permeabilize the outer membrane to cytochrome *c* and adenylate kinase. The transfer of sphingolipids is bidirectional and non-specific as erythrocyte membranes can also receive the ceramide. The ER that is tightly-associated with isolated mitochondria can produce enough ceramide to permeabilize the outer membrane. Thus this ceramide exchange obviates the need for a complete ceramide *de novo* pathway in mitochondria in order for cells to use ceramide from this pathway to activate mitochondria-mediated apoptosis.

INTRODUCTION

Apoptosis, or programmed cell death, is a mechanism by which cells perish without harming surrounding cells. An important form of apoptosis is mediated by mitochondria (Crompton, 1999; Birbes *et al.*, 2002). There are many known factors that contribute to the induction of mitochondria-mediated apoptosis, but the factors responsible for the crucial decision-making step, the release of proteins from the intermembrane space, are still debated. In addition to generally accepted factors such as Bax and Bak, the sphingolipid, ceramide, was shown to be able to form large, stable, protein-accessible channels in planar phospholipid membranes (Siskind and Colombini, 2000), in isolated mitochondria (Siskind *et al.*, 2002) and in liposomes (Stiban *et al.*, 2006). We believe that elevated ceramide levels in mitochondria are important for cells to undergo apoptosis.

In the cell, ceramide has diverse roles (Hannun and Obeid, 2002; Futerman and Hannun, 2004). The two major pathways of ceramide generation are *de novo* synthesis and sphingomyelin hydrolysis. *De novo* ceramide synthesis occurs on the outer leaflet of the endoplasmic reticulum (Mandon *et al.*, 1992) even though there are some ceramide metabolic enzymes in mitochondria (Bionda *et al.*, 2004). The *de novo* synthesis (for reviews, see Perry, 2002; Merrill, 2002) involves the condensation of serine with a fatty acyl-CoA (normally, palmitoyl-CoA). The reaction, catalyzed by serine palmitoyltransferase complex (Gable *et al.*, 2000; Hannun *et al.*, 2001) produces ketosphinganine, which, after being reduced to sphinganine (dihydrosphingosine), is converted to dihydroceramide by ceramide synthase. The oxidation of dihydroceramide at the 4,5 position by dihydroceramide desaturase produces ceramide.

Many reports (reviewed in Perry, 2002) point to the importance of the *de novo* pathway in producing the ceramide that is involved in mitochondria-mediated apoptosis. Although ceramide generated by sphingomyelinase action has been shown to lead to the release of cytochrome *c* from mitochondria (Birbes *et al.*, 2001) most reports favor the *de novo* pathway. Yet, it is clearly established that a critical enzyme in the *de novo* pathway, the dihydroceramide desaturase (DDase), is located in the ER (Morell *et al.*, 1970; Hirschberg *et al.*, 1993) facing the cytosol (Michel and van Echten-Deckert, 1997). Thus the production of ceramide from the *de novo* pathway for use in mitochondria requires either the presence of a mitochondrial DDase or a mechanism for transfer of ceramide from the ER to mitochondria. Another source of ceramide could be the reacylation of sphingosine by mitochondrial ceramide synthase. However, sphingosine is produced by hydrolyzing ceramide and thus this reacylation is a salvage pathway. In this study, we provide evidence that the location of ceramide production in a cell does not necessarily coincide with its functional site. We find that mitochondria lack significant DDase activity but the well established intimate interaction between ER and mitochondria (Pickett *et al.*, 1980; Meier *et al.*, 1981; Katz *et al.*, 1983; Vance, 1990; Marsh *et al.*, 2001) allows the transport of ceramide from one compartment to another. Sufficient transfer occurs to cause functional changes in mitochondria permeabilizing them to pro-apoptotic proteins.

MATERIALS AND METHODS

Experiments with rats were approved by the University of Maryland Institutional Animal Care and Use Committee.

Materials - C_2-dihydroceramide, C_8-ceramide, C_8-dihydroceramide C_{16}-ceramide, C_{16}-dihydroceramide and sphinganine were purchased from Avanti Polar Lipids (Alabaster, AL). Analytical-grade DMSO (from Fisher) was used to dissolve C_2-dihydroceramide whereas isopropanol (from Acros Organics) was used to dissolve C_8-ceramide, C_8-dihydroceramide, C_{16}-ceramide and C_{16}-dihydroceramide. Horse heart cytochrome *c* and bovine serum albumin (BSA, fatty acid depleted) and cholesterol were from Sigma (St. Louis, MO). Sodium ascorbate, sodium borate and 99.9% dry ethylacetate were bought from Acros Organics. Radiolabelled ^{14}C-octanoic acid and ^{3}H-sphingosine were from American Radiolabeled Chemicals (St. Louis, MO). *N*-hydroxysuccinimide and dicyclohexylcarbodiimide were from Fisher. Thin layer chromatography was performed on Analtech (Newark, DE) silica gel plates.

Preparation of radiolabeled C_8-dihydroceramide – Sphinganine was acetylated using 1-^{14}C-octanoic acid (Fig. 4.1), according to the procedure of Schulze *et al*. (1999). Four sealed, septum-topped, argon-flushed reaction vials were used to minimize any contact with water vapor or air. In the first vial, 5 μL containing 5 μCi of radioactive octanoic acid (specific activity 55 mCi/mmol) was mixed with 13 μL of 10 mg/mL cold octanoic acid (in ethylacetate). In total, 1 μmol of octanoic acid was used. The solvent was flushed with a stream of argon and then 1 mL of 99.9% ethylacetate was added to the vial. After mixing for 5 minutes, the solution was transferred to the second reaction vial which already contained 4 μL of 0.25 M *N*-hydroxysuccinimide (1 μmol) and had been flushed

with argon. After mixing for 5 minutes, the solution was injected in the third vial. The latter had 1 μmol of dicyclohexylcarbodiimide (1 μL of 1 M). This reaction was carried overnight. The next day, the solution was transferred into the fourth vial which contained 0.87 g (2.6 μmol) of sphinganine. The acylation reaction was carried over 3 days and at the end of the third day, the reaction mixture was dried under nitrogen and the products were dissolved in 30 μL of $CHCl_3$:CH_3OH (1:1, v/v) and applied to a TLC plate. The vial was rinsed with 30 μL of $CHCl_3$:CH_3OH (1:1, v/v) three times and also applied to the TLC plate (to recover essentially all the radioactivity). The developing system was $CHCl_3$:CH_3OH:H_2O (80:10:1, v/v/v). On the TLC, the radioactive band was recovered after being detected with a Geiger-Müller counter, scrapped off the gel and extracted in $CHCl_3$:CH_3OH (2:1, v/v). Multiple extractions of the gel were made. Further separation of octanoic acid from the product *N*-[1-^{14}C]octanoyl-$_D$-erythro-sphinganine, or C_8-dihydroceramide (DHC_8^*) was not needed since the reaction apparently consumed all reactants (as evident by the visualization of the gel in a PhosphorImager screen). The final concentration of 1-^{14}C-DHC_8 was 18 μM in isopropanol.

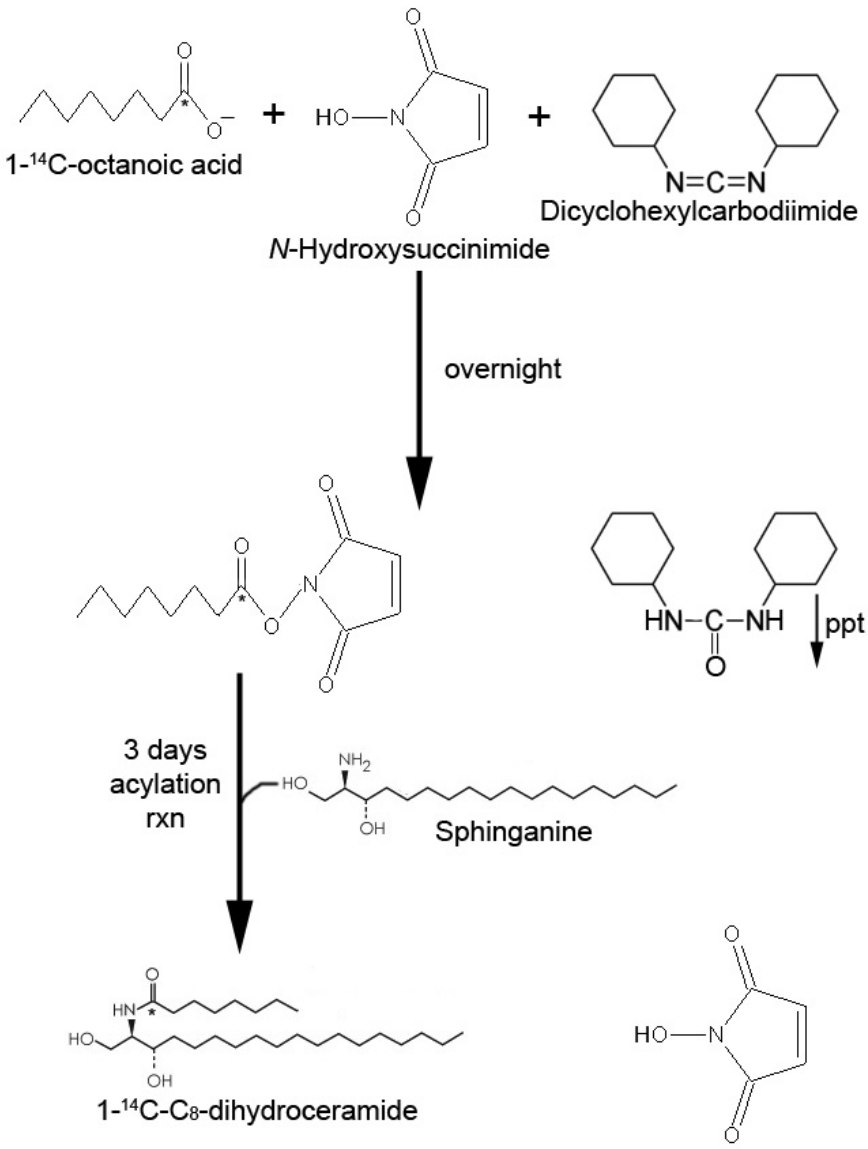

FIG. 4.1. Synthesis of radiolabeled DHC_8. 1-^{14}C-octanoic acid and *N*-hydroxysuccinimide were mixed and activated by dicyclohecylcarbodiimide. In an overnight reaction, dicyclohexylurea precipitated and sphinganine was added to the activated fatty acid. The aceylation reaction took 3 days to be completed forming DHC_8 and retrieving the *N*-hydroxysuccinimide.

