Ceramide synthesis in the endoplasmic reticulum can permeabilize mitochondria to proapoptotic proteins

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Abstract **Increased mitochondrial ceramide levels are associated with the initiation of apoptosis. There is evidence that ceramide is causal. Thus, the conversion of the precursor, dihydroceramide, to ceramide by the enzyme dihydroceramide desaturase may be important in preparing the cell for apoptosis. Ceramide can initiate apoptosis by permeabilizing the mitochondrial outer membrane to apoptosis-inducing proteins. However, the mitochondrion's ability to produce ceramide may be limited by its proteome. Here, we show that ceramide synthesized in isolated mammalian endoplasmic reticulum (ER) vesicles from either C_8-dihydroceramide or sphingosine to produce long-chain ceramide can transfer to isolated mitochondria. The rate of transfer is consistent with a simple collision model. The transfer of the long-chain ceramide is faster than expected for an uncatalyzed process. Sufficient ceramide is transferred to permeabilize the outer membrane to cytochrome *c* and adenylate kinase. The mitochondria-associated membranes, ER-like membranes that are tightly associated with isolated mitochondria, can produce enough ceramide to permeabilize the outer membrane transiently. Thus, this ceramide exchange obviates the need for a complete ceramide de novo pathway in mitochondria to increase ceramide levels to the critical value required for functional changes, such as ceramide channel self-assembly followed by protein release.—** Stiban, J., L. Caputo, and M. Colombini. **Ceramide synthesis in the endoplasmic reticulum can permeabilize mitochondria to proapoptotic proteins. *J. Lipid Res.* 2008.** 49: **625–634.**

Supplementary key words lipid transport • mitochondria-associated membranes • ceramide channels • apoptosis • dihydroceramide desaturase

Apoptosis is a form of programmed cell death by which cells perish without harming surrounding cells. Mitochondria can initiate apoptosis by releasing key proteins (1, 2). Many factors are known to contribute to the initiation of mitochondria-mediated apoptosis, but the factors responsible for the crucial, decision-making step, the formation of the protein release pathway in the outer membrane, are still debated. In addition to generally accepted factors such as Bid, Bax, and Bak, the sphingolipid ceramide was shown to be able to form large, stable, protein-permeable channels in planar phospholipid membranes (3), in isolated mitochondria (4), and in liposomes (5). Ceramide channel formation depends, in part, on the steady-state level of free ceramide in the membrane; thus, increased ceramide levels in mitochondria lead to channel formation and protein release (6).

Ceramide generation varies depending on location, forming different pools of ceramide that serve diverse functions (7, 8). The highly insoluble nature of ceramide means that there is a large energy barrier inhibiting the movement of ceramide from one membrane to another, thus tending to isolate the different pools. The two major pathways of ceramide generation are sphingomyelin hydrolysis and de novo synthesis. The former occurs in a variety of membranous systems, including the plasma membrane (9), whereas the latter is mainly found in the outer leaflet of the endoplasmic reticulum (ER) (10). The de novo synthesis (for reviews, see Refs. 11, 12) involves the condensation of serine with a fatty acyl-CoA (normally, palmitoyl-CoA). This reaction, catalyzed by serine palmitoyltransferase complex (13, 14), produces ketosphinganine, which, after being reduced to sphinganine (dihydrosphingosine), is *N*-acylated to dihydroceramide by ceramide synthase. The oxidation of dihydroceramide at the 4,5 position by dihydroceramide desaturase (DDase) produces ceramide.

Many reports (reviewed in Ref. 11) suggest the importance of the de novo pathway in producing the ceramide that is involved in mitochondria-mediated apoptosis. The source of this ceramide is unclear. Ceramide synthase has been found in mitochondria (15). In addition, ceramidase has also been demonstrated there, and this enzyme can drive the synthesis of ceramide through a reverse ceramidase activity (15–17). Moreover, mitochondria are closely associated with smooth membranous sacs that resemble the ER membranes. These ER-like membranes are referred to as mitochondria-associated membranes (MAMs) (18–22). Thus, perhaps ceramide is made

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Abbreviations: DDase, dihydroceramide desaturase; DHC_{16}, *N*-palmitoyl-D-*erythro*-sphinganine; 1-^{14}C-DHC_8, *N*-[1-^{14}C]octanoyl-D-*erythro*-sphinganine; ER, endoplasmic reticulum; MAM, mitochondria-associated membrane.

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in these membranes and transferred to mitochondria. A soluble protein called CERT has been described that carries ceramide from the ER to the Golgi apparatus (23). Perhaps some unknown protein might deliver ceramide to mitochondria. Finally, the demonstration that sphingomyelinase targeted specifically to mitochondria induces apoptosis (24) indicates that the sphingomyelinase pathway is not important in mitochondria, because normally there is insufficient sphingomyelinase activity to induce apoptosis.

In this study, we provide evidence that ceramide made in ER membranes can move to mitochondria and that sufficient amounts are translocated to result in the permeabilization of the outer membrane to proteins.

MATERIALS AND METHODS

Rats (Sprague-Dawley males) were euthanized in conformity with Public Health Service policy, and the protocol was 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). Horse heart cytochrome *c*, 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 (Geel, Belgium). [^{14}C]octanoic acid and [^{3}H]sphingosine were from American Radiolabeled Chemicals (St. Louis, MO). *N*-Hydroxysuccinimide and dicyclohexylcarbodiimide were from Fisher Scientific. Thin-layer chromatography was performed on Analtech (Newark, DE) silica gel plates. Analytical-grade DMSO (Fisher Scientific) was used to dissolve C_2-dihydroceramide, whereas isopropanol (Acros Organics) was used to dissolve C_8-ceramide, C_8-dihydroceramide, C_{16}-ceramide, and C_{16}-dihydroceramide.

