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ORIGINAL PAPER

Ceramide and activated Bax act synergistically to permeabilize the mitochondrial outer membrane

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Abstract A critical step in apoptosis is mitochondrial outer membrane permeabilization (MOMP), releasing proteins critical to downstream events. While the regulation of this process by Bcl-2 family proteins is known, the role of ceramide, which is known to be involved at the mitochondrial level, is not well-understood. Here, we demonstrate that Bax and ceramide induce MOMP synergistically. Experiments were performed on mitochondria isolated from both rat liver and yeast (lack mammalian apoptotic machinery) using both a protein release assay and real-time measurements of MOMP. The interaction between activated Bax and ceramide was also studied in a defined isolated system: planar phospholipid membranes. At concentrations where ceramide and activated Bax have little effects on their own, the combination induces substantial MOMP. Direct interaction between ceramide and activated Bax was demonstrated both by using yeast mitochondria and phospholipid membranes. The apparent affinity of activated Bax for ceramide increases with ceramide content indicating that activated Bax shows enhanced propensity to permeabilize in the presence of ceramide. An agent that inhibits ceramide-induced but not activated Bax induced permeabilization blocked the enhanced MOMP, suggesting that ceramide is the key permeabilizing entity, at least when ceramide is present. These and previous findings that anti-apoptotic proteins disassemble ceramide channels suggest that ceramide channels, regulated by Bcl-2-family proteins, may be responsible for the MOMP during apoptosis.

Keywords Apoptosis · Mitochondria · Ceramide · Channel · Bcl-2 · Protein release

Introduction

Apoptosis, a type of programmed cell death, is a regulated process where unwanted or damaged cells are eliminated. The permeabilization of the mitochondrial outer membrane (MOM) to key intermembrane space (IMS) proteins such as cytochrome *c*, Smac/Diablo, and apoptosis-inducing factor, is an irreversible and decision-making step in apoptosis, which leads to the execution phase involving effector caspase activation. Ceramide, a pro-apoptotic sphingolipid, has been reported [1, 2] to act as a second messenger in several cellular processes, including apoptosis. It can also form channels [3] and thus can directly permeabilize the MOM without the aid of ancillary proteins [4]. Physiological studies show that ceramide is both an extracellular stimulus and intracellular mediator [1, 2] of mitochondrial apoptosis. Several lines of evidence indicate that cellular levels of ceramide become elevated [5–9], most importantly in mitochondria [9–14]. We have shown that Bcl-xL and Bcl-2 can inhibit permeabilization induced by ceramide in isolated rat liver mitochondria [4].

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This is achieved by the anti-apoptotic proteins inhibiting ceramide channel formation and disassembling pre-formed ceramide channels.

The mechanism by which Bax increases MOMP is cause for much debate [15–18]. Bax is located in the cytosol or loosely associated with the MOM in the monomeric form. Upon an apoptotic signal, Bax inserts into the MOM and becomes activated and oligomerized. Activated Bax and/or Bak are proposed to be directly responsible for the release of intermembrane space proteins by forming channels in cells and indeed, in phospholipid membranes, Bax treated with β-octyl glucoside (activated Bax) is capable of forming channels [19, 20]. Yet whether these channels are large enough to allow the release of all the IMS proteins is unclear [16]. In yeast cells that lack the mammalian apoptotic machinery, expression of Bax still leads to leads to cytochrome *c* release and cell death indicating that Bax channels may suffice despite the presence of ceramide. However, the effect of Bax is inhibited by co-expression of sphingomyelin synthase, which consumes ceramide [21]. In mammalian cells, ceramide-induced cytochrome *c* release does not require Bax [4, 22] but Bax enhances apoptosis induced by ceramide [23]. Also, ceramide has been found to activate monomeric Bax in the presence of the MOM [10, 24]. When combined, these publications can be interpreted to indicate that Bax and ceramide may interact cooperatively to generate the MOMP. This is clearly not the view of most investigators in the field but it is a logical conclusion from published experiments. The present work provides direct evidence of a synergistic interaction between activated Bax and ceramide resulting in enhanced MOMP to proteins. We also provide mechanistic insights into this interaction.

Experimental procedures

Reagents

C_{16}-ceramide was bought from Avanti Polar Lipids. Antimycin A, 2,4-dinitrophenol (DNP), horse heart cytochrome *c*, fatty acid-depleted bovine serum albumin (BSA), and sodium ascorbate were bought from Sigma Chemical Co. Other chemicals were reagent grade.

Isolation of mitochondria

Rat liver mitochondria were isolated essentially as previously described [3]. The isolation buffer was 210 mM mannitol, 70 mM sucrose, 0.1 mM EGTA, 0.05% fatty acid free BSA, 5 mM HEPES, pH 7.4. To achieve a high level of intactness, mitochondria were sedimented at 5600 RCF (average). Finally, the BSA was removed by sedimenting the mitochondria in isolation buffer without BSA. These were suspended in sucrose-free medium (280 mM mannitol, 0.1 mM EGTA, 2 mM HEPES, pH 7.4), typically to a protein concentration of 9–10 mg/ml and stored on ice.