Mitochondria – Mitochondria were isolated from male Sprague-Dawley rat liver as described by Parsons *et al.* (1966) as modified by (Siskind *et al.*, 2002 and Sitban *et al.*, 2006). Briefly, livers from overnight-fasted young rats (generally 200 to 300g) were obtained and cut in ice-cold isolation buffer (210 mM Mannitol, 70 mM sucrose, 10 mM HEPES, 0.1 mM 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). Differential centrifugation followed, starting at 760 ×g for 10 minutes at 4 ^{o}C. To recover the mitochondria, the supernatant was then spun for 10 minutes at 9000 ×g. The supernatant of this spin was used to isolate ER (see below). This sequence was repeated but the second high-speed spin was performed with HB-buffer lacking BSA (i.e. H-buffer). Finally the mitochondrial pellet was gently resuspended in H-buffer.

The protein concentration of the mitochondrial suspension was measured by using a spectroscopic method (Clarke, 1976). Reduced cytochrome *c* was prepared by mixing 5.5 mg of cytochrome *c* with 2 mg sodium ascorbate in 0.25 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 (after elution, the concentration of reduced cytochrome *c* is about 1 mM).

Ultra-pure Mitochondria Isolation – Mitochondria isolated by differential centrifugation were layered on top on a 28 % self-generating Percoll gradient according to (Holden and Colombini, 1993) and the tube was spun at 39,000 $\times g_{max}$ for 30 minutes. The mitochondrial fraction was collected and diluted in H-buffer and spun at 9000 ×g for 10 minutes to remove the Percoll.

The Endoplasmic Reticulum – Here, we use the term ER for the isolated microsomes which have different components but are mostly composed of endoplasmic reticulum membranes. The ER was separated following the modified procedure (Schulze *et al.*, 1999). Briefly, the first 9000 ×g supernatant from the mitochondrial isolation was spun down at 105,000 ×g for 1 hour. The pellet was suspended in H-buffer. To separate some attached mitochondria, another spin of 9000 ×g for 10 minutes followed and the protein concentration in the supernatant was determined using the BCA protein assay kit from Pierce (Rockford, IL).

Red Blood Cells – Blood from a decapitated rat was collected in a tube pre-filled with an anticoagulation buffer (AB) containing 150 mM NaCl, 4 mM EGTA and 5 mM HEPES, pH 7.4. Red blood cells were collected by centrifugation of 1 mL of the blood at 600 ×g for 10 minutes. The cells were resuspended in AB and pelleted 3 times. After the third spin, the cells were osmotically shocked in double distilled water and the membranes were spun at 12,000 ×g for 10 minutes. This step was also repeated 3 times. After the last run, the membranes were resuspended in AB and the protein concentration was measured by the aforementioned assay.

Radioactive Desaturase Assay – Following the protocol of (Schulze *et al.*, 1999) radioactive desaturase assays were performed on mitochondrial and endoplasmic reticular fractions. One modification was the use of isopropanol to dissolve ceramides and dihydroceramides. This solubilization protocol and the concentration of dihydroceramide used were effective in preventing the sedimentation of dihydroceramide micelles when

the solution was centrifuged at 105,000 ×g to sediment endoplasmic reticulum (more than 99 % of the radioactivity remained in the supernatant). In contrast, solubilizing ceramide in DMSO or ethanol at higher concentrations has problems in its delivery to mitochondria (Siskind *et al.*, 2006).

Mitochondrial Inner Membrane Desaturase Assay – 300 µL of 21 mg/mL mitochondria were mildly shocked in 0.9 mL of double distilled water and were kept on ice for 5 minutes. Osmolarity was restored by adding 0.9 mL of R×2 buffer (0.6 M mannitol, 20 mM NaH_2PO_4, 10 mM $MgCl_2$, 20 mM KCl pH 7.2). The final concentration of mitochondria was 3 mg/mL. To assess the DDase activity, to 1 mL of the shocked mitochondria was added 36 µL of the M/G buffer (70 mM malate, 70 mM glutamate, K^+ salt pH 7.3) to generate NADH in the matrix, 40 µL of 0.1 M NADH (to supply the NADH to the medium along with 10 µL of 1-^{14}C-DHC_8). The reaction was run for 45 minutes at 37^{o}C before being stopped by the addition of 1.5 mL of $CHCl_3$:CH_3OH (2:1, v/v). In the control experiment, the reaction was stopped immediately after the addition of the radiolabeled compound. The lipids were isolated as described below.

Separation of C_8 from DHC_8 – The protocol of (Schulze *et al.*, 1999) was followed. Basically, a TLC plate impregnated with borax was used to separate ceramide from dihydroceramide. A 70 mM suspension of sodium borate in methanol was poured on a silica gel TLC plate and incubated for 30 minutes. The plates were removed and dried at room temperature for 2 hours. After the lipids were extracted, they were spotted on these borax-impregnated plates. The developing system for the separation was 9:1 (v/v) $CHCl_3$:CH_3OH.

Visualization of the radiolabeled bands – After the TLC plates were developed, they were air dried and then stored in contact with a PhosphorImager screen (Amersham Biosciences) in a dark cassette for a minimum of 3 days. The screen was then scanned in a Storm PhosphorImager.

Medium-chained Lipid Transfer Experiments – The transfer of radiolabeled lipids from the ER to mitochondria was measured as follows. 750 µL of 3 mg/mL ER was incubated with 10 µL of 1-^{14}C-DHC$_8$ and 40 µL of 0.1 M NADH for 45 minutes. An equivalent amount of mitochondria was added resulting in a total volume of 1.5 mL. The tube was vortexed and incubated at 37^{o}C for 2, 5 or 10 minutes before being spun down at 9000 ×g for 10 minutes. Aliquots were taken from the pellet (mitochondria) and supernatant (ER) to measure total radioactivity, then $CHCl_3$:CH_3OH (2:1, v/v) was added to each fraction to isolate the lipids. The lipids were extracted and run on a TLC plate. The radioactive bands were visualized as indicated above.

Long-chained Lipid Transfer Experiments – ER preparations (8.1 mL of 3 mg/mL protein) were mixed with 100 µM palmitoyl CoA (900 µL of 1 mM) and 1.5 µL of ^{3}H-sphingosine (1.5 µCi) (specific activity 20 Ci/mmol) and incubated at 37^{o}C for 15 minutes (Wang and Merrill, 1999). After incubation, excess sphingosine was removed by the addition of 63 mg of fatty acid free BSA and spinning down the ER at 105,000 ×g for 1 hour. After resuspending the ER pellet in 9 mL of H-medium and distributing it in 3 tubes, mitochondria (3 mL of 3 mg/mL protein) were gently mixed with the ER preparations carrying the radiolabeled ceramide and the tubes were incubated for 0, 5 and

10 minutes at room temperature before spinning at low speed (9000 ×g for 10 minutes) to recover mitochondria and then high speed (105,000 ×g for 1 hour) to retrieve the ER. Aliquots from the pellets were taken to measure the ER marker enzyme. The lipids were extracted from the pellets by $CHCl_3$:CH_3OH (2:1, v/v) and samples were counted in a scintillation counter. The rest of the extracted lipids were dried down under nitrogen and house vacuum over night and applied to a TLC. The mobile phase used was 99:1 (v/v) diethylether:CH_3OH (Wang and Merrill, 1999). The ceramide and sphingosine bands were then scrapped and counted to determine the percent conversion.

Functional Desaturase Assay – Mitochondria (50 µL of 3 mg protein/mL) were suspended in 0.75 mL of isotonic H-buffer and incubated with 20 µL of 4 mM NADH (0.1 mM) and 10 µL of 1 mg/mL DHC_{16} (19 nmol) for various times. After the incubation, the mitochondria were spun down at 6500 rpm for 5 minutes (at 4 ^{o}C) before being gently resuspended in 0.75 mL of H-buffer supplemented with 2.2 µM antimycin A and 2 mM 2,4-dinitrophenol (HAD-buffer). The reduction of absorbance of added 15 µL of 1 mM reduced cytochrome *c* at 550 nm was used as a measure of the permeabilization of the mitochondrial outer membrane (Siskind *et al.*, 2002; Stiban *et al.*, 2006). Vehicle controls were performed as appropriate.

Monitoring Outer Membrane Permeability After Exposure to ER – Isolated ER (2 mg protein) were preincubated with 3 µmol NADH and 28 nmol DHC_{16} in 1 mL of H-buffer for 0, 20, 45 and 80 minutes. The control lacked NADH and DHC_{16}. At each time point, 50 µL of the ER (control or experimental) was mixed with 50 µL of 0.5 mg protein/mL mitochondria in 0.75 mL of HAD-buffer and incubated for 10 minutes before 15 µL of 1

mM reduced cytochrome *c* was added to measure the cytochrome *c* oxidation rate (an indication of the outer membrane permeability to cytochrome *c*). The difference in absorbance at 550 nm and 600 nm was used to record the cytochrome *c* oxidation because adding ER to mitochondria caused an increase in the light scattering with time (increasing the baseline at 600 nm). Also, incubating the ER by itself (no mitochondria) had a baseline of cytochrome *c* oxidation, indicating some contamination with mitochondria. The baseline rate of cytochrome c oxidation by ER vesicles was subtracted from the rate measured in each experimental treatment with ER

The Release of Adenylate Kinase –Isolated mitochondria (200 μg protein) were incubated in 3 mL of H-buffer and 1 μL of 5 mg/mL leupeptin, 1 μL of 5 mg/mL aprotinin and 1 μL of 5 mg/mL pepstatin. This was the control, untreated sample. At time points 0, 5 and 15 minutes, 1 mL was taken out and spun at 14,000 rpm. 900 μL of the supernatant was recovered and put on ice. In each experiment, 300 μL of the supernatant was mixed with 700 μL of the reaction mixture (RM) containing: 50 mM Tris (pH 7.5), 5 mM $MgSO_4$, 10 mM glucose, 1 mM ADP and 0.2 mM $NADP^+$. After waiting for a minute, 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. In the experimental trials, 2.4 mM NADH or 74 nmol DHC_{16} or 2.4 mM NADH + 74 nmol DHC_{16} were added to the control mixture. The rest of the procedure was similar.

ER Marker Enzyme Assay – The marker enzyme for the ER was NADPH:cytochrome *c* reductase and the assay was according to (Ernster *et al.*, 1962).