Preparation of radiolabeled C_8-dihydroceramide

Sphinganine was acetylated using [1-^{14}C]octanoic acid, according to the procedure of Schulze, Michel, and van Echten-Deckert (25). 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 min, 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 min, 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 mg (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 of the radioactivity). The developing system was $CHCl_3/CH_3OH/H_2O$ (80:10:1, v/v/v). By TLC, the radioactive band was recovered after being detected with a Geiger-Müller counter, scrapped off the silica gel, and extracted in $CHCl_3/CH_3OH$ (2:1, v/v). Multiple extractions of the silica gel were made. Further separation of octanoic acid from the product *N*-[1-^{14}C]octanoyl-D-*erythro*-sphinganine (1-^{14}C-DHC$_8$), or C_8-dihydroceramide, was not needed because the reaction apparently consumed all reactants (as shown by the visualization of the plate in a PhosphorImager). The final concentration of 1-^{14}C-DHC$_8$ was 18 μM in isopropanol.

Mitochondria

Mitochondria were isolated from male Sprague-Dawley rat liver as described by Parsons, Williams, and Chance (26) as modified (4, 5). Briefly, livers from overnight-fasted young rats (generally, 200–300 g) 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 fatty acid-free BSA, pH 7.4; HB buffer). The liver was minced and homogenized in a motorized Potter homogenizer with a loose Teflon pestle (two passes). Differential centrifugation followed, starting at 760 *g* for 10 min at 4°C. To recover the mitochondria, the supernatant was then spun for 10 min at 9,000 *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 using a spectroscopic method (27). Reduced cytochrome *c* was prepared by mixing 5.5 mg of cytochrome *c* with 2 mg of sodium ascorbate in 0.25 ml of buffer Q (200 mM HEPES and 10 mM EGTA, pH 7.5). The reduced cytochrome *c* was separated from the ascorbate on a Sephadex G-10 gel filtration column preequilibrated with buffer Q (after elution, the concentration of reduced cytochrome *c* is ~1 mM).

Ultrapure mitochondria isolation

Mitochondria isolated by differential centrifugation were layered on top on a 28% self-generating Percoll gradient according to Holden and Colombini (28), and the tube was spun at 39,000 g_{max} for 30 min. The mitochondrial fraction was collected and diluted in H buffer and spun at 9,000 *g* for 10 min to remove the Percoll.

The ER

Here, we use the term ER for the isolated microsomes, which have different components but are mostly composed of ER membranes. The ER was separated according to a slight modification of a published procedure (25). Briefly, the first 9,000 *g* supernatant from the mitochondrial isolation was spun down at 105,000 *g* for 1 h. The pellet was suspended in H buffer and centrifuged again at 9,000 *g* for 10 min. The supernatant represents the ER fraction. The protein concentration in the supernatant was determined using the bicinchoninic acid protein assay kit from Pierce (Rockford, IL).

Desaturase assay

Desaturase activity was assayed in mitochondrial and ER fractions according to a published procedure (25). One modification was the use of isopropanol to dissolve dihydroceramides before dispersal into the aqueous phases. We chose conditions and concentrations of these agents that resulted in no significant sedimentation of dihydroceramide micelles (>99% of the radioactivity remained in the supernatant when the solution was centrifuged at 105,000 *g*, the speed used to sediment ER). By

contrast, dispersal of ceramide dissolved in either ethanol or DMSO results in its sedimentation unless special precautions are taken (6).

Mitochondrial inner membrane desaturase assay

Three hundred microliters of 21 mg/ml mitochondria was mildly shocked by adding 0.9 ml of double distilled water and kept on ice for 5 min. Osmolarity was restored by adding 0.9 ml of R×2 buffer (0.6 M mannitol, 20 mM NaH_2PO_4, 10 mM $MgCl_2$, and 20 mM KCl, pH 7.2). The final concentration of mitochondria was 3 mg/ml. To assess DDase activity, to 1 ml of the shocked mitochondria was added 36 μl of M/G buffer (70 mM malate and 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), and 10 μl of 1-^{14}C-DHC_8. The reaction was run for 45 min at 37°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, Michel, and van Echten-Deckert (25) 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 min. The plates were removed and dried at room temperature for 2 h. After the lipids were extracted, they were spotted on the 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-chain lipid transfer experiments

The transfer of radiolabeled lipids from the ER to mitochondria was measured as follows. A total of 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 min. An equivalent amount of mitochondria was added, resulting in a total volume of 1.5 ml. The tube was vortexed and incubated at 37°C for 2, 5, or 10 min before being spun down at 9,000 *g* for 10 min. 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-chain lipid transfer experiments

ER preparations (8.1 ml of 3 mg/ml protein) were mixed with 1.5 μl of [^{3}H]sphingosine (1.5 μCi; specific activity, 20 Ci/mmol) with and without 100 μM palmitoyl-CoA (900 μl of 1 mM) and incubated at 37°C for 15 min (29). 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 h. This step was repeated twice. After resuspending the ER pellet in 9 ml of H medium and distributing it in three 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 min at room temperature before spinning at low speed (9,000 *g* for 10 min) to recover mitochondria and then at high speed (105,000 *g* for 1 h) to retrieve the ER. For the control (no palmitoyl-CoA), the incubation with mitochondria was performed for 10 min only. Aliquots from the pellets were taken to measure the ER marker enzyme. The lipids were extracted from the pellets with $CHCl_3/CH_3OH$ (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 overnight and applied to a TLC plate. The mobile phase used was 99:1 (v/v) diethylether/CH_3OH (29). The ceramide and sphingosine bands were scraped off and counted.