Yeast mitochondria were prepared and isolated as previous described [25] until the washed spheroplast pellet was obtained. Twice, the pellet was resuspended in H-medium (0.6 M mannitol, 0.1 mM EGTA, 10 mM HEPES, pH 7.2) and spun at 700 RCF for 5 min to yield the mitochondrion-containing supernatants. The supernatants were spun at 5600 RCF for 10 min and the pellets were retained. The pellets were resuspended in H-Medium, combined and spun at 700 RCF for 5 min. The supernatant was spun at 5600 RCF for 10 min yielding the final mitochondrial pellet which was resuspended in H-medium.

The intactness of the mitochondria, as measured by comparing their rate of cytochrome *c* oxidation with that of osmotically shocked mitochondria [3], was more than 85% for rat mitochondria and over 93% for yeast mitochondria.

Preparation of Bax

Recombinant human Bax was produced as described previously [26] except the Bax eluted from the chitin column (New England Biolabs Inc.) was dialyzed in 12,000 MW cut-off dialysis membranes at 4°C for 24 h in 3 L (1 mM EDTA and 20 mM Tris–HCl pH 8.0) and then changed into 5 L (20 mM Tris–HCl pH 8.0) for 48 h. The Bax was then filter sterilized through a 0.2 μm filter and 0.5 ml aliquots were made. Glycerol (10%) was added to some of these and they were rapidly shell-frozen in ethanol and dry ice and stored at −80°C. The protein content was typically 10–25 μg/ml, assayed by the Micro BCA Protein Kit (Pierce Chemical Co.), and SDS/PAGE analysis showed a 95% pure m-Bax band (S2). No detergents were used during the protein isolation. It must be noted that monomeric Bax, when stored in solution at 4°C shows a tendency to oligomerize over time, which is why most of the protein was immediately frozen after purification and only thawed on ice just before use. This method of freezing did not alter protein activity. The native Bax was oligomerized by adding 10% β-octyl glucoside to a final concentration of 0.7% and incubated for 30 min on ice. The proper folding of the detergent treated Bax was confirmed by its partial resistance to trypsin (S3), as published elsewhere [27]. Note that the concentrations of activated Bax reported in the paper were based on the molecular weight of monomeric Bax (21 kDa) because the activated form is heterogeneous.

Purification of N/C Bid

Recombinant N/C Bid (cleaved human Bid) was purified as previously described [28]. Once the Bid was bound to the

glutathione-sepharose beads, 50 U/ml thrombin in PBS was loaded onto the column and allowed to incubate overnight at room temperature. The addition of thrombin directly to the column allowed us to elute only N/C Bid without the contamination of the GST tag in one step. The elution was then dialyzed overnight against 50 mM Tris–HCl pH 8.0, filter sterilized, aliquoted and rapidly frozen as described above. The yield was typically 3 mg/ml as assayed by the Micro BCA Protein Kit (Pierce Chemical Co.) and SDS/PAGE analysis showed an over 95% pure N/C Bid band. Wherever N/C Bid was used, protease inhibitors were added to prevent any deterioration of protein activity. The final concentration of the protease inhibitors was: 1.5 μM pepstatin, 16.5 μM chymostatin, 2.2 μM leupeptin, .6 μM aprotinin and 20 μM PMSF.

Cytochrome *c* accessibility assay

Shortly before use, mitochondria were diluted in the isolation buffer to 0.5 mg protein/ml and stored on ice. Once diluted, mitochondria lose function more rapidly and so the diluted suspension is used within an hour. In a typical experiment, 50 μl of this dilution was dispersed in 650 μl of room temperature incubation buffer (the sucrose-free buffer supplemented with 5 mM DNP and 5 μM antimycin A and pH 7.25 [3] to final protein content of 25 μg in 700 μl). Unless otherwise stated, this was done for all experiments. In most experiments, when Bax was added, it was added immediately. Then the mitochondria were incubated for 10 min at room temperature to allow them to acclimate and interact with Bax. In experiments where N/C Bid and monomeric Bax were used, the mitochondria were incubated with the permeabilizing agents for 30 min at 30°C in a rotary shaker set for gentle mixing. Ceramide was generally added at this point from a 1 mg/ml solution in isopropanol. It was added while simultaneously vortexing the microfuge tube vigorously so as to achieve rapid and effective dispersal of the sphingolipid (controls show that this does not damage the outer membrane). After dispersal, the mixture was incubated for 10 min followed by measurement of the outer membrane permeability. Reduced cytochrome *c* [3] was added to the mitochondrial suspension (10 μl; final concentration approx. 25 μM) and the absorbance at 550 nm was measured immediately for 2 min. The initial rate was used to assess the permeability of the MOM to cytochrome *c*. The extinction coefficient of 18.5 mM^{-1} cm^{-1} ($\Delta\epsilon_{Red.\text{-}Ox.}$) was used to convert absorbance units to μM units.

Assessment of apparent dissociation constant of ceramide channels for activated Bax

The oxidation rate was used as the functional parameter to evaluate the apparent dissociation constant of ceramide channels for activated Bax. The data was plotted using the Hill formalism for cooperative binding.

$$\log \frac{\text{rate}_{\text{Bax}}}{\text{rate}_{\text{max}}} = \log(K) + n \log[\text{Bax}]$$

where the rates are the initial activated Bax-stimulated rates of oxidation at a specific ceramide concentration, above the rate observed with ceramide alone. Rate_{Bax} is the rate at any (activated Bax) and rate_{max} is the maximal rate. K and n are the parameters of the Hill formalism. The inherent variability in the experiments forced us to combine these parameters into one. Since K and n increased simultaneously with amount of ceramide used, we found it more informative to combine these two parameters into one term:

$$K_{0.5} = [1/K]^{1/n}$$

$K_{0.5}$ is the Bax concentration at which the extent of permeabilization (as measured by the rate of cytochrome *c* oxidation) is half-maximal. It is a measure of the apparent dissociation constant, the reciprocal of the apparent affinity.