RESULTS

A dihydroceramide desaturase capable of using cytosolic NADH is not found in ultra pure mitochondria

Previous studies showed that some ceramide metabolic enzymes are found in mitochondria. We looked for the activity of the DDase in isolated mitochondria. Isolated mitochondria prepared by differential centrifugation, ultra pure mitochondria and microsomes were assessed for their ability to convert 1-^{14}C-DHC$_8$ to 1-^{14}C-C$_8$-ceramide using the DDase assay of Schulze *et al.* (1999). Unlabeled sphingolipids were added to the samples at the end of the incubation period to act as carriers and to confirm separation on the TLC. Microsomes, largely composed of endoplasmic reticulum (ER) vesicles (Fig. 4.2, ER) showed the largest conversion (44 ± 5 % in 45 minutes, average and standard deviation of 23 experiments). Mitochondria isolated by differential centrifugation (DC-mitochondria) also had some significant conversion indicating that mitochondria might have a small amount of the enzyme or some associated ER vesicles (as previously reported, Pickett *et al.*, 1980 and Katz *et al.*, 1983) (Fig. 4.2, M). In the Percoll gradient-purified mitochondria the DDase activity was not seen. The Percoll step seems able to disassociate the two organelles. The results for these ultra pure mitochondria indicate that the enzyme is not found in mitochondria in a detectable amount (Fig. 4.2, UP) but the tight interaction between mitochondria and ER is what causes this conversion in the DC-mitochondrial fraction. Following an ER marker enzyme assay (NADPH:cytochrome *c* reductase), the DC-mitochondria had double the activity (13.5 μM mg^{-1}min^{-1}) of the ultra pure (5.8 μM mg^{-1}min^{-1}) but significantly less than the microsomes (426 μM mg^{-1}min^{-1}).

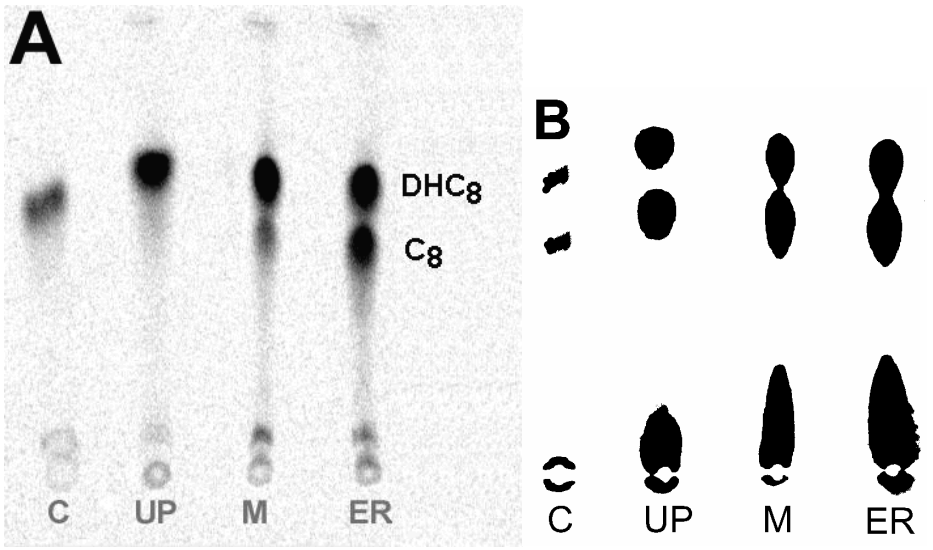

FIG. 4.2. DDase capable of using cytosolic NADH was not found in mitochondria. (**A**) A PhosphorImager screen of a TLC gel showing the conversion of added radioactive 1-^{14}C-DHC_8 to radioactive 1-^{14}C-C_8 in different cellular fractions: no organelles (C), ultra pure mitochondria (UP), mitochondria obtained by differential centrifugation (M), and endoplasmic reticulum (ER). (**B**) Iodine staining of the same gel. Cold DHC_8 and C_8 was added to each sample prior to extraction so that it would act as a carrier for the label and to clearly indicate the location of the two compounds on the TLC plate. The lipids were extracted from the same amounts of organellar protein (0.6 mg of protein per lane, except for "C"). All the extracted lipid was applied to the TLC plate. Mitochondria were purified on a Percoll gradient.

Ultra pure mitochondria isolated from rat liver have minimal DDase activity in both membranes

To check whether the DDase is found in the inner membrane of mitochondria utilizing matrix NADH, NADH was produced in the matrix using the malate/glutamate shuttle (Rickwood *et al.*, 1987). The mitochondria used here were Percoll-purified with a broken outer membrane to allow maximal accessibility to the inner membrane. Thus 4 mM NADH was present in the medium while NADH was maintained high in the matrix by the malate/glutamate shuttle. The TLC (Fig. 4.3**A**) shows virtually no difference

between zero min of incubation (BM_{cont}) and 45 min of incubation (BM). Subtraction of the curves (Fig. 4.3**B**) shows a slight radioactive ceramide peak consistent with about 8.4 ± 1.3 % conversion. This is 3.7 % of the specific activity in the ER. For comparison, the NADPH:cytochrome *c* reductase marker enzyme activity was 3.2 % and 1.4 % of the specific activity of the ER in DC- and Percoll-treated mitochondria, respectively. Recovered ER from the Percoll-treated mitochondria showed the same conversion of dihydroceramide to ceramide, ruling out that Percoll inhibits this enzyme.

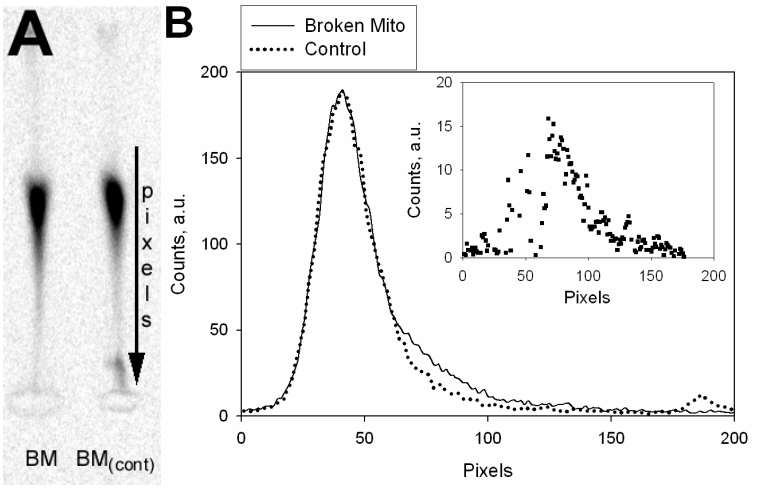

FIG. 4.3. The DDase associated with mitochondria is minimal. (**A**) A PhosphoImager screen of a TLC plate showing minimal conversion of 1-^{14}C-DHC_8 to 1-^{14}C-C_8^* in Percoll-purified mitochondria with broken outer membrane supplied with NADH externally and maintained in the matrix by providing malate/glutamate. Sample "BM" was incubated for 45 min and sample "BM_{cont}" was incubated for zero time. The traces were quantified as a function of migration distance in pixels and plotted in (**B**). The inset shows the subtraction of the zero-time control from the experimental sample, BM. A peak possibly attributable to ceramide was detected whose area corresponds to 8.4 ± 1.3 % conversion in 45 minutes per 3 mg of protein. This is the average of 3 independent experiments ± standard deviation (in contrast, the microsomal fraction achieved 44 ± 5 % conversion in 45 minutes with 0.6 mg of protein).

Medium-chain DHC_8 and C_8 and long-chain C_{16} in the ER can be transferred to mitochondria in a time dependent manner

The deficiency in mitochondrial DDase activity would indicate that mitochondria would have difficulty in generating ceramide from the *de novo* synthetic pathway. However, the proximity of ER and mitochondria raises the possibility of transfer of lipids between these compartments. To investigate this possibility, radiolabeled ceramide was generated in the ER by incubating these with NADH and 1-^{14}C-DHC_8. After 45 min these were incubated with mitochondria for 10 min followed by separation of the organelles by differential centrifugation. The ER marker enzyme assay was used to determine the effectiveness of separating ER from mitochondria and the separation was effective (only 1.4 % of the ER marker enzyme activity was recovered in mitochondria). Lipid extractions were performed followed by separation of ceramide from dihydroceramide on TLC (Fig. 4.4**A**). Ceramide and dihydroceramide bands were present in both mitochondria and ER. The transfer of these lipids occurred with a half-life of 5-10 minutes (Fig. 4.4**B**). A similar rate of transfer was seen in the reverse direction (data not shown). In order to test for specificity of transfer between ER and mitochondria, we tested the transfer to erythrocyte membranes (Fig. 4.4**C**). The rate was essentially the same indicating that we are observing the rate of spontaneous transfer of C_8-ceramide between membranes. This rate has not been reported in the literature, so we decided to estimate it from the published rates of transfer of C_{16}-ceramide. The rate of transfer of ceramide from the donor membrane can be calculated from the results of Simon *et al.* (1999) by using Eyring rate theory and an energy deficit of 3 kJ/methylene molecule. Thus, for C_8-ceramide, the rate constant for this process is 1.6 min^{-1}. So, an exchange of 15 % should occur within 70 seconds. This is consistent with our observations

considering that there are other steps in the exchange process. These become significant when the transfer rate out of the membrane is fast.

We examined the possibility that a mechanism might be present to accelerate ceramide transfer from ER to mitochondria. The rapid spontaneous transfer of C_8-ceramide did not allow us to detect such a mechanism; therefore we examined the transfer rate of C_{16}-ceramide. Exchange experiments were performed with endogenously-produced ^{3}H-C_{16}-ceramide. ^{3}H-sphingosine and palmitoyl coenzyme A were added to ER preparations to endogenously synthesize ^{3}H-C_{16}-ceramide in the ER membrane using the enzyme ceramide synthase. Excess sphingosine was removed with BSA. Fig. 4.4**D** shows that the transfer of the ceramide from ER to mitochondria occurs in a time-dependent manner at a rate much faster than that of spontaneous transfer (35 h for 20 % transfer) (Simon *et al.*, 1999). Therefore, the ER must contain some system to catalyze the egress of ceramide from its membrane.