Permeabilization of the mitochondrial outer membrane after NADH and dihydroceramide addition

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 *N*-palmitoyl-D-*erythro*-sphinganine (DHC_{16}; 19 nmol) for various times. After the incubation, the mitochondria were spun down at 6,500 rpm for 5 min (at 4°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). At time zero, 15 μl of 1 mM reduced cytochrome *c* was added. The initial rate of the reduction of absorbance at 550 nm of the added cytochrome *c* was used as a measure of the permeabilization of the mitochondrial outer membrane (4, 5). It was expressed as a percentage of the rate measured with the same amount on hypotonically shocked mitochondria. Vehicle control treatments were performed as appropriate.

Monitoring outer membrane permeability after exposure to ER

Isolated ER (2 mg of protein) was preincubated with 3 mM NADH and 28 nmol DHC_{16} in 1 ml of H buffer for 0, 20, 45, and 80 min. 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 min before 15 μl of 1 mM reduced cytochrome *c* was added to measure the cytochrome *c* oxidation rate. The difference in absorbance at 550 and 600 nm was used to record the cytochrome *c* oxidation, because adding ER to mitochondria caused an increase in 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.

Release of adenylate kinase

Isolated mitochondria (200 μg of 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 min, 1 ml was taken out and spun at 14,000 rpm. A total of 900 μl of supernatant was recovered and put on ice. In each experiment, 300 μl of the supernatant was mixed with 700 μl of the reaction mixture 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 1 min, 9 μl of the enzyme mixture (25 units of hexokinase and 25 units of glucose-6-phosphate dehydrogenase) was added, and the production of NADPH at 340 nm was monitored. In the experimental trials, 2.4 mM NADH, 74 nmol of DHC_{16}, or 2.4 mM NADH + 74 nmol DHC_{16} was added to the control mixture. The spectrophotometer was blanked with the appropriate solution.

ER marker enzyme assay

The marker enzyme for the ER was NADPH:cytochrome *c* reductase, and the assay was according to Ernster, Siekevitz, and Palade (30).

RESULTS

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

It has been shown that the addition of ceramide to isolated mitochondria leads to the permeabilization of the outer membrane to proteins (4–6). Mitochondria isolated by differential centrifugation were incubated with NADH and long-chain DHC_{16}. At the indicated times (**Fig. 1A**), they were centrifuged to remove the excess NADH and tested for their ability to oxidize exogenously added cytochrome *c*. The rate of cytochrome *c* oxidation is a measure of the permeabilization of the outer membrane to proteins and is expressed as a percentage of the rate measured after damaging the outer membrane by hypotonic shock. The permeabilization was plotted as a function of time of incubation with NADH and DHC_{16} (Fig. 1A). The permeabilization of the outer membrane to cytochrome *c* that