Electrophysiological experiments

The monolayer method was used as described previously [29] to make planar phospholipid membranes. Calomel electrodes were used to interface with the aqueous solutions (1.0 M KCl, 1 mM $MgCl_2$, 5 mM PIPES pH 6.95] on either side of the membrane. The voltage was clamped and the current recorded. The lipid solution contains 0.5% (w/v) 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine, 0.5% (w/v) asolectin, and 0.05% (w/v) cholesterol dissolved in hexane. To form a ceramide channel, a stock solution of 0.05 mg/ml C_{16}-ceramide was made in isopropanol and 10–20 μl amounts were added to the cis side of the membrane while stirring for 15 s to induce an initial conductance. The channel was then allowed to enlarge and stabilize before the addition of proteins or other compounds. To obtain an activated Bax conductance, activated Bax was added to the cis side of the membrane only.

Adenylate kinase assay

As described earlier [3], mitochondria were resuspended in the incubation buffer (60 mM potassium lactobionate, 180 mM mannitol, 0.1 mM EGTA and 2 mM HEPES and pH 7.4) to a final mitochondrial protein concentration of 160 μg/ml. After adding ceramide and Bax, as done for cytochrome *c* oxidation assay, to 1 ml aliquots, mitochondria were incubated at 30°C for 30 min. The mitochondria were sedimented at 14,000 RCF for 5 min at 4°C and 300 μl of the supernatant was combined with 700 μl adenylate kinase reaction buffer (50 mM Tris, 5 mM

$MgSO_4$, 10 mM glucose, 5 mM ADP, 0.2 mM NADP pH 7.5) that had been preincubated for 2 min with 5 µl of enzyme mixture (2.5 units of hexokinase and 8.7 units of glucose-6-phosphate dehydrogenase). The absorbance of the mixture at 340 nm was recorded immediately and the initial rate used as a measure of the adenylate kinase activity. Maximal release of adenylate kinase was achieved by exposing the mitochondria to an osmotic shock. An aliquot of the mitochondrial suspension (usually 10–20 µl containing 160 µg mitochondrial protein) was added to 1 ml distilled H_2O (50- to 100-fold shock) and incubated on ice for 10 min.

Statistics

The Student's *t* test was used to determine the probability values.

Results

Activated Bax enhances ceramide-induced MOMP

To evaluate the influence of Bax on ceramide permeabilization of the MOM, we used a dynamic cytochrome *c*