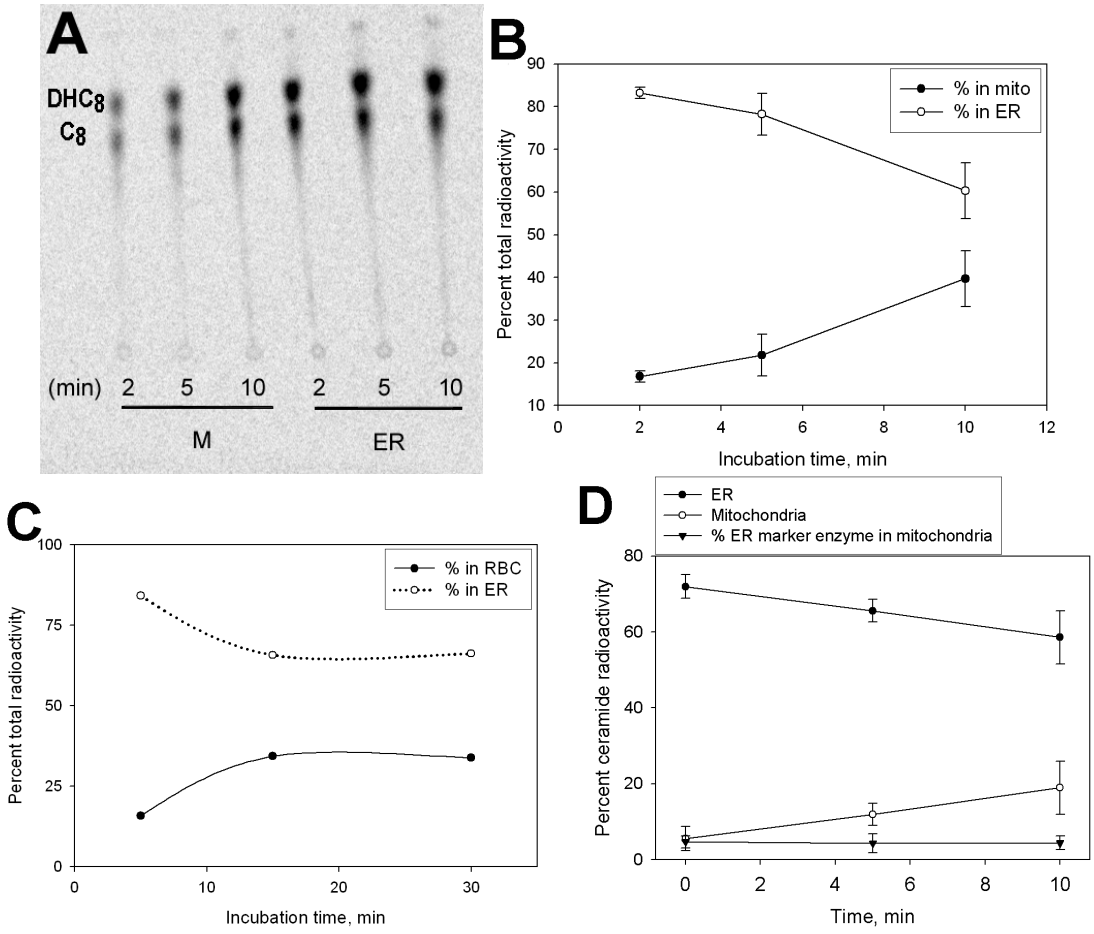

FIG. 4.4. Both medium and long-chain ceramide transfer from ER to mitochondria or RBC in a time dependent fashion. (**A**) A PhosphorImager screen of a TLC showing the transfer of medium-chain ceramide from ER to mitochondria. After the production of ceramide in the ER, Percoll-treated mitochondria were incubated with the ER for the indicated times and then the organelles were separated by centrifugation. The lipids were extracted from the low speed spin pellet (M) and the high speed spin pellet (ER). Equal amounts of protein were used in these experiments. (**B**) The percentage total radioactivity recovered in the mitochondria (●) and ER (○) is shown as an average of 4 separate experiments ± S.E.M. (**C**) ER vesicles containing labeled medium-chained ceramide and dihydroceramide were incubated with RBC membranes for the indicated times followed by separation and quantitation of the radioactivity. (**D**) ER vesicles were preloaded with radiolabeled long-chain ceramide and incubated with mitochondria at room temperature for 0, 5 and 10 min resulting in a slow but steady transfer of ceramide from the ER (●) to mitochondria (○). Mitochondria were checked for the percent ER marker enzyme contamination (▼). The result represents the average ± S.E.M. of 3 replicates.

The transfer of long-chain ceramide into mitochondria depends on the concentrations of organelles used

Determining the mechanism of this transfer was critical. If the transfer of ceramide between ER and mitochondria occurred because of random collision between these organelles then the transfer rate should depend on the concentration of these organelles in the assay. A bimolecular process depends on the product of the concentrations of the reactants. Thus reducing the concentrations of each of the interacting organelles by half, the transfer rate should drop by a factor of 4. Other processes would predict different outcomes. For example if the organelles adhered to each other then the dilution would have a much weaker effect. The results obtained were 334 ± 36 DPM for the undiluted sample and 99 ± 14 DPM for the diluted sample. This result is not significantly different from a four-fold dilution effect. It is significantly different from both a 2-fold effect ($P<0.02$) and from no dilution effect ($P<0.005$). The results represent the average and standard error of 3 experiments. Hence, this shows that the transfer occurs by random collision of the organelles.

Incubating mitochondria with NADH and long-chain dihydroceramide permeabilizes the outer membrane to adenylate kinase and cytochrome *c*

The addition of ceramide to isolated mitochondria leads to the permeabilization of the outer membrane to proteins (Siskind *et al.*, 2002; Stiban *et al.*, 2006, Siskind *et al.*, 2006). Since we have shown that ceramide produced in the ER can rapidly transfer to mitochondria, mitochondria with associated ER may be capable of acting as a functional unit. Dihydroceramide conversion in the mitochondria-associated ER may permeabilize the mitochondrial outer membrane to proteins. Mitochondria isolated by differential

centrifugation were incubated with NADH and long-chain DHC_{16} and samples tested as a function of time for permeability to cytochrome *c* (Fig. 4.7**A**) or the release of adenylate kinase (Fig. 4.5**B**). As adenylate kinase leaks out it accumulates in the medium and thus even low or transient permeabilities will result in its release. The cytochrome *c* oxidation assay measures the permeability of the outer membrane to cytochrome *c* as the rate of cytochrome c oxidation. Thus this assay monitors the actual permeability as a function of time. Note that the permeability peaks followed by a reduction possibly due to the movement of ceramide into other non-conducting structures. Incubating the mitochondria with NADH alone, NAD^+, or NAD^+ plus DHC_{16} did not induce permeabilization more than the control, untreated mitochondria (Fig. 4.5**A**, **C**) indicating that the permeability is due to the production of long-chain C_{16}-ceramide by the DDase in the ER and its transfer to mitochondria. Short-chain dihydroceramide (DHC_2) was ineffective and it is known not to be a substrate for the DDase (Schulze *et al.*, 1999).

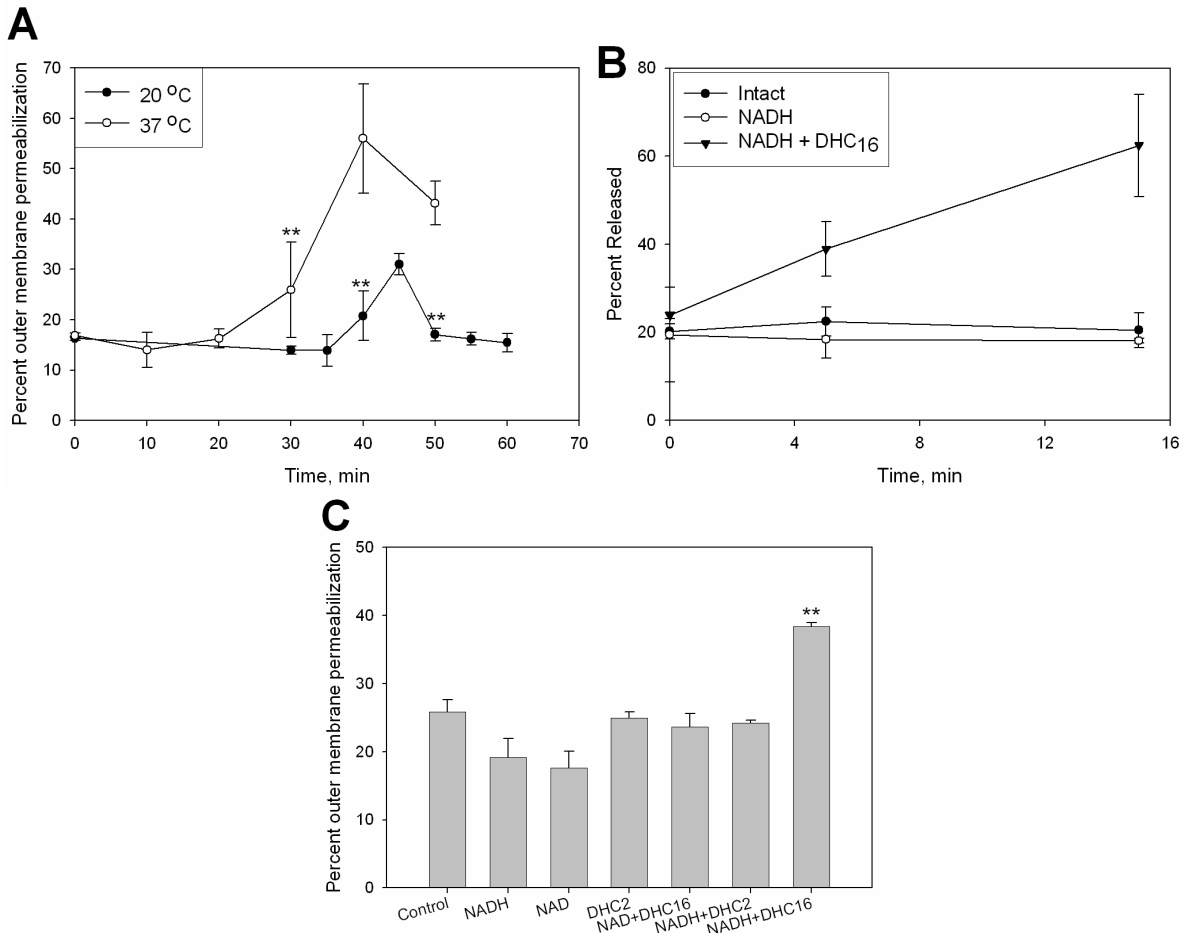

FIG. 4.5. Permeabilization of mitochondrial outer membranes to cytochrome *c* and adenylate kinase following generation of ceramide by associated ER vesicles. (**A**) The outer membrane permeabilization to cytochrome *c* was measured by recording the cytochrome *c* oxidation rate and expressed as a percent of the rate observed with hypotonically shocked mitochondria. The cytochrome *c* oxidation rate was measured after incubation of rat liver mitochondria for the indicated time at 20°C (●) and 37°C (○). The amounts of NADH and DHC_{16} are identical to the amounts in (**C**). (**B**) The release of adenylate kinase increases when the mitochondria are incubated with NADH and DHC_{16}. Mitochondria were incubated for the indicated amount of time with either NADH alone (○) NADH plus DHC_{16} (▼) or no treatment (●) followed by quantitating the amount adenylate kinase released into the medium. Treating the mitochondria with DHC_{16} alone (not shown) did not lead to adenylate kinase activity in the supernatant. This is expressed as a percent of the total activity released following hypotonic shock. (**C**) The production of ceramide by dihydroceramide desaturase in DC-mitochondria permeabilizes the mitochondrial outer membrane to cytochrome *c*. 50 µL of 3 mg/mL rat liver mitochondria obtained by differential centrifugation were incubated with the indicated compounds for 30 min followed by centrifugation to remove the supernatant (especially the NADH). This extra step caused some mitochondrial damage (high control). Treatment with 0.1 mM NADH and 19 nmoles DHC_{16} (but not DHC_2) resulted in the permeabilization of the outer membrane. The percent permeabilization is the rate of cytochrome *c* oxidation as a percent of that observed with hypotonically shocked mitochondria. All the results are representative of the mean reading of three experiments ± standard deviation. **, $P < 0.01$.