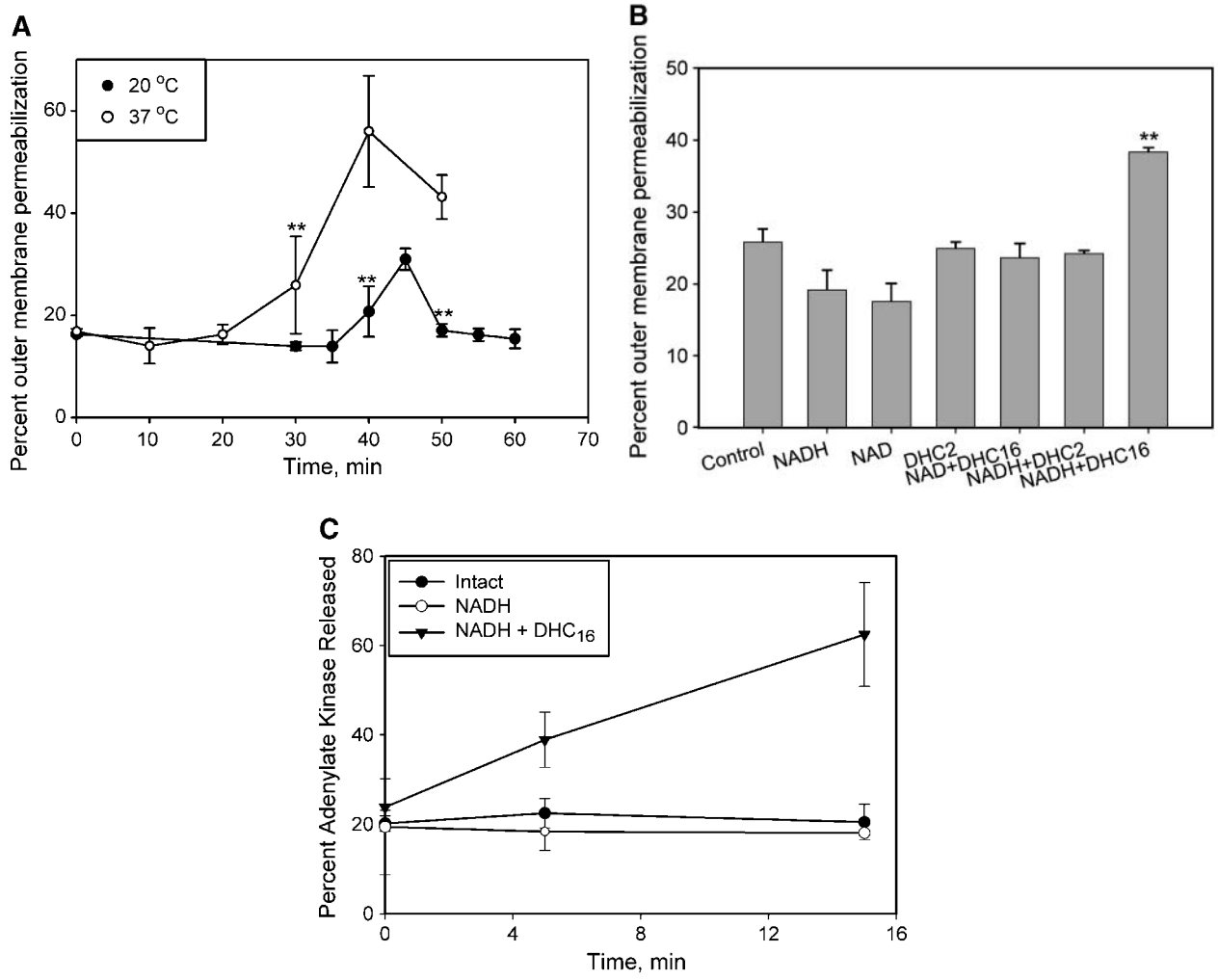

Fig. 1. Permeabilization of mitochondrial outer membranes to cytochrome *c* and adenylate kinase by incubating mitochondria with NADH and dihydroceramide. A: Mitochondria were exposed to NADH and *N*-palmitoyl-D-*erythro*-sphinganine (DHC_{16})-ceramide at 20°C (closed circles) and 37°C (open circles) for the indicated times, and then their ability to oxidize cytochrome *c* was measured to estimate the degree of permeabilization of the outer membrane to proteins. The cytochrome *c* oxidation rate is expressed as a percentage of the rate observed with hypotonically shocked mitochondria. B: The experiment in A was performed under a variety of conditions. Fifty microliters of 3 mg/ml rat liver mitochondria obtained by differential centrifugation was 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 nmol of DHC_{16} (but not DHC_2) resulted in the permeabilization of the outer membrane. For A and B, the results are means of three experiments ± SD. ** $P < 0.01$. C: The release of adenylate kinase increases when the mitochondria are incubated with NADH and DHC_{16}. Mitochondria were incubated for the indicated times with NADH alone (open circles), NADH plus DHC_{16} (closed triangles), or no treatment (closed circles), followed by quantitating the amount of adenylate kinase released into the medium. Treating the mitochondria with DHC_{16} alone did not release significant amounts of adenylate kinase (not shown). The results are expressed as a percentage of the total activity released after hypotonic shock of the mitochondria. Error bars indicate SD of three experiments.

occurs after the addition of ceramide saturates after 10 min (4). Here, with dihydroceramide there is a 30 min lag followed by a transient increase in permeability. This lag could be attributed to a number of factors: metabolic rate, rate of transfer of ceramide from MAM to mitochondria, and the ability of dihydroceramide to interfere with ceramide channel formation (5). The latter effect was predicted to cause a sudden increase in outer membrane permeability as dihydroceramide levels decline while ceramide levels increase (5). Figure 1 shows a sudden rapid increase.

The permeabilization seems to be the result of DDase activity. It could not be achieved with NADH alone, if NADH was substituted by NAD^+, or if short-chain DHC_2-ceramide was used instead of DHC_{16}-ceramide (Fig. 1B). The DDase requires NADH and does not desaturate short-chain dihydroceramide (25).

When adenylate kinase release was used to monitor outer membrane permeabilization (Fig. 1C), the kinetics were much faster. This is not surprising, because adenylate kinase is released even by the formation of transient pathways. It accumulates in the medium, and no further release occurs once the concentrations in the intermembrane space and the medium are equal. Thus, the amount of enzyme in the medium is the integral of the rate of efflux times the time. By contrast, the cytochrome *c* oxidation assay measures the permeability of the outer membrane to cytochrome *c* at one point in time as the rate of cytochrome *c* oxidation. Thus, this assay monitors the actual permeability at that particular moment in time and cannot detect the effect of permeability increases at other times. Note that the permeability peaks were followed by a reduction, possibly caused by the movement of ceramide into other nonconducting structures or ceramide hydrolysis.

The most straightforward interpretation of these results is that isolated rat liver mitochondria contain a DDase activity. Is this located in the mitochondria proper or the associated MAM?

A DDase capable of using cytosolic NADH is not found in ultrapure mitochondria isolated from rat liver

We looked for DDase activity in mitochondria isolated by differential centrifugation (DC-mitochondria) by adding radiolabeled dihydroceramide (1-^{14}C-DHC_8) and looking for conversion to ceramide. Samples were resolved on a TLC plate, and the amount of radioactivity in ceramide and dihydroceramide is visualized in **Fig. 2A**. 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 plate (Fig. 2B). Significant conversion of dihydroceramide to ceramide was observed (Fig. 2, M). However, because mitochondria isolated by differential centrifugation have associated membranes (MAM), we removed these using a Percoll gradient as described previously (18, 20) to generate ultrapure mitochondria. Percoll somehow dissociates these membranes from the mitochondria. These ultrapure mitochondria had no significant DDase activity (Fig. 2, UP). The ER fraction