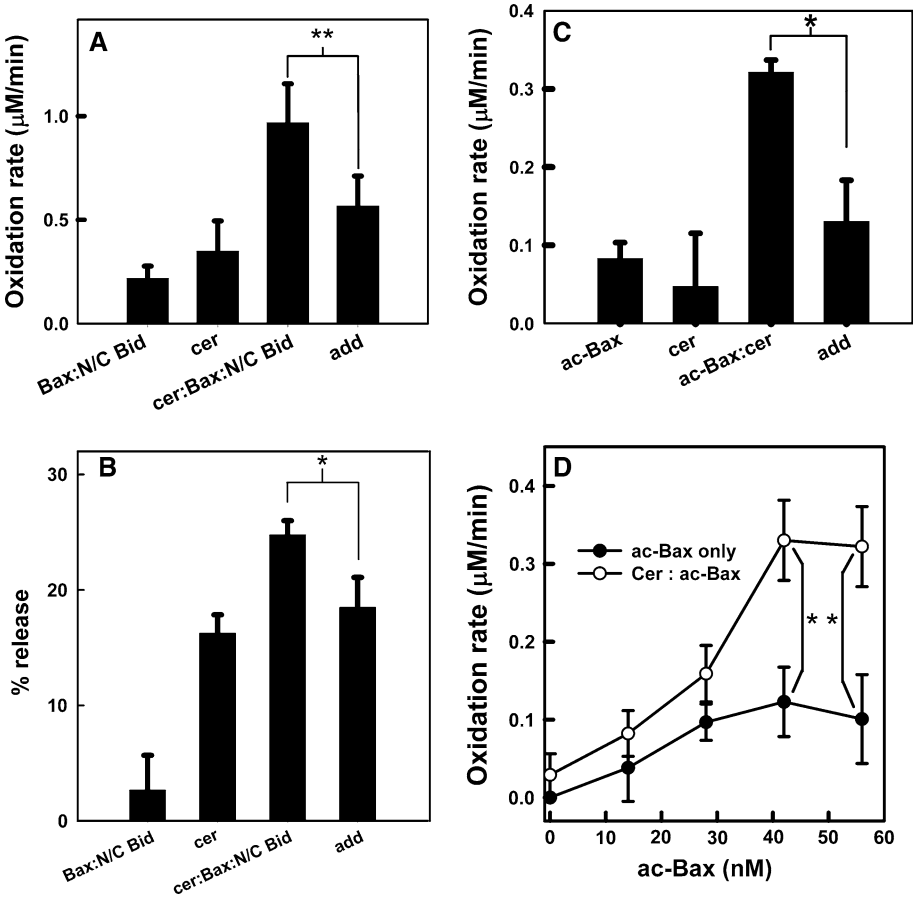

Fig. 1 Ceramide (*cer*) and activated Bax increase the MOMP in a cooperative fashion assessed by the cytochrome *c* accessibility assay (**a**, **c**, **d**) or adenylate kinase release (**b**). **a** Isolated rat liver mitochondria diluted to 250 µg protein/ml were pre-treated with both 45 nM N/C Bid and 40 nM monomeric Bax. Sub-aliquots containing 25 µg of mitochondria were either treated with 5 µl isopropanol (as a vehicle control) or 5 µl of 1 mg/ml ceramide (in isopropanol) and incubated at room temperature for 10 min before assaying for cytochrome *c* accessibility. Respective vehicle controls were subtracted from the data shown. The "add" *bar* is the sum of the rates measured with ceramide alone and Bid/Bax alone. The results are the means ± SD of three experiments. The respiration rate of the combined treatment differed from the sum of the individual rates with $P < .01$. **b** Rat liver mitochondria were treated either with 10 µg ceramide or a combination of 40 nM monomeric Bax and 245 nM N/C Bid or both and the released adenylate kinase activity was measured. The "add" *bar* is the sum of the results of individual treatments minus their product divided by the maximal possible release (to correct for both agents acting on the same mitochondrion). The combined treatment exceeded the sum of individual treatments, "add", with $P < .05$. **c** Isolated rat liver mitochondria were treated with either 1 µg ceramide, 15 nM detergent activated Bax (ac-Bax), or both. The results are mean ± SE of three experiments. The combined treatment exceeded the sum of individual treatments, "add", with $P < .05$. **d** Mitochondria isolated from wild-type *S. cerevisiae* were used and treated with 2 µg of ceramide and activated Bax as shown. The results are means ± SE of three to five experiments. Statistical tests yielding $P < .05$ compared the results of the combined treatment of ceramide and ac-Bax with the numerical addition of the results of treatments with ceramide alone and ac-Bax alone

accessibility assay, as previously described [3]. This is a dynamic measurement of permeability and permeability changes in the MOM to cytochrome *c* in real-time. While treatment of isolated mitochondria with low levels of ceramide or N/C Bid-monomeric Bax induced a small amount of membrane permeabilization, when the same amounts were added together, there was a synergistic increase in MOMP (Fig. 1a). This synergistic induction of permeabilization was also observed using the adenylate kinase release assay that evaluates release of proteins from the intermembrane space. (Fig. 1b). N/C Bid alone did not have any effect on ceramide-induced MOMP at these levels. It must be noted that processed Bid itself is capable of permeabilizing mitochondria [30], possibly by acting on Bak. Also, the degree of Bax activation varies with the amount of N/C Bid. In order to eliminate the effects of N/C Bid alone on ceramide and differences in degree of Bax activation at different concentrations of N/C Bid, we used chemically activated Bax for subsequent experiments. Bax activated chemically with detergent has been found to permeabilize the MOM in similar manner to physiologically activated Bax [28, 31] and shows similar restricted accessibility to trypsin [27]. A similar synergistic enhancement of permeabilization was also observed with ceramide and chemically activated Bax (Fig. 1c). Under these conditions, there was no swelling of the mitochondria and thus the permeabilization was not secondary to inner-membrane swelling.

One possible interpretation for the synergistic interaction between ceramide and activated Bax is that activated Bax could be binding to anti-apoptotic proteins thus removing any inhibitory effect on ceramide induced MOMP. To test if the anti-apoptotic Bcl-2 proteins are involved in this interaction, ceramide induced MOMP enhancement by activated Bax was tested using yeast mitochondria, which are devoid of Bcl-2 family proteins. We found that activated Bax and ceramide interact synergistically to induce MOMP in yeast mitochondria also, suggesting that their interaction could be direct and not indirectly mediated by elimination of inhibition by anti-apoptotic members (Fig. 1d). This also suggests that the interaction between activated Bax and ceramide does not require processed Bid or Bak.

Bax mediated enhancement of ceramide induced permeabilization can be inhibited by trehalose, a disaccharide that disassembles ceramide channels

Since both Bax and ceramide are channel formers and each of them is capable of permeabilizing the MOM in the absence of the other, we wanted to test whether, in this synergistic interaction, Bax is enhancing ceramide channels or vice versa. Some insight was gained by using trehalose, a disaccharide that inhibits ceramide channels causing partial disassembly. The same dose of trehalose inhibited both the ceramide induced MOM permeabilization and the enhancement of this permeabilization induced by activated Bax (Fig. 2a). In this experiment, activated Bax alone had essentially no effect. Using a higher concentration of activated Bax (inset) a significant MOMP was achieved and trehalose had no effect on this MOMP induced by activated Bax alone. The simplest interpretation is that the enhanced MOMP has the properties of ceramide channels. Activated Bax could be acting by enhancing the permeabilization induced by ceramide, perhaps by favoring the growth of ceramide channels. This observation can also be explained by a complex structural assembly of ceramide and activated Bax that is sensitive to trehalose. However, this observation is inconsistent with the possibility that ceramide is simply enhancing Bax activation. In addition, if the enhanced MOMP were the result of ceramide monomers enhancing channels formed by activated Bax then it is hard to see how trehalose could interfere with this process because trehalose cannot remove ceramide from the membrane.