Mitochondrial outer membrane permeabilization by interaction with long-chain ceramide-containing ER vesicles

Aliquots of mitochondria were mixed either with ER vesicles pretreated with NADH and DHC_{16} to preload them with ceramide, or untreated ER vesicles, or medium alone. The mitochondria-ER combinations were incubated for the indicated times (Fig. 4.6) and then the cytochrome *c* oxidation rate was measured. The permeabilization of the outer membrane to cytochrome *c* is once again expressed as a percent of the rate observed with shocked mitochondria. Only mitochondria exposed to ER vesicles preloaded with ceramide developed a permeabilized outer membrane to proteins.

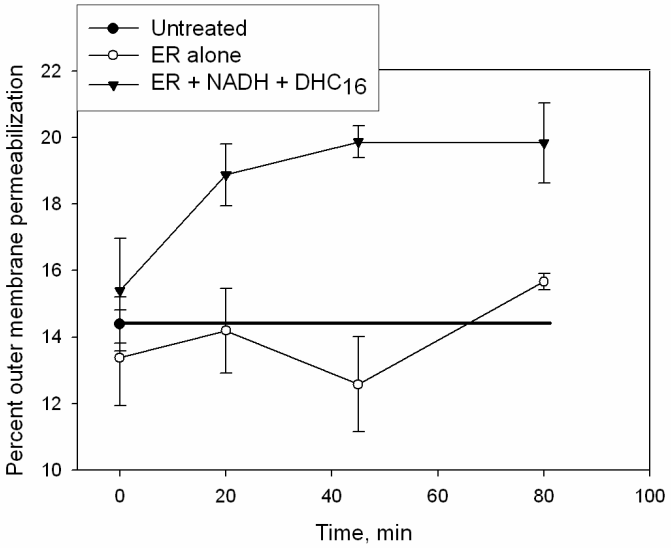

FIG. 4.6. The addition of ceramide-containing ER to DC-mitochondria induced permeabilization to cytochrome *c*. Mitochondria were incubated for the indicated amount of time with either untreated ER vesicles (○), ER vesicles pretreated with NADH and DHC_{16} (▼) or no treatment (●) followed by measurement of their ability to oxidize cytochrome *c*. The permeabilization is the cytochrome *c* oxidation rate expressed as a percent of the total oxidation rate following hypotonic shock. The results represent the mean value ± standard deviation of 3 experiments.

DISCUSSION

In the cell, lipid biosynthesis sometimes requires that intermediates move from one cell compartment to another. For instance, the endoplasmic reticulum is the main site of lipid biogenesis but mitochondria also play a role. Most of the enzymes of the phospholipid synthetic pathway reside in the ER (Bishop and Bell, 1988). However, some enzymes such as phosphatidylserine decarboxylase are located in mitochondria (Dennis and Kennedy, 1972). This enzyme is critical for the formation of phosphatidylethanolamine (PE) from phosphatidylserine (PS) (Dennis and Kennedy, 1972; Voelker, 1989; Vance, 1991). Thus, the synthesis of PE requires that PS, made in the ER, move into mitochondria where it is decarboxylated forming PE which returns to the ER (Vance, 1990; Vance, 1991; Shiao *et al.*, 1995; Gaigg *et al.*, 1995; Vance and Shiao, 1996).

The mechanism of PE and PS translocation between the ER and mitochondria has been investigated extensively and, while questions remain, the evidence indicates that membrane contact between the ER and mitochondria is responsible for rapid lipid exchange (reviewed comprehensively in Daum and Vance, 1997 and Voelker, 2005) rather than vesicular transport (Voelker, 1989; Vance *et al.*, 1991). A close interaction between ER and mitochondria has been inferred from functional studies (Meier *et al.*, 1981; Vance, 1990) and observed ultrastructurally (Pickett *et al.*, 1980; Katz *et al.*, 1983; Marsh *et al.*, 2001). Thus direct exchange of lipids between these compartments is the most likely mechanism and may be a general process.

The import of other phospholipids from the ER into mitochondria has also been studied (reviewed in Daum and Vance, 1997) but the exchange of sphingolipids has not. Sphingolipids are known to be transferred from the ER to Golgi and plasma membrane

by vesicular transport (reviewed in van Meer and Lisman, 2002; Futerman and Riezman, 2005) but it is not known how and whether sphingolipids travel between ER and mitochondria. Ceramide, the critical sphingolipid which induces apoptosis in a variety of ways, is synthesized in the ER by the *de novo* pathway but is also found in mitochondria (Bionda *et al.*, 2004). There, ceramide permeabilizes the outer membrane to apoptotic proteins signaling the start of irreversible mitochondria-based apoptosis (De Maria, 1997). Some enzymes in the ceramide synthetic pathway are found in mitochondria such as ceramide synthase (Bionda *et al.*, 2004) and neutral ceramidase (El Bawab *et al.*, 2000) but typically the former enzyme produces dihydroceramide, the inactive precursor. We found that the critical dihydroceramide desaturase (DDase) needed to generate ceramide from dihydroceramide is lacking in both mitochondrial membranes, thus indicating that complete *de novo* synthesis in mitochondria requires sphingolipid exchange with the ER. However, it is possible that mitochondrial DDase activity is absent or suppressed under normal conditions but becomes introduced or activated under special circumstances, e.g. during the initiation of apoptosis.

We found that both ceramide and dihydroceramide can exchange between ER and mitochondrial membranes. Using the DDase to oxidize 1-^{14}C-DHC$_8$ to 1-^{14}C-C$_8$ in the ER, we were able to find substantial transfer of both lipids to mitochondrial membranes. Since 1-^{14}C-C$_8$ was made in the ER membrane, all radiolabeled ceramide in mitochondria must have come from the ER. Of the total added 1-^{14}C-DHC$_8$, 75 % inserts in the ER membrane and the rest remains in the buffer. The reverse was also true. Incubating mitochondria with 1-^{14}C-DHC$_8$ before mixing them with ER led to an equal distribution of radioactivity in both membranes after 10 minutes of incubation. Furthermore, long-chain ^{3}H-C$_{16}$-ceramide, made in the ER by radiolabeled ^{3}H-sphingosine and palmitoyl

CoA, can also transfer to mitochondria, although slower than the medium-chained ceramide, as expected.

The mitochondrial ceramide level is important because elevation of ceramide levels in the outer membrane leads to the release of pro-apoptotic proteins. In a variety of systems, the steady state level of ceramide increases in mitochondria when the cell is preparing to undergo mitochondria mediated apoptosis (De Maria *et al.*, 1997; Zamzami *et al.*, 1995; Castedo *et al.*, 1996; Susin *et al.*, 1997; Pettus *et al.*, 2002). This increase coincides with the permeabilization of the outer membrane to intermembrane space proteins. This is not merely a correlation because the addition of ceramide to isolated mitochondria induces the release of cytochrome *c* from the intermembrane space (Arora *et al.*, 1997; Ghafourifar *et al.*, 1999; Di Paola *et al.*, 2000). The permeability of the mitochondrial outer membrane to proteins is enhanced by the incubation with ceramide in a dose- and time-dependent manner (Siskind *et al.*, 2002). This release of pro-apoptotic proteins (cytochrome *c*, Smac/DIABLO, AIF, etc.) starts the execution phase of apoptosis (Saelens *et al.*, 2004).

Among the various pro-apoptotic activities of ceramide (reviewed in Siskind, 2005), direct channel formation in the outer membrane accounts for the protein release. Ceramide forms channels traversing that membrane (Siskind *et al.*, 2002; Stiban *et al.*, 2006; Siskind *et al.*, 2006) and forms large, stable and highly organized channels in planar phospholipid membranes (Siskind and Colombini, 2000; Siskind *et al.*, 2003) and liposomes (Stiban *et al.*, 2006). Ceramide channels were also reported to be able to enhance solute efflux (Montes *et al.*, 2002) and release chloride ions (Pajewski *et al.*, 2005) from lipid vesicles. Molecular dynamics simulations support the stability and validity of the proposed ceramide channel structure (Anishkin *et al.*, 2006).

The formation of ceramide channels in mitochondria requires the elevation of steady state levels of ceramide. We show that *de novo* ceramide synthesis in the ER is sufficient to induce mitochondrial outer membrane permeability. Not only is ceramide exchanged quickly between ER and mitochondria, but also the small amount of ER associated with mitochondria isolated using differential centrifugation is able to produce ceramide and permeabilize the outer membrane to cytochrome *c* and adenylate kinase when the substrates of the DDase are provided. Thus, ER and mitochondria form a functional unit for ceramide generation and the release of proteins from mitochondria.

This functional unit obviates the need for a duplicate set of metabolic enzymes in both membrane systems. The ER has a complete *de novo* ceramide metabolic pathway so why are there any enzymes of this pathway in mitochondria? Ceramide synthase can convert both sphingosine and sphinganine to ceramide and dihydroceramide, respectively. In the *de novo* pathway, sphinganine is the precursor of ceramide and sphingosine is the product of ceramide hydrolysis. However, in the mitochondria the synthase may be used to recycle sphingosine into ceramide. Thus the activities of mitochondrial ceramide synthase and ceramidase may act to bias the system toward or away from apoptosis by controlling the steady-state ceramide level. This is a distinct function from the ER *de novo* pathway.

The mechanism of exchange of sphingolipids between ER and mitochondria likely occurs through membrane contact resulting from the close apposition of these organelles. Other mechanisms such as vesicular trafficking and protein-mediated exchange seem unlikely under the conditions of the experiments reported here but may well occur in intact cells. The quick transfer seen here tends to rule out the need for a vesicular transport system. An enzyme like CERT (Perry and Ridgway, 2005; Hanada,

2006) might be responsible for such a lipid trafficking if it were not for the fact that the differential centrifugation process removed the cytosolic proteins. Moreover, we showed that lipids can be transferred at similar rates from ER to either erythrocyte membranes or mitochondria. This non-specificity is consistent with simple membrane contact. In vivo, the cellular organization determines which membranes come in contact and thus where exchange occurs.

In conclusion, the elevation of mitochondrial ceramide levels that leads to protein release and end-stage apoptosis may arise from the synthesis of ceramide in the ER followed by rapid exchange to mitochondria at sites of close contact between these two organelles. The mitochondria lack the requisite DDase needed for independent complete *de novo* ceramide synthesis but has enzymes that may regenerate ceramide from the hydrolysis product, sphingosine. This ER-mitochondria collaboration is another example of cellular functions requiring the cooperative activity of both and leads us to support the notion of an ER-mitochondria functional unit.