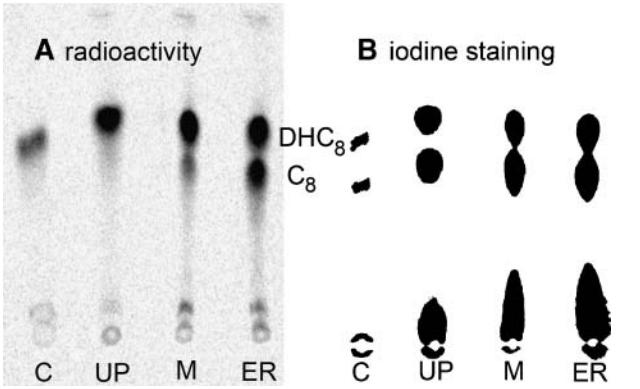

Fig. 2. Dihydroceramide desaturase (DDase) capable of using cytosolic NADH was not found in mitochondria stripped of mitochondria-associated membranes (MAMs). A: PhosphorImager screen of a TLC plate showing the conversion of added radioactive *N*-[1-^{14}C]octanoyl-D-*erythro*-sphinganine (1-^{14}C-DHC_8) to radioactive *N*-[1-^{14}C]-octanoyl-D-*erythro*-sphingosine (C_8) with different additions: no organelles (C), ultrapure mitochondria (UP), mitochondria obtained by differential centrifugation (M), and endoplasmic reticulum (ER). B: Iodine staining of the same plate. Unlabeled DHC_8 and C_8 were added to each sample before 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 of the extracted lipids were applied to the TLC plate. Ultrapure mitochondria were purified on a Percoll gradient.

(Fig. 2, ER) showed the largest conversion (44 ± 5% in 45 min, average and SD of 23 experiments). The results for these ultrapure mitochondria indicate that the enzyme is not found in mitochondria from rat liver in a detectable amount (Fig. 2, UP), but the tight interaction between mitochondria and ER is what causes this conversion in the DC-mitochondrial fraction. After an ER marker enzyme assay (NADPH:cytochrome *c* reductase), the DC-mitochondria had double the activity (13.5 μM/mg/min) of the ultrapure (5.8 μM/mg/min) but significantly less than the ER (426 μM/mg/min). Although, by this enzyme, one might conclude that some MAM membranes are still associated with the ultrapure mitochondria despite the loss of DDase activity, no marker is perfect and there is a small amount of NADPH:cytochrome *c* reductase in mitochondria (31).

The ability of added dihydroceramide and NADH to permeabilize the mitochondrial outer membrane to proteins, as illustrated in Fig. 1, seems to arise from ceramide generation in the MAM and its subsequent transfer to the mitochondrial outer membrane.

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

Membrane compartmentation may have prevented the detection of DDase activity in mitochondria. To check whether the DDase is found in the inner membrane of mitochondria using matrix NADH, NADH was produced in the matrix using the malate/glutamate shuttle (32).

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, whereas NADH was maintained high in the matrix by the malate/glutamate shuttle. The TLC results (**Fig. 3A**) show virtually no difference between 0 min of incubation (BM_{cont}) and 45 min of incubation (BM). Subtraction of the curves (Fig. 3B) shows a slight radioactive ceramide peak consistent with ~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 the notion that Percoll inhibits this enzyme.

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 straightforward interpretation from the experiments illustrated in Figs. 1 and 2 is that dihydroceramide is converted to ceramide in the MAM and then transferred to the mitochondrial outer membrane, resulting in ceramide channel formation and permeabilization to cytochrome *c* and adenylate kinase. A direct demonstration of the ability of ceramide to translocate from ER membranes to mitochondria was undertaken to test this hypothesis. Radiolabeled ceramide was generated in ER vesicles 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. 4A**). Ceramide and dihydroceramide bands were present in both mitochondria and ER. The transfer of these lipids occurred with a half-life of 5–10 min (Fig. 4B). A similar rate of transfer was seen in the reverse direction (data not shown). 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, Holloway, and Gear (33) using Eyring rate theory and an energy deficit of 3 kJ/methylene group. Thus, for C_8-ceramide, the rate constant for this process is calculated to be 1.6 min^{-1}. Therefore, an exchange of 15% should occur within 70 s. This is consistent with our observations, considering that there may be other steps in the exchange process that could slow the measured rate. Nevertheless, the exchange observed with the C_8-ceramide can be accounted for by a simple physical exchange process.

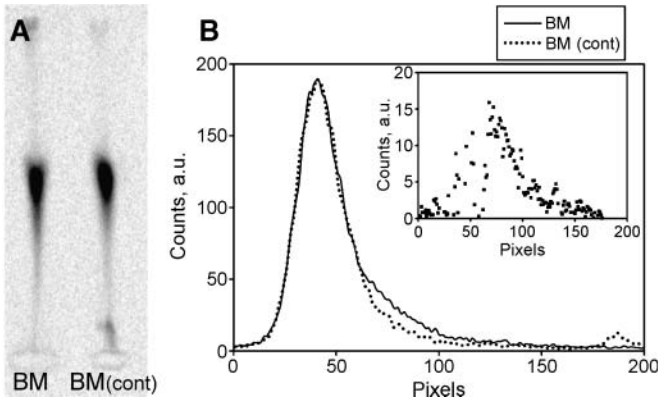

Fig. 3. The DDase associated with mitochondria is minimal. A: PhosphorImager screen of a TLC plate showing minimal conversion of 1-^{14}C-DHC_8 to C_8 in Percoll-purified mitochondria with broken outer membrane supplied with NADH externally and maintained in the matrix by providing malate/glutamate. Sample BM was incubated for 45 min, and sample BM_{cont} was incubated for zero time. B: The traces were quantified as a function of migration distance in pixels and plotted. The inset shows the subtraction of the zero-time control from the experimental sample, BM. A peak possibly attributable to ceramide was detected. In three independent experiments, the area was found to represent 8.4 ± 1.3% (±SD) conversion in 45 min per 3 mg of protein. In contrast, the ER fraction achieved 44 ± 5% conversion in 45 min with 0.6 mg of protein. a.u., arbitrary units.