Bax expands ceramide induced conductance in planar phospholipid membranes

Planar phospholipid membranes are membranes consisting only of phospholipids and cholesterol. Being a defined system, one can clearly demonstrate interactions without the influence of other constituents found in natural membranes. The ability of ceramide to form channels in planar membranes has been established [32]. To assess the effect of activated Bax on ceramide channels in an environment free of other membrane components, ceramide channels were formed in the planar membranes and then activated

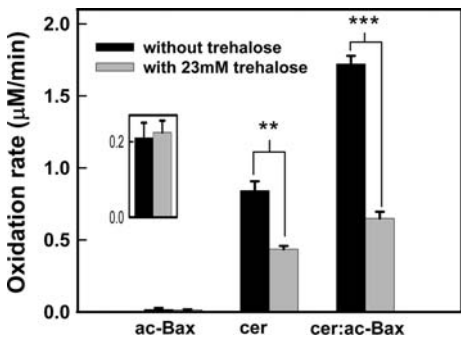

Fig. 2 Trehalose (23 mM final) inhibits MOMP induced by ceramide alone and that induced by the combination of ac-Bax and ceramide. In the main figure, samples were treated with 30 μg of ceramide and 10 nM ac-Bax as indicated. In the *inset* 43 nM ac-Bax was used to obtain a high enough MOMP. The cytochrome *c* oxidation rates are means ± SE of three to four experiments. ** Represents $P < .01$ and *** represents $P < .001$

Bax was added. Activated Bax caused a large increase in conductance while monomeric Bax (m-Bax) did not significantly affect the channel (Fig. 3a). This is typical of many experiments. These show that the interaction between activated Bax and ceramide is a direct one and does not require other proteins.

Trehalose also inhibited the conductance of a ceramide channel formed in a phospholipid membrane (data not shown). In Fig. 3b, a small ceramide conductance was greatly increased by the addition of activated Bax to a final calculated size of 50 nm. At the reduced scale used to show the experiment, the initial ceramide conductance is barely visible. Once the conductance stabilized, trehalose was added resulting in an immediate decline in conductance. Thus the activated Bax-enhanced conductance was reduced by trehalose (Fig. 3b). Trehalose has no effect on the conductance produced by activated Bax alone (Fig. 3c). Thus, trehalose is useful to effectively distinguish between a permeability formed by activated Bax and one formed by ceramide. When the Bax/ceramide conductance was