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CHAPTER 5

A CANDIDATE FOR MITOCHONDRIAL OUTER MEMBRANE PERMEABILITY DURING APOPTOSIS: LARGE ORGANIZED CERAMIDE CHANNELS VISUALIZED

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Running title: ***TEM of ceramide channels***

KEYWORDS:

Transmission electron microscopy, ceramide channels, liposomes, planar membrane, structure, osmium tetroxide staining.

ABSTRACT

The method by which mitochondrial intermembrane space proteins are released into the cytosol to start the process of programmed cell death, or apoptosis, is still debated. The sphingolipid ceramide is a major player that mediates intrinsic apoptosis. One method of ceramide action that was proposed by our group involves the formation of organized ceramide channels in the mitochondrial outer membrane. In this study, we validated this claim by visualizing ceramide pores in liposomes using transmission electron microscopy. Those channels were of various sizes, averaging 10 nm in diameter. Using electrophysiological techniques, the estimated size of ceramide channels, judged from the conductance in a planar phospholipid membrane, was again around 10 nm in diameter. Moreover, those channels are large enough to allow the pro-apoptotic intermembrane space proteins to be released to initiate apoptosis; hence ceramide channels are a good candidate for the pathway of pro-apoptotic protein release from mitochondria.

INTRODUCTION

Mitochondria are at the center of cell viability (production of energy) and mortality (programmed cell death, or apoptosis). The release of intermembrane space proteins from mitochondria is a key step in the initiation of the apoptotic pathway. There is recent evidence that this release occurs through a large pore because the release occurs simultaneously for many intermembrane space proteins, such as cytochrome *c*, Omi, Smac and adenylate kinase-2 (Muñoz-Pinedo *et al.*, 2006). The pore is also proposed to be open for a long time because even though the onset of the release of AIF occurred with the rest of the proteins, it was slower (Muñoz-Pinedo *et al.*, 2006). The nature of this pore, however, is still under investigation. In concert, the sphingolipid ceramide was shown to initiate apoptosis and release intermembrane space proteins.

In the past decade, the interest in the sphingolipid, ceramide, and its role in ceramide-mediated apoptosis resulted in a large amount of research (for example: Hannun, 1996; Decaudin *et al.*, 1998; Jacotot *et al.*, 1999; Tomassini and Testi, 2002; Taha *et al.*, 2006). Many mechanisms of action of ceramide have been proposed (reviewed in Hannun and Obeid, 2002 and more recently Siskind, 2005).Whereas it is established that ceramide can interact with different cellular components and change or attenuate their function, its direct effect on mitochondria is becoming widely accepted. Our group was first to introduce the notion of large, ceramide-based channels being able to be formed in planar phospholipid membranes (Siskind and Colombini, 2000). Since ceramide was shown to lead to the release of pro-apoptotic proteins from mitochondria, the ceramide channel hypothesis was tested and validated on isolated mitochondria (Siskind *et al.*, 2002). We followed by the biophysical characterization of these channels (Siskind *et al.*, 2003). The ability of ceramide to permeabilize unilamellar liposomes was

also demonstrated (Stiban *et al.*, 2006). Molecular dynamics simulation of the proposed structure of self-assembled ceramide channels showed that such a structure can exist in a biological environment (Anishkin *et al.*, 2006).

Functional studies of ceramide permeabilizing lipid bilayers (Siskind and Colombini, 2000), mitochondria (Siskind *et al.*, 2002) and liposomes (Stiban *et al.*, 2006) as well as molecular dynamics simulations (Anishkin *et al.*, 2006) indicate that these channels are valid structures. Nevertheless, direct structural evidence on the existence of these channels was still lacking. Here we show that using osmium tetroxide fixation and uranyl acetate negative staining of diphytanoyl phosphatidylcholine (DPhPC)-cholesterol liposomes incorporating ceramide allows the visualization of these structures under a transmission electron microscope as stained spots on liposomes.

MATERIALS AND METHODS

Materials

Diphytanoyl phosphatidylcholine (DPhPC), asolectin (soybean phospholipids polar extract) and ceramides were from Avanti Polar Lipids (Alabaster, AL). Cholestrol was from Sigma (St. Louis, MO).

Electrophysiological Recordings

Using the planar membrane technique (Montal and Mueller, 1972; Schein *et al.*, 1976) as modified (Colombini, 1987), ceramide channels were studied. The lipids used to form the membrane were hexane-based containing 5:5:1 DPhPC, asolectin, cholesterol (w/w/w). Lipid monolayers were used to form a bilayer. The current is measured as the voltage across the membrane is clamped. The aqueous solution contained 100 mM KCl, 5mM $MgCl_2$, 5mM PIPES (pH 7.0). All the experiments were performed at room temperature. The voltage is controlled in the *cis*-side. Ceramide was added to both sides of the chamber, typically 25 μL of 0.1 mg/mL or 2.5 μL of 1 mg/mL. The ceramide solution was made in DMSO.

Conversion between Conductance and Size

The conversion between the conductance (reciprocal of the resistance) and the radii is according to the equation (Hille, 2001):

$$R_{channel} = \left(l + \frac{\pi a}{2} \right) \frac{\rho}{\pi a^2}, \qquad \text{Eq. (1)}$$

where $R_{channel}$ is the resistance of the channel (which equals 1/conducatance of the channel); l is the length of the channel (about 5 nm, the length of the bilayer); a is the

radius in nm of the channel and ρ is the resistivity of the solution (which is equal to 0.775 nm/nS for 0.1 M KCl solution). The radius of a channel can therefore be written as a function of the conductance by solving the quadratic equation and using the positive root:

$$a = G + \sqrt{\frac{G^2 + 32.8G}{5.16}}. \qquad \text{Eq. (2)}$$

Conversion between Molecular Weight and Size

The conversion between the molecular weight of a globular protein and its radius is according to the equation (Tinoco *et al.*, 2002):

$$R = \sqrt[3]{\left(\frac{3M_r \bar{\upsilon}}{4\pi N_A}\right)}. \qquad \text{Eq. (3)}$$

where R is the radius of the sphere, M_r is the molecular weight, υ is the partial specific volume of proteins (about 0.74 cm^3/g) and N_A is Avogadro's number.

Liposome Formation

DPhPC:cholesterol (94:6 mole percent) liposomes were made. Breifly, 500 µL of 4 mg/mL DPhPC were mixed with 60 µL of 1 mg/mL cholesterol were mixed and dried under nitrogen. The dried lipids were left in house vacuum overnight.

Using 10 mM NaCl, 10 mM HEPES, 1 mM EDTA, pH 7.2, the liposomes were reconstituted. After letting the liposomes swell for 3 minutes, a cycle of freeze-thaw-sonication (4 times) was performed followed by a freeze-thaw and extrusion (17 times through a 0.2 µm polycarbonate membrane) cycle.

The extruded liposomes were then diluted to 8 mL with buffer. Then, they were divided into 4 vials. Ceramide (C_{16}) and dihydroceramide (DHC_{16}) were added as 40 µL of 1

mg/mL solution in isopropanol. The control contained 40 µL of isopropanol. Each vial was then diluted to 8 mL with buffer and spun for 105,000 g for 4 hours at 4°C. The supernatant was removed gently and the pellet was suspended in the final drop of supernatant.

Liposome Staining

An electron microscope grid was floated on a drop of liposomes for 5 minutes, and then was blotted dry using a filter paper. The grid was then floated on a drop of 2% OsO_4 for 5 minutes before blotting it. Finally, the grid was put on top of a 1% aqueous uranyl acetate solution for 1 minute. The grid was blotted and visualized by TEM.

Transmission Electron Microscopy

Electron microscopy was performed in a Zeiss EM following the protocols by the manufacturer.

Mitochondrial Isolation

Mitochondria were isolated by differential centrifugation per previously published methods (Parsons *et al.*, 1966; Siskind *et al.*, 2002; Stiban *et al.*, 2006).

Protein Release, SDS-PAGE, BN-PAGE and Gel Filtration

Mitochondria (2.4 mg protein) were treated with protease inhibitors (20 µM PMSF, 1 µg/mL leupeptin, 1 µg/mL pepstatin, 10 µg/mL chymostatin, 4 µg/mL aprotinin). The mitochondria were then incubated with 240 µg C_{16}-ceramide (120 µL of 2 mg/mL in isopropanol), 40 µg C_{16}-ceramide or 120 µL isopropanol (control) or shocked

in double distilled water. After 10 minutes at room temperature, the mitochondria were spun down (30,000 rpm for 30 minutes at 4^{o}C) and the supernatant was collected and concentrated in Amicon centricon tubes (5,000 g for about 1.5 hours). The concentrated proteins were collected in volumes between 70 and 80 µL. Those proteins were then loaded separately on a Sephacryl S-200 gel filtration column (30 mL total bed volume). Proteins in the eluted volume were detected by a UV-light monitor measuring the absorbance at 280 nm and the traces were recorded on the computer by Clampfit software.

Alternatively, the proteins were assayed using SDS-PAGE on 12% pre-made gels from Pierce (Rockford, IL) using Tris-HEPES as the buffer system per the manufacturer's instructions.

RESULTS AND DISCUSSION

Ceramide channels are visualized by TEM

Figure 5.1A and B show two fields of liposomes with ceramide channels (arrows), whereas in C a control group of liposomes that does not have ceramide (no observable stained spots) is shown. Many controls were done using vehicle (isopropanol) only or dihydroceramide (a non-apoptogenic, non-channel former) and they indeed show no stained spots similar to the ones observed with ceramide (all bars represent 100 nm).