The energy barrier for long-chain ceramide would be much greater than that for C_8-ceramide, and a simple physical exchange could not account for the results illustrated in Fig. 1. We examined the possibility that a mechanism might be present to accelerate the transfer of long-chain ceramide from ER to mitochondria. Exchange experiments were performed with endogenously produced [^{3}H]palmitoyl-D-*erythro*-sphingosine-ceramide. [^{3}H]sphingosine and palmitoyl-CoA were added to ER preparations to endogenously synthesize [^{3}H]palmitoyl-D-*erythro*-sphingosine-ceramide in the ER membrane using the enzyme ceramide synthase. Excess sphingosine was removed with BSA. Figure 4C 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) (33). Controls lacking palmitoyl-CoA resulted in essentially no production of radiolabeled ceramide and essentially no ceramide in the mitochondrial fraction, despite comparable amounts of sphingosine transport (Fig. 4D). Thus, the translocation of sphingosine to mitochondria, under these conditions, did not result in a significant conversion to ceramide in the mitochondrial membranes. Therefore, ceramide was transferred rapidly from ER vesicles to mitochondria, and thus, the ER must contain some system to catalyze this translocation process.

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

To further test the theory that random collisions between ER vesicles and mitochondria might account for the transfer of ceramide, a dilution experiment was performed to determine whether the transfer rate depended