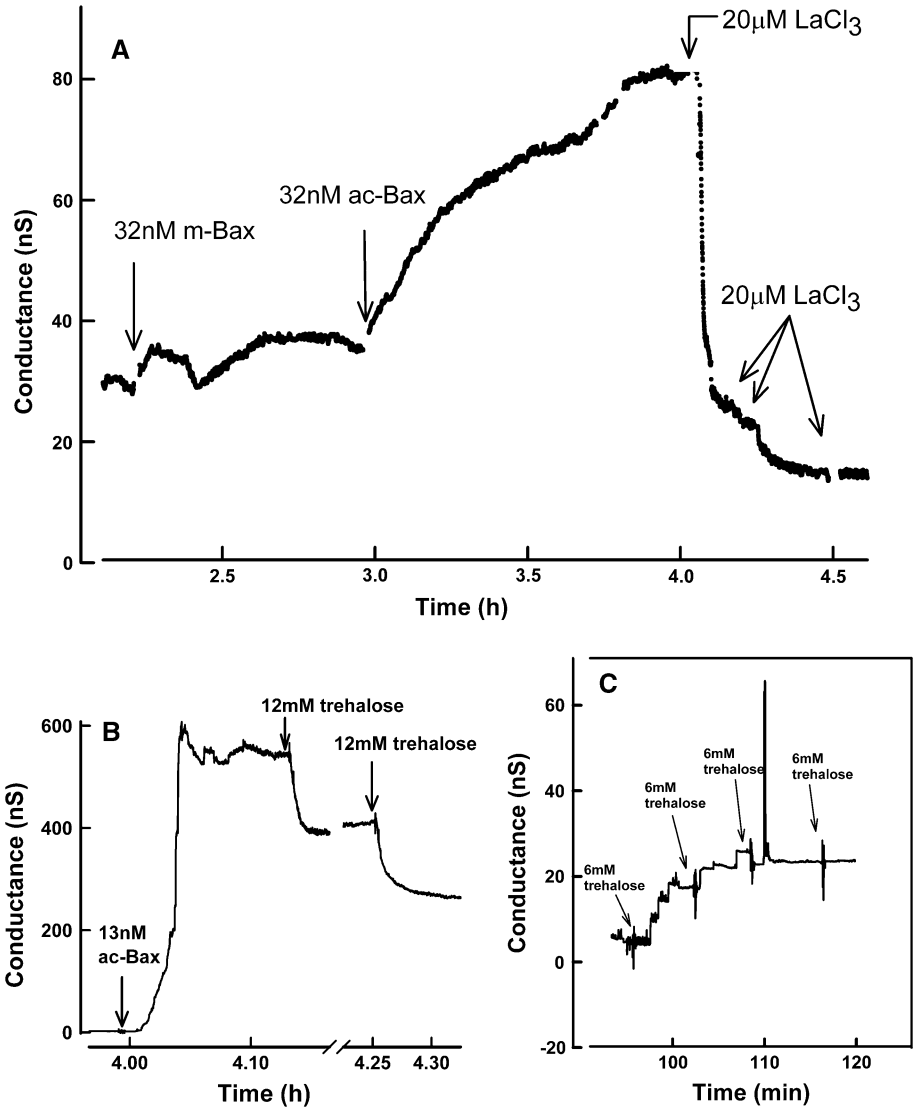

Fig. 3 Addition of activated Bax to a ceramide channel in a planar phospholipid membrane causes it to enlarge. **a** The initial conductance was formed by the addition of 16 μg ceramide to a high-resistance phospholipid membrane. Once the conductance had stabilized first monomeric Bax then activated Bax were added to both sides of the membrane. Once the conductance rise had restabilized at a higher level $LaCl_3$ was added to the final concentrations indicated in the figure. The $LaCl_3$ additions were added to one side in the sequence: *cis*, *cis*, *trans*, *trans*. The experiment shown is representative of more than eight experiments. Amounts of added ceramide and ac-Bax were different between experiments. Bax enhancement of a ceramide channel was seen in over 50 experiments. **b** A small ceramide channel was formed followed by the addition of ac-Bax at the indicated final concentration. Trehalose was added as indicated. **c** Activated Bax conductances were induced in a planar phospholipid membrane in the experiment illustrated in the figure. The total amount of activated Bax added was equivalent to 42 nM. Trehalose was added as indicated stepping up the final concentration by 6 mM at each addition. This is typical of more than three experiments

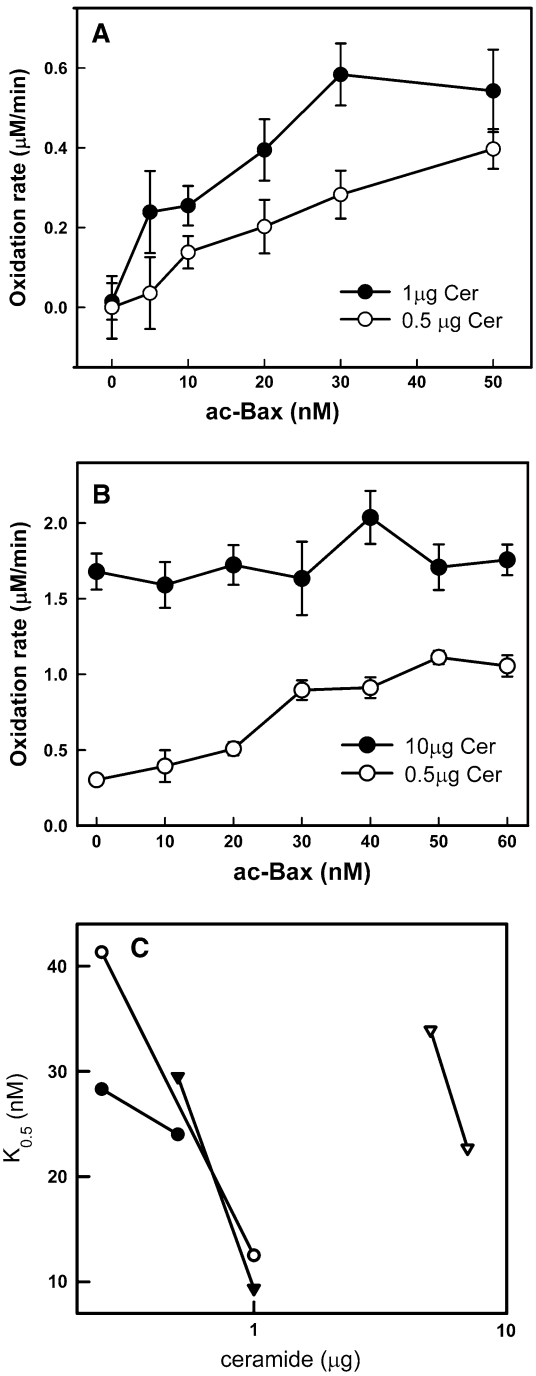

Fig. 4 Dose–response curves of the enhancement of ceramide mediated MOMP by the addition of activated Bax to rat liver mitochondria. **a**, **b** Experiments were performed by preincubating the mitochondria with the indicated amount of activated Bax for 10 min followed by addition of the indicated amount of ceramide. **a** 0.5 and 1 μg of ceramide were used. These results are mean ± SE of four experiments. **b** 0.5 and 10 μg of ceramide were used. These results are mean ± SE of three to four experiments. Experiments within one panel were performed on the same batch of mitochondria. **c** Summary of the results of four independent experiments. Each set of data points defining each line was determined from a set of experiments performed on the same mitochondrial isolation. The figure shows the increase in apparent affinity of activated Bax for ceramide channels with increase in ceramide content. $K_{0.5}$ is defined in the "Experimental procedures" section. These results were obtained from experiments such as those illustrated in **a** and **b**. The indicated ceramide was added to 0.7 ml

inhibited using lanthanum ions, there was stochastic delay in channel disassembly, suggesting that a singular structure is responsible for the conductance observed. Ceramide and activated Bax may be forming a unified structure or activated Bax might enhance the size of the ceramide channel (S1). These experiments also suggest that metabolism of ceramide to other sphingolipids like sphingosine is not necessary for interaction with activated Bax and the consequent changes in permeability.