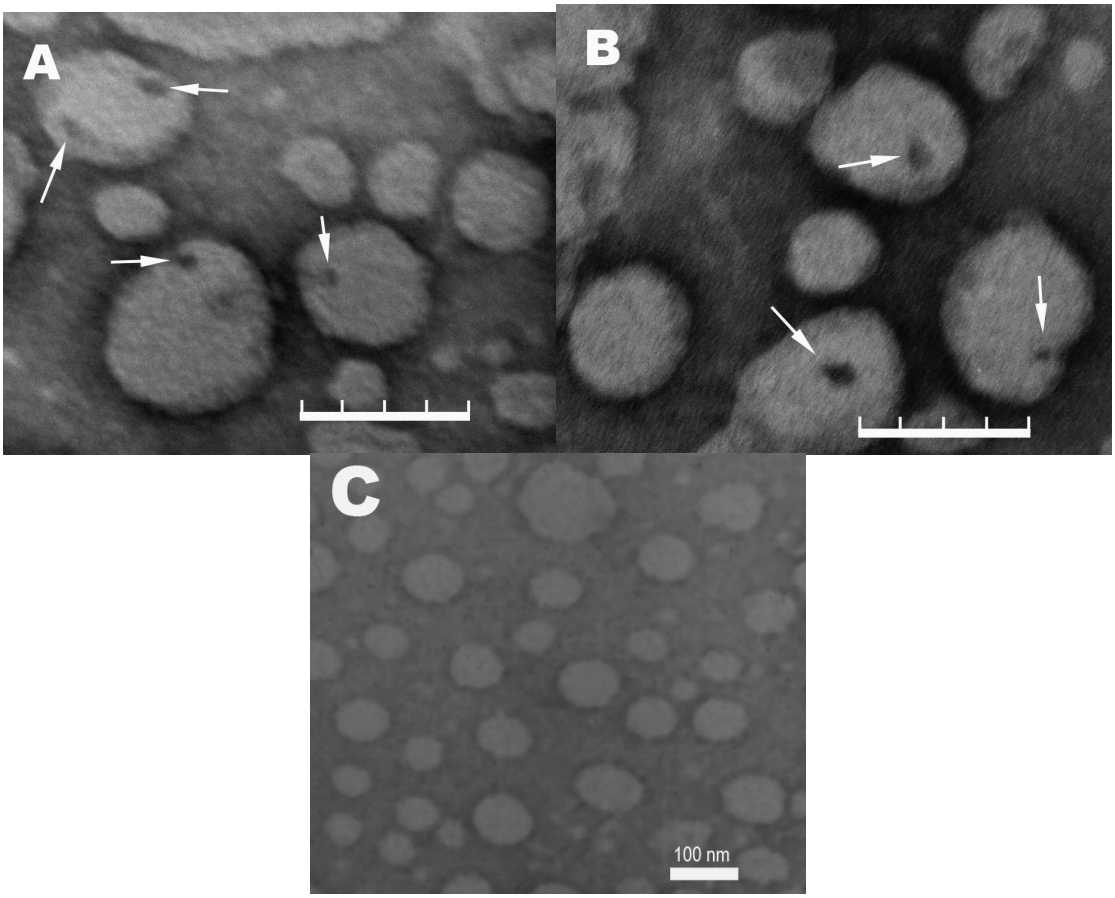

FIG. 5.1. Ceramide channels are visualized by TEM. DPhPC liposomes with (**A** and **B**) or without (**C**) ceramide were fixed with 2% OsO_4 and stained with 1% uranyl acetate. Ceramide channels were stained and can be seen by the pointed arrows. The bars represent 100 nm.

The diameter of ceramide channels is around 10 nm

Using the same lipid DPhPC combined with asolectin (a soy bean extract of lipids) planar phospholipid membranes were tested for the capability of ceramide to induce pores in them. As previously reported from our group (Siskind and Colombini, 2000), both long and short chain ceramides are able to form pathways (channels) across planar phospholipid membranes that allow metabolites and proteins to pass through. These channels can be very large, according to their conductance. In a typical planar membrane experiment (Fig. 5.2), short- or long-chain ceramide is added to a zero-conductance membrane. After some time, ceramide self-assembles into forming a channel in the membrane allowing current to flow and the conductance grows.

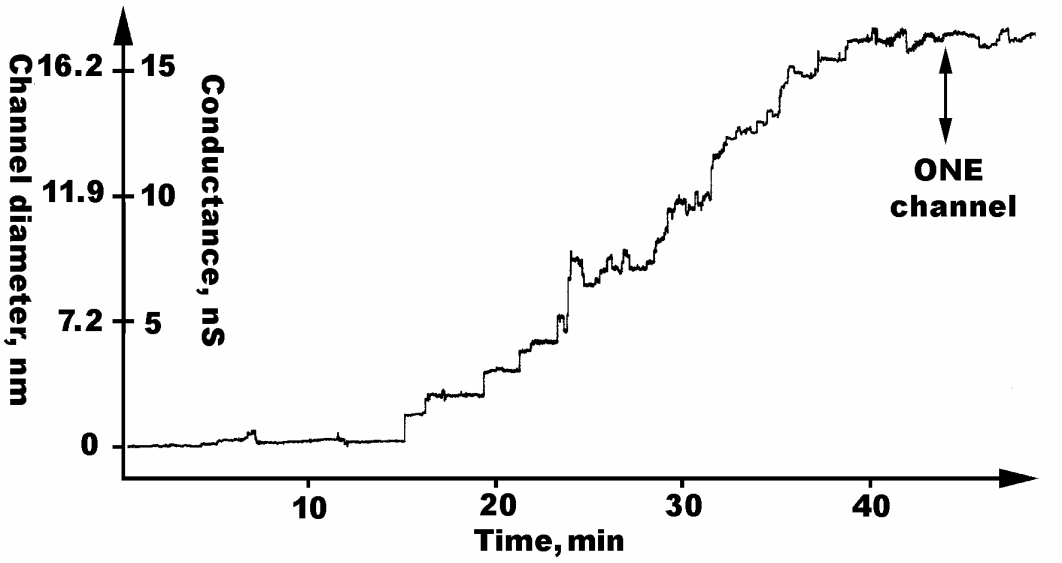

FIG. 5.2. Ceramide channel growth in a planar phospholipid membrane. A trace of ceramide conductance increase in a planar phospholipid bilayer as a function of time. Also, the channel diameter is plotted on the y-axis and this was calculated from the equation as explained in materials and methods. The ceramide was added at time = – 40 min.

As shown previously (Siskind *et al.*, 2003), the conductance represents a single channel growing in size rather than a set of small pores in the planar membrane. Therefore, the total membrane conductance of this channel can be indicative of its diameter. After the membrane has reached a steady level of conductance, this conductance is measured and, from equation 2, the diameter was calculated. The range of ceramide channel radii in planar membrane experiments varied from less than 1.3 nm (<1 nS of total membrane conductance) to about 88.3 nm (220 nS). The diameter of the channels was determined for many records achieving maximal conductance and the data were pooled in a histogram (Fig. 5.3 (bottom and inset)). From the electrophysiological recordings, the channel size distribution followed a Gaussian with a preference for a diameter around 5 to 15 nm. The inset to this figure is the full trace of all the diameters of channels observed. Jointly, many pictures were taken for ceramide channels visualized by TEM. The diameter of the stained spot was measured and it varied from channel to channel. Again, the distribution of diameters adheres to a Gaussian with an average size of 15 nm (Fig. 5.3 (top)). This is a large channel (considering the bilayer thickness of 5 nm and the size of the large outer membrane channel VDAC of 3 nm).

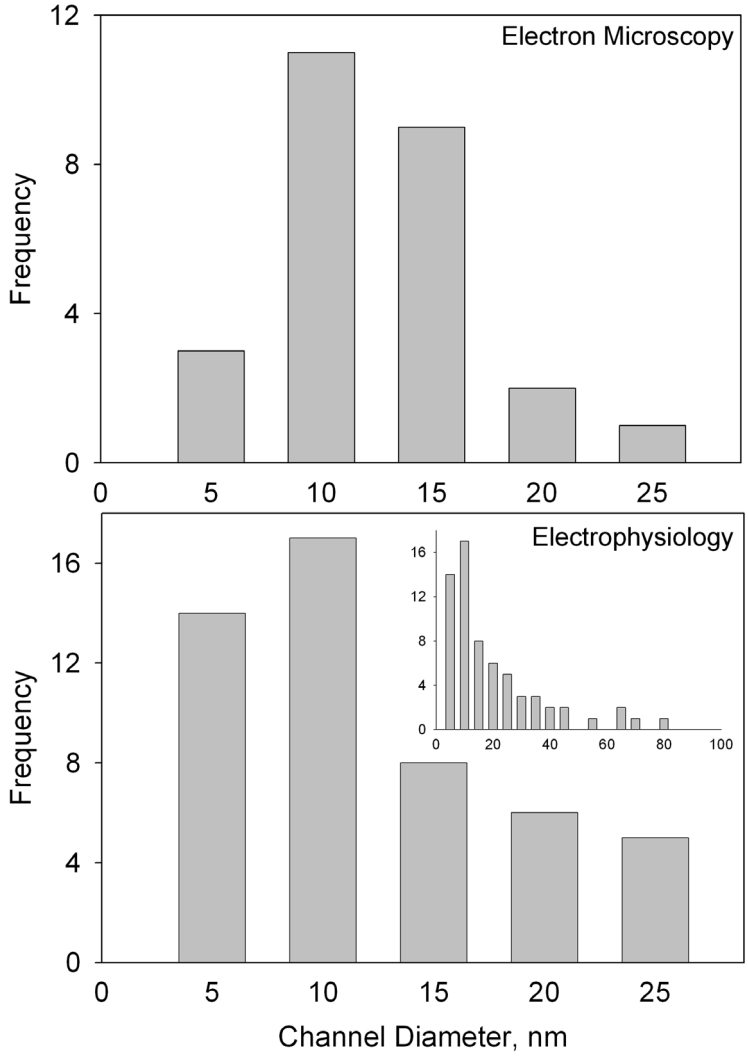

FIG. 5.3. The preferable diameter of ceramide channels is between 5 and 15 nm. Histograms of channel diameters measured by electron microscopy (top) and electrophysiology (bottom) are shown. In synthetic systems (liposomes, top and planar membranes, bottom), ceramide channels have a preference for diameters between 5 and 15 nm.

Ceramide channels have a cutoff size between 115 – 135 kDa

After determining the size of the channel in 2 synthetic systems, we decided to determine the physiological size of the channel by looking at the size of the proteins that are released from mitochondria following ceramide addition. Previously, our lab showed that ceramide added to mitochondria caused a release of proteins up to a molecular weight of 60 kDa (Siskind *et al.*, 2002). Ceramide was added from an ethanol stock solution and only 15 % of the total intermembrane space proteins were released. Here we optimized the addition and insertion of ceramide into mitochondria and had a percent release of about 60-90 %, using isopropanol as the vehicle and adding ceramide while vortexing. The cutoff size of ceramide channels in mitochondria was measured by two methods: SDS-PAGE and gel filtration. Mitochondria were either shocked by dilution in double distilled water to release all the intermembrane space proteins, or treated with different doses of C_{16}-ceramide. The released proteins were concentrated and run as native proteins on a 30×1.5-cm Sephacryl S-200 gel filtration column or denatured into polypeptides and separated on SDS-PAGE. In the gel filtration experiment, the eluted proteins were quantitated by measuring the absorbance at 280 nm. Figure 5.4A shows the absorbance profiles of the intermembrane space proteins released by hypotonic shock and 1:10 ceramide:mitochondrial protein (w/w) (samples from vehicle-treated mitochondria were subtracted from both traces) as a function of molecular weight. Figure 5.4B presents the proteins released by ceramide as a percentage of the proteins released by the hypotonic shock. A cutoff of around 135 kDa is observed from these profiles.