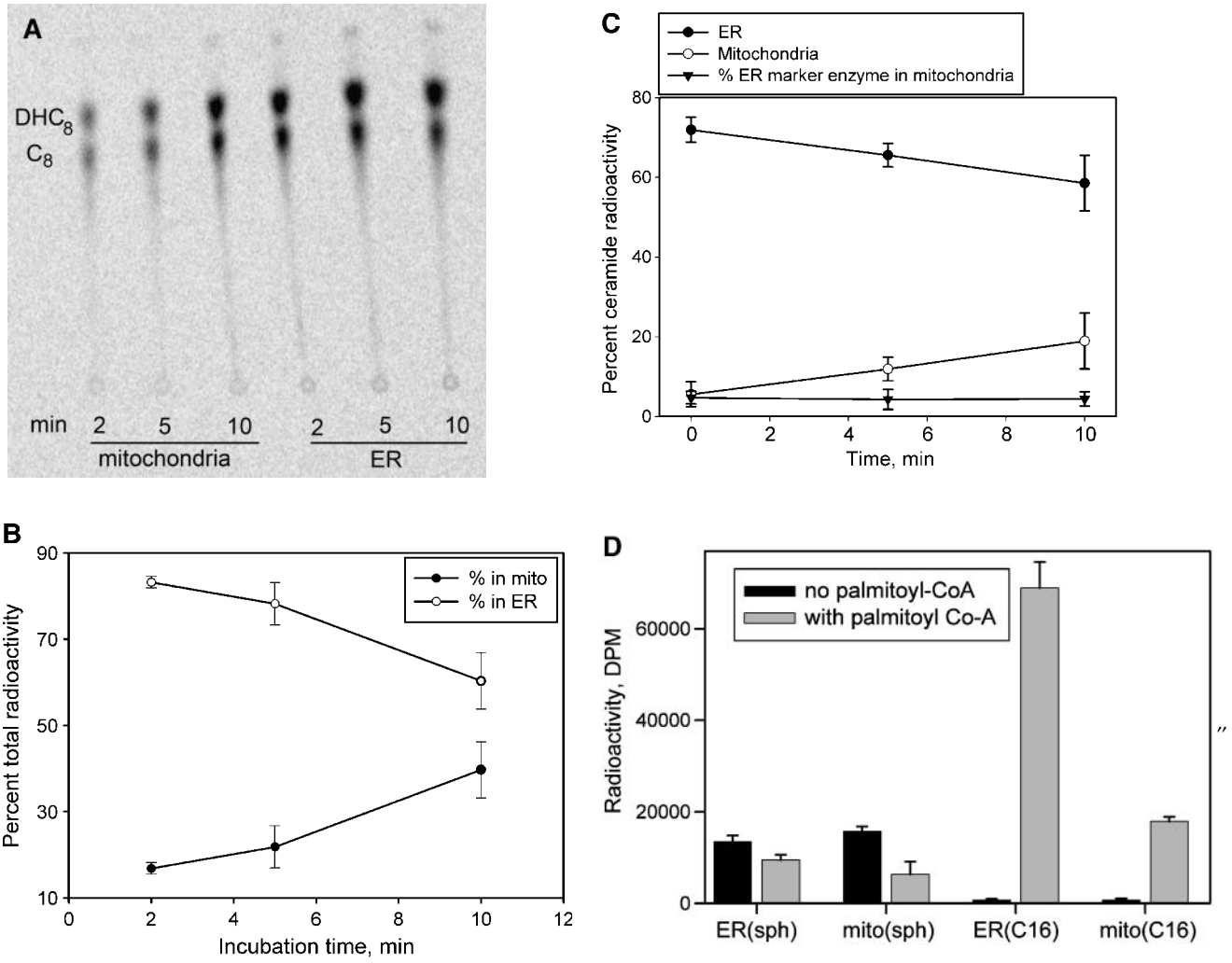

Fig. 4. Both medium- and long-chain ceramide transfer from ER to mitochondria in a time-dependent manner. A: PhosphorImager screen of a TLC plate showing the transfer of medium-chain ceramide from ER to mitochondria. After the production of ceramide in the ER, Percoll-treated mitochondria were incubated 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 (closed circles) and ER (open circles) is shown as an average of four separate, independent experiments ± SEM. C: ER vesicles were preloaded with radiolabeled long-chain ceramide using [^{3}H]sphingosine and palmitoyl-CoA. Preloaded ER vesicles were incubated with mitochondria at room temperature for 0, 5, and 10 min, resulting in a slow but steady transfer of ceramide from the ER (closed circles) to mitochondria (open circles). Mitochondria were checked for the percentage of ER marker enzyme contamination (closed triangles). The results represents averages ± SEM of three replicates. D: The addition of palmitoyl-CoA is vital for the development of a ceramide band in the mitochondrial extract. ER vesicles were incubated with [^{3}H]sphingosine with and without palmitoyl-CoA, washed, and then incubated with mitochondria for 10 min. A transfer of both sphingosine (sph) and ceramide (C16) is observed. However, only when palmitoyl-CoA is present in the ER to produce ceramide by ceramide synthase is a ceramide band detected in mitochondria (mito). Results represent averages ± SD of three independent experiments.

on the concentration of organelles. A bimolecular process depends on the square of the concentrations of the reactants. Thus, by reducing the concentrations of each of the interacting organelles by half, the transfer rate should decline 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. ER vesicles were incubated with [^{3}H]sphingosine and palmitoyl-CoA (similar to the experiments outlined above). Mitochondria were added, and after 10 min of incubation, the organelles were separated and the lipids were extracted and run on a TLC plate. The results obtained for long-chain ceramide were 334 ± 36 dpm for the undiluted sample and 99 ± 14 dpm for the diluted sample. This result is not significantly different from a 4-fold dilution effect. It is significantly different from both a 2-fold effect ($P < 0.02$) and no dilution effect ($P < 0.005$). These results represent the average and SEM of three experiments. Hence, this indicates that the transfer occurs by random collision of the organelles.

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

The transfer of ceramide from ER membranes to mitochondria is no more than a curiosity unless it results in functional changes. Aliquots of mitochondria were mixed with ER vesicles pretreated with NADH and DHC_{16} to preload them with ceramide, or with untreated ER vesicles, or with medium alone. The mitochondria-ER combinations were incubated for the indicated times (**Fig. 5**), and then the cytochrome *c* oxidation rate was measured. The permeabilization of the outer membrane to cytochrome *c* is once again expressed as a percentage of the rate observed with shocked mitochondria. Only mitochondria exposed to ER vesicles preloaded with ceramide developed an outer membrane permeabilized to proteins. Note that the development of permeability occurred at a much faster rate than that in Fig. 1A, where ceramide could only be generated by the relatively small amount of membranes associated with the mitochondria.

DISCUSSION

There is general consensus that the increase of ceramide levels in cells is associated with or leads to the onset of apoptosis (34). Ceramide is increased in different membrane compartments and has a variety of effects. We are interested in its ability to increase the permeability of the mitochondrial outer membrane to proteins (4) and the consequential release of proapoptotic proteins into the cytosol. The increase of ceramide levels in mitochondria, which has been reported early in apoptosis (35–37), begs the question: where does it come from?

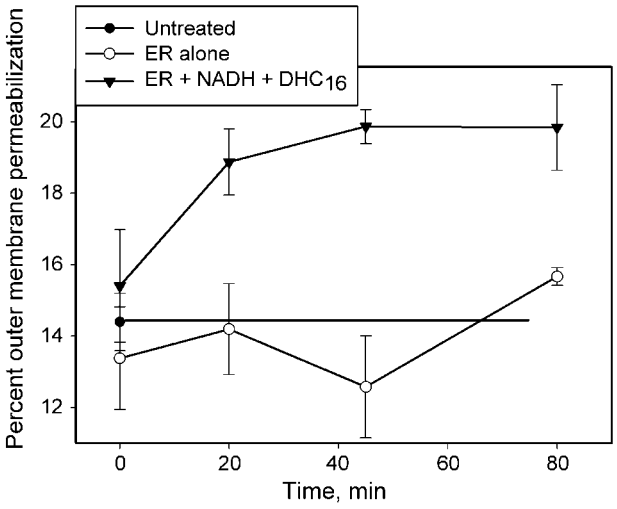

Fig. 5. The addition of ceramide-containing ER to mitochondria isolated by differential centrifugation induced permeabilization to cytochrome *c*. Mitochondria were incubated for 10 min with untreated ER vesicles (open circles), ER vesicles pretreated with NADH and DHC_{16} for the indicated times (closed triangles), or no treatment (closed circles), followed by measurement of their ability to oxidize cytochrome *c* as a function of time after the treatment. The no-treatment measurement was only done after the 10 min mock incubation. Its line is extended to later times to better visualize differences between the different ER treatments. Permeabilization is the cytochrome *c* oxidation rate expressed as a percentage of the rate after hypotonic shock. The results are expressed as means ± SD of three experiments.

The conversion of dihydroceramide, existing in the outer membrane, to ceramide is one possibility. In mitochondria isolated from rat liver, we detected substantial DDase activity when the mitochondria were associated with MAM but only trace levels of this activity in mitochondria purified with Percoll to dissociate the MAM. These results are consistent with previous reports (38). However, other sources of mitochondria seem to have substantial amounts of DDase activity that may not be accounted for by MAM (39). The mitochondrial proteome varies from tissue to tissue, and it is also possible that DDase is activated or introduced into mitochondria under specific physiological conditions, such as early in apoptosis. Thus, the conversion of existing dihydroceramide to ceramide is one possible pathway.