The influence of activated Bax on MOMP depends on the amount of added ceramide

At a low level of added ceramide 0.5 μg, the amount of activated Bax needed to enhance the MOMP to half maximal was 30 nM. At a higher dose of 1.0 μg ceramide, less activated Bax was needed to achieve a half-maximal effect (12 nM) (Fig. 4a). Both of these results were obtained on the same mitochondrial preparation, eliminating variability between mitochondrial isolations. This difference can be interpreted as a change in apparent affinity between activated Bax and a ceramide channel as the size of the ceramide channel increases. In a separate set of experiments (Fig. 4b) low levels of ceramide still show a dose-dependence but at high levels (10 μg), the enhancement by activated Bax was not observed (Fig. 4b). The lack of further stimulation was not due to the achievement of maximal cytochrome *c* accessibility to cytochrome oxidase because higher levels of ceramide did produce still higher rates of cytochrome *c* oxidation, closer to those observed with hypotonically shocked mitochondria. This indicates that the effect of activated Bax on ceramide induced-MOMP is a saturable function of the amount of ceramide. This saturation could be interpreted as activated Bax favoring an optimal size of the ceramide channel and if the channel is already at the optimal size there is no further change in MOMP. A Hill plot revealed changes in both the measure of cooperativity, *n*, and the constant, *K*. The Hill parameters were combined (see "Experimental procedures") to evaluate the apparent dissociation constant, $K_{0.5}$ (the Bax concentration at which half-maximal enhanced permeabilization is achieved). The resulting apparent dissociation constant of activated Bax for ceramide channels in the MOM decreases as the ceramide concentration increases (Fig. 4c). This is highly reproducible, despite the variation in intrinsic sensitivity of isolated mitochondria to added ceramide. Each line represents the results of sets of

experiments performed on one isolated batch of mitochondria. These results indicate a relationship between the structure of activated Bax and the structure formed in the presence of ceramide. An increase in affinity indicates a better fit. The increase in MOMP may result from an increase in diameter of the Bax-ceramide assembly resulting is a better match to the structure of activated Bax. In this way activated Bax could result in the formation of a channel of a particular size.

Discussion

The release of proteins from mitochondria is a critical, decision-making step in apoptosis and thus the identification of the structure responsible for this release has many important implications. A favorite candidate for the release pathway is activated Bax since Bax is pro-apoptotic, can form channels and its activation on the MOM leads to protein release. Interestingly, ceramide is also pro-apoptotic, can self-assemble to form channels [1, 2, 32] and its delivery to the MOM leads to protein release [2, 10, 33, 34]. Here we show that activated Bax and ceramide act synergistically to permeabilize the MOM.

The nature of the channels formed in the presence of both ceramide and activated Bax is not known and could be a structure fundamentally different from that formed by either substance alone. Some findings in this work, however, are easily interpreted in terms of activated Bax controlling the structure of ceramide channels. The apparent affinity of activated Bax for ceramide-induced MOM permeability increases with the amount of ceramide added. In other words, as the amount of added ceramide was increased, lesser amounts of Bax were required to achieve half-maximal permeabilization. This could be due to more ceramide increasing the degree of Bax activation but at high levels of ceramide, where ceramide by itself produced a large permeability, there was no further stimulation by activated Bax (even though additional amounts of ceramide would have increased MOMP). This observation is not easily compatible with ceramide activating Bax or the two channels mutually enhancing each other. It is more naturally explained by activated Bax favoring the growth of a ceramide channel up to a size designated by the structure of activated Bax.

Activated Bax seems to enhance the size of ceramide channels up to some critical level, probably an optimum channel size. The increased radius of curvature may offer a better fit for activated Bax. Regardless of the site of interaction, if the interaction energy increases with channel size, then the binding of activated Bax to a smaller channel could generate stress on the channel that would be relieved by the channel growing in size (Fig. 5). The dynamic equilibrium between ceramides in the channel and non-

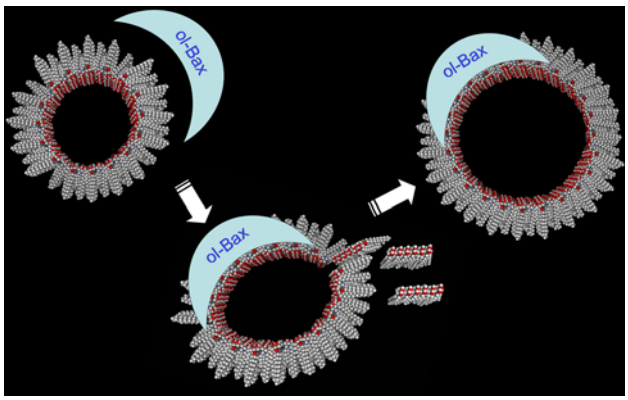

Fig. 5 Illustration of how activated Bax might increase the size of the ceramide channel. On the *left*, a 32-column ceramide channel has a small radius of curvature than ac-Bax. When ac-Bax binds (*center*) it distorts the channel disturbing the equilibrium between ceramide aggregates on the monolayer and ceramides forming the channel. The insertion of more ceramide columns increases the channel size until its curvature matches that of activated Bax (*right*), forming a 48-column channel

conducting ceramide in the membrane would be shifted toward ceramide insertion into the channel and thus growth of the channel size. Once the optimal size is achieved, activated Bax would have no further effect and this was observed. It is possible that the physical shape of the activated Bax in the membrane might act as a molecular mold to drive the ceramide channel to an optimum size.