The SDS-PAGE analyzed protein fractions (figure 5.4C) show a smaller cutoff. With increasing concentrations, the ceramide is able to release more proteins until it approaches the total amount of protein present (as in shocked). Vehicle controls were

used and subtracted as background. In figure 5.4D, the intensity of the released proteins from the gel is plotted against the molecular weight of each band. There is a clear cutoff of about 115 kDa. This is inconsistent with the cutoff measurement by our lab (Siskind *et al.*, 2002), however, the delivery method of ceramide is different (more efficient with isopropanol as a solvent). The two methods agree that the size of the ceramide channel in mitochondria within 10 minutes of incubation is sufficient to release large molecular weight intermembrane space proteins. This corresponds to a diameter of around 6.5 nm (Eq. 3). This is on the lower side of the sizes measured in electrophysiology or TEM experiments. However, since mitochondrial membranes have various proteins and/or lipids that may have an impact on channel formation and growth, this result does not seem too surprising. The possibility that mitochondria can regulate the ceramide channel size may be important in the regulation of the starting phase of apoptosis. If, for instance, the factors that limit the enlargement of the channel are active, ceramide might not be able to release intermembrane space proteins, even though the concentration of ceramide is sufficient. On the other hand, if those factors are inactivated the channel may grow and the proteins can be released to start apoptosis.

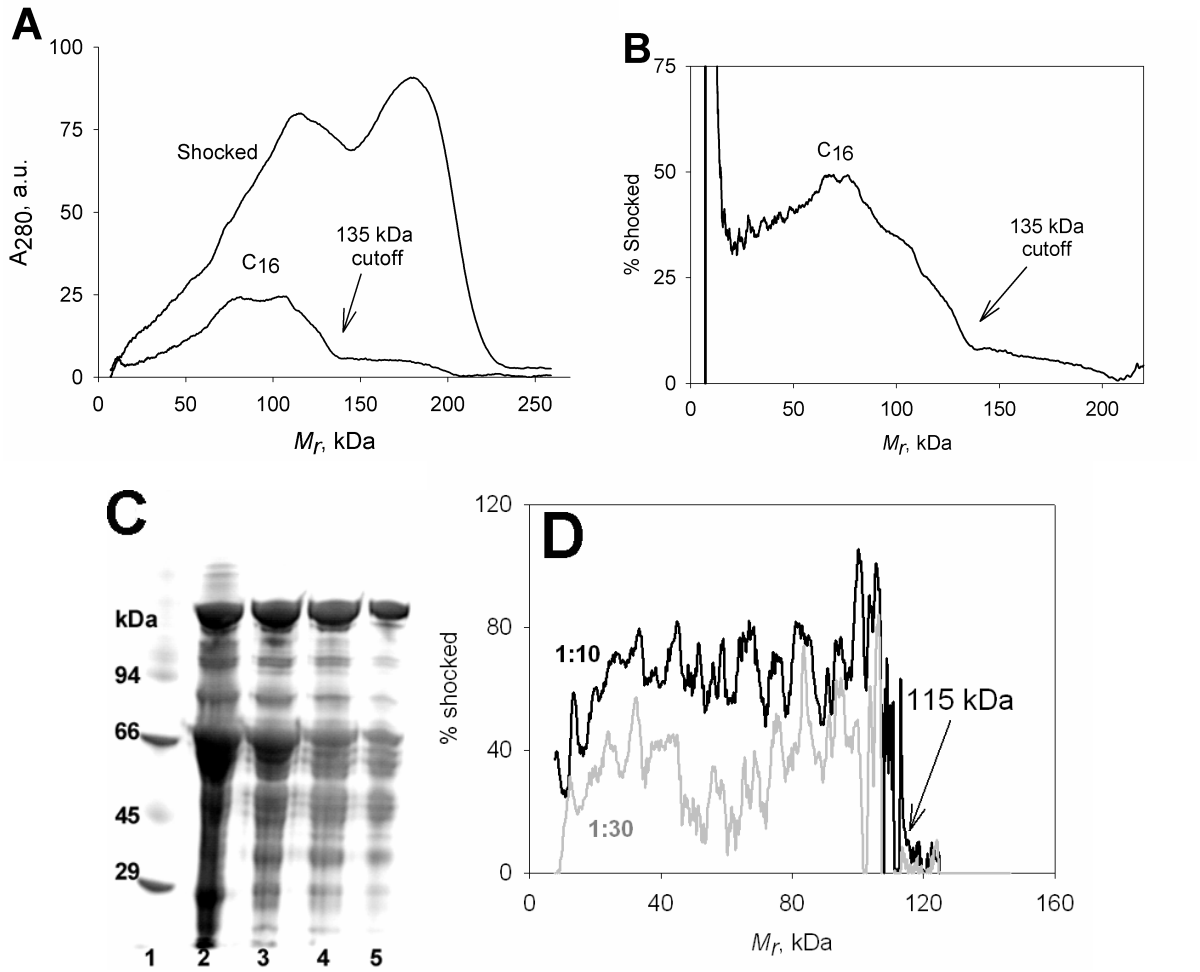

FIG. 5.4. Ceramide forms channels in mitochondria with a cutoff of between 115 and 135 kDa. (**A**) Gel filtration size-exclusion chromatography of released proteins shows a cutoff size of around 135 kDa. Intermembrane space proteins released by hypotonic shock and by 1:10 C_{16}-ceramide:mitochondrial protein (w/w) are detected as they come out of the column by an absorbance monitor. The background, isopropanol treated mitochondrial trace was subtracted from each. The ceramide-treated mitochondria show a scale-down version of the total protein release, however, there is a clear lack of release by ceramide beyond the 135 kDa point. The void volume of the column corresponded with M_r of 215 kDa. (**B**) The ceramide trace is plotted as the percentage of the shocked. The cutoff is evident at 135 kDa. (**C**) An SDS-PAGE gel of intermembrane space proteins released by **2**. hypotonic shock, **3**. 1:10, **4**. 1:30, and **5**. vehicle treatment. Lane **1**. shows the standards with the corresponding M_r in kDa. (**D**) A densitometry plot showing the percent protein released by the 2 doses of ceramide (lanes **3** and **4**) compared to the total proteins (lane **2**). The background (lane **5**) was subtracted.

Of the various pathways proposed to release proteins from mitochondria to initiate the execution phase of apoptosis (Crompton *et al.*, 1998; Basañez *et al.*, 1999; Pastorino *et al.*, 1999; Antonsson *et al.*, 2000; Belaud-Rotureau *et al.*, 2000; Saito *et al.*, 2000; Antonsson *et al.*, 2001; Pavlov *et al.*, 2001; Kuwana *et al.*, 2001), only the ceramide channel is now well characterized. There is a proposed structure that has been shown to be stable by molecular dynamics simulation (Anishkin *et al.*, 2006). Here we present direct visualization of the channels. Ceramide levels increase in mitochondria early in apoptosis (Garcia-Ruiz *et al.*, 1997; Matsko *et al.*, 2001; Dai *et al.*, 2004; Birbes *et al.*, 2005) to levels sufficient to generate ceramide channels (Siskind *et al.*, 2006). Thus a strong case can now be made for ceramide channels being the pathway for mitochondrial protein release.

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CHAPTER 6

FUTURE DIRECTIONS

In this dissertation, I presented my work on ceramide, its metabolism, its transport and its channel-formation ability. Ceramide is a very interesting lipid to study because of its unusual characteristics and its importance for the life and death of the cell. Future studies on this subject are currently under way, so in this chapter I will illustrate feasible directions to study the ceramide phenomenon.

Membrane-specificity of ceramide channel formation

As was previously mentioned, ceramide can form channels in planar phospholipid bilayers, synthetic lipid vesicles and mitochondrial outer membranes. Moreover, ceramide cannot permeabilize the inner membrane of mitochondria, nor does it form channels in the plasma membrane. This raises the question of membrane specificity. The lipid environment could be very crucial in allowing ceramide molecules to form energetically favorable channels.

A future work regarding the lipid specificity should be investigated using different lipid compositions to test channel formation activity in carboxyfluorescein-DPX-filled liposomes. This work may help illustrate the requirement of certain lipids over others to favor channel formation. This could lead to further mechanistic studies showing the interactions between membrane lipids and ceramide molecules.

ER permeabilization by ceramide

Since the ceramide is almost exclusively made in the endoplasmic reticulum, it is important to see whether it can form channels there. Certainly, this goal coincides with the pervious one of testing the lipid dependence of channel formation. Nevertheless, the complexity of the ER system and the variety of proteins and factors available on that membrane may influence the results of pure lipid dependence experiments.

Isolated microsomes can be assessed for their intactness (in other words, permeability) using a substance called 4-methylumbelliferryl α-glucoside (4MαG) taking advantage of the ER enzyme α-glucosidase (Heritage and Wonderlin, 2001). The accessibility of this substance to the enzyme will lead to an increase in the fluorescence of the product, 4-methylumbelliferrone. Hence, the permeability of the ER membranes can be proportional to the rate of hydrolysis of 4MαG.

A dose dependence, as well as time dependence, of exogenously-added ceramide permeabilization of ER membranes can be done to see if ceramide is able to form channels in the membrane in which it is synthesized. Provided that ceramide can form channels in the ER, a protein analysis of the released ER proteins should follow. Another experiment one could think of is to see whether the endogenously-made ceramide (by the *de novo* synthesis, using dihydroceramide and NAD(P)H as substrates) can also permeabilize the ER.

Ceramide and VDAC collaboration?

The main mitochondrial outer membrane channel is the Voltage-Dependent Anion Channel, VDAC. The role of VDAC in the programmed cell death still remains controversial. VDAC is too small to allow proapoptotic proteins to pass to initiate

apoptosis. There are some indications that VDAC may be a part of the permeability transition pore complex, PTPC (Szabo and Zoratti, 1993; Szabo *et al.*, 1993; Crompton *et al.*, 1998; Marzo *et al.*, 1998). Other results rule out the presence of VDAC in PTPC (Halestrap *et al.*, 2002). However, more convincing experiments show that the closure of VDAC is important to initiate the cytochrome *c* dependent apoptotic pathway (Doran and Halestrap, 2000; Vander Heiden *et al.*, 2000). Can the closure of VDAC and the elevation of ceramide levels in mitochondria be integrated? Does the closure of VDAC allow the recruitment of ceramide to the mitochondria? Ceramide was shown to permeabilize yeast mitochondria lacking VDAC1, but is there a common ground between these two, very different, outer membrane channels?

Interaction between ceramide and Bcl-2 family proteins

Ceramide's interaction with Bcl-2 family proteins, whether anti-apoptotic (for instance Bcl-xL and Bcl-2) or pro-apoptotic (e.g. Bax) is currently being studied. Our lab has shown that Bcl-2 and Bcl-xL are able to disassemble ceramide channels in planar phospholipid membranes. More mechanistic experiments designed to see if there is actual binding of ceramide to these proteins should be done in order to specifically deduce that the action of these proteins is directly projected on ceramide.

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