Another pathway is the generation of ceramide from sphingosine either by ceramide synthase or by the reverse action of ceramidase, both reported to be present in mitochondria. However, cellular sphingosine levels are quite low (40), and one might wonder if this source could be sufficient. Unlike ceramide, sphingosine is quite promiscuous. Having a single hydrocarbon acid chain, it moves from one membrane to another quite readily. Thus, if sphingosine were generated in one part of the cell, the mitochondrial enzymes mentioned above could consume it and thus increase mitochondrial ceramide levels.

We present a whole different pathway: direct exchange of ceramide from ER or MAM to mitochondria. We showed that this can occur and, for the physiologically present long-chain ceramide, does so at a much faster rate than expected from published experiments with liposomes (reviewed in Ref. 41). Thus, the transfer appears to be catalyzed. Regardless, we have raised the possibility that mere proximity between mitochondria and ER membranes containing ceramide can result in the delivery of ceramide to mitochondria. The amount delivered is sufficient to permeabilize the outer membrane to proteins and therefore has functional significance. The transfer is sufficient to increase the steady-state levels of ceramide in mitochondria to the point that channels are formed. Thus, this transfer must, in some way, overwhelm the ability of mitochondrial ceramidase to hydrolyze ceramide.

Because ceramide is routinely generated in the ER for sphingomyelin synthesis and transfer to the plasma membrane, what keeps this ceramide from moving to mitochondria and initiating inappropriate apoptosis? We propose that what is required is intimate contact with mitochondrial membranes. It is well established that the proteins and lipids are sorted in the ER; thus, ceramide must be sorted in such a way that, under normal conditions, it does not travel to mitochondria or else does so at a very slow rate. Indeed, the ER-like membranes, the MAM, that are in intimate contact with mitochondria have different lipid and protein compositions from those of the ER in general. Thus, guided delivery of ceramide to

mitochondria might occur only during the initial phases of apoptosis.

The ability of lipids to travel from the ER to mitochondria is not a novel finding. For instance, most of the enzymes of the phospholipid synthetic pathway reside in the ER (42). However, some enzymes, such as phosphatidylserine decarboxylase, are located in mitochondria (43). This enzyme is critical for the formation of phosphatidylethanolamine from phosphatidylserine (43–45). Thus, the synthesis of phosphatidylethanolamine requires that phosphatidylserine, made in the ER, move into mitochondria, where it is decarboxylated, forming phosphatidylethanolamine, which then returns to the ER (21, 45–48). Experimental evidence indicates that membrane contact between the ER and mitochondria is responsible for rapid lipid exchange (reviewed comprehensively in Refs. 49, 50) rather than vesicular transport (44, 51).

A close interaction between ER and mitochondria has been inferred from functional studies (19, 21). This conclusion is strongly supported by ultrastructural observations (18, 20, 22). These show intimate contact between ER-like vesicles and mitochondria. Thus, our proposal of the direct exchange of ceramide between these compartments is well supported by the literature.

Sphingolipids are known to be transferred from the ER to the Golgi and plasma membrane by vesicular transport (reviewed in Refs. 52, 53), but it is not known how and whether sphingolipids travel between the ER and mitochondria by this process. To date, there has been no evidence of the involvement of mitochondria in any vesicular transport. The soluble protein, CERT (23, 54), has been shown to transport ceramide from the ER to the Golgi. Although similar methods might be used to transport ceramide to mitochondria, these methods are unlikely to play a role in the in vitro studies presented here. The purification of mitochondria and ER vesicles would have washed away soluble factors. The BSA used in the early isolation steps was washed out in the later steps. Thus, the results are consistent with a catalyzed exchange through simple membrane contact. In vivo, the cellular organization limits contact between organelles. Thus, the structure of the cell may determine which membranes come in contact and where exchange occurs.

Based on our results, we propose that the mitochondrion and its associated MAM form a functional unit for ceramide generation and the subsequent release of proteins from mitochondria. A similar idea of a functional unit was proposed for calcium ion fluxes and their role in mitochondrial function (for recent review, see Ref. 55). This functional unit could allow for greater regulation by providing more points of control. It also obviates the need for a duplicate set of metabolic enzymes in both membrane systems. If so, then why is there a ceramide synthase in mitochondria? Perhaps the mitochondrial ceramide synthase is a salvage pathway. It converts sphingosine produced from ceramide hydrolysis back into ceramide. 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.

As already stated, increases in mitochondrial ceramide levels often occur early in apoptosis (35–37), and these are consistent with the formation of ceramide channels (6). However, such increases are not mandatory, as changes in the level of antiapoptotic proteins also change the propensity for ceramide channel formation (56). Thus, in cases in which cells undergo apoptosis without measurable changes in mitochondrial ceramide levels, other factors may result in ceramide channel formation.

In conclusion, we have shown that ceramide generated in ER membranes from dihydroceramide or sphingosine can be rapidly transferred to mitochondria and result in the permeabilization of the outer membrane to proteins. This result contributes to a growing body of evidence that ceramide channels formed in the mitochondrial outer membrane initiate the execution phase of apoptosis by releasing proapoptotic proteins from mitochondria. This and other sources of mitochondrial ceramide can increase the steady-state level of ceramide to the point at which it self-assembles into channels. We propose that mitochondria surrounded by MAM form a functional unit serving a number of functions, including the ceramide-induced release of proteins from mitochondria.

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