The results obtained with phospholipid membranes argue that the interaction between activated Bax and ceramide channels must be direct. Furthermore, these demonstrate that activated Bax increases the size of the single existing ceramide channel because the disassembly of the resulting enhanced permeability shows stochastic properties consistent with a unified structure. If the added Bax were to form a separate structure then the disassembly would show two separate processes rather than just one. This is in harmony with the natural interpretation of the mitochondrial experiments that measured the apparent affinity of activated Bax as described above.

Experiments on isolated mitochondria raise the possibility of indirect effects. Ceramide could act on another mitochondrial component and thus indirectly influence the ability of activated Bax to permeabilize the outer membrane. Indeed, a product of ceramide metabolism could be the active species. Mitochondria have ceramidases that could convert ceramide to sphingosine [35, 36]. Although the conversion rate was shown to be minimal under the conditions of our experiments [37], even a small conversion might be important. Sphingosine was shown to be capable of interacting with and influencing the formation of ceramide channels [37] and thus might influence the channel-forming ability of activated Bax. Although this possibility cannot be excluded, the experiments performed

in planar membranes demonstrate a direct functional synergism between ceramide and activated Bax in terms of channel formation. The close parallelism between the results obtained with the two experimental approaches provides strong confidence that both are reporting the same synergistic permeabilization.

It is not clear whether the release of IMS proteins in vivo is due to transient openings or sustained permeability. Many publications [30, 38] have reported a Bax-induced release of cytochrome *c* from isolated mitochondria over a period of hours without significant increases in the MOM permeability to cytochrome *c*. Transient channel openings are a likely explanation. Does this mean that the Bax/ceramide sustained permeabilization of the MOM and thus rapid release of protein is unnecessary or unphysiological? It seems unlikely that the Bax/ceramide synergism is an interaction that is not specific and not maintained by natural selection, especially since anti-apoptotic proteins act precisely in the opposite manner [4]. Further, Kluck et al. [30], have shown that, in the presence of a putative apoptotic component of the cytosol (which would be expected to be available to apoptotic mitochondria in vivo), Bax induces sustained enhancement of permeability. Munoz-Piendo et al. [39] report that a non-specific pore showing prolonged permeability is responsible for protein release during apoptosis. Thus the sustained permeability observed in the presence of activated Bax and ceramide is not an aberration.

It must be emphasized that the experiments were performed with physiologically relevant doses of ceramide and Bax. The ceramide used in this study is C_{16}-ceramide which is one of the common, naturally occurring, long-chain ceramides. The amounts of ceramide used seem high but, only about 5% of the ceramide added to isolated mitochondria inserts into mitochondrial membranes [14], requiring the addition of larger amounts of ceramide to the mitochondrial suspension to achieve MOMP. Nevertheless, as shown previously [10, 14], we are working at mole fractions of ceramide typically found in mitochondria early in apoptosis. The amount of activated Bax used in these experiments is also at physiological levels. Enhancement of ceramide induced MOMP was achieved with levels of activated Bax in the low nM range. At these levels, activated Bax often has little or no effect on MOMP, indicating that its action on ceramide channels may be more important.

Activation of Bax in cells is thought to be mediated by N/C Bid. Apart from N/C Bid, non-ionic detergents, at their CMC, have also been found to activate Bax [27, 40]. This detergent-activated Bax shows similar reactivity to conformation specific antibody [40] and restricted accessibility to trypsin digestion [27]. We have used detergent activated Bax for most of our experiments to focus on the Bax/ceramide interaction. This is just part of a bigger, more complicated, picture. For example, Bcl-2 family proteins have been shown to regulate the formation of ceramide also, during apoptosis [41].

These and earlier studies with the anti-apoptotic Bcl-2 family proteins [4] underscore that natural selection has favored mechanistic interactions between some Bcl-2 family proteins and ceramide resulting in functional outcomes. These functional interactions could be pivotal in regulating MOMP under circumstances where both ceramide and the Bcl-2 family proteins are present.

Concluding remarks

Experiments with isolated mitochondria and phospholipid membranes show that low levels activated Bax and ceramide interact to enhance the membrane permeability to a level greater that each agent alone. The results are best interpreted as activated Bax enhancing ceramide channels but other interpretations are not excluded. These results are in harmony with published results showing that anti-apoptotic proteins disassemble ceramide channels. These show that the Bcl-2 family of proteins that regulate protein release from mitochondria early in apoptosis also interact with ceramide and ceramide channels to influence MOMP. This result is consistent with an emerging picture that ceramide channels may be a pathway by which proteins are released from mitochondria, initiating the execution phase of apoptosis.

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