The oxidized phospholipid PazePC modulates interactions between Bax and mitochondrial membranes

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Activation of the pro-apoptotic protein Bax under intracellular oxidative stress is closely related to its association with the mitochondrial outer membrane (MOM) system, ultimately resulting in cell death. The precise mechanism by which this activation and the subsequent structural changes in the protein occur is currently unknown. In addition to triggering the onset of apoptosis, oxidative stress generates oxidized lipids whose impact on mitochondrial membrane integrity and the activity of membrane-associated Bax is unclear. We therefore devised a model system that mimics oxidative stress conditions by incorporating oxidized phospholipids (OxPls) into mitochondria-like liposomes, and studied the OxPls’ impact on Bax-membrane interactions. Differential scanning calorimetry (DSC) was used to study membrane organization and protein stability, while conformational changes in the protein upon contact with lipid vesicles were monitored using far-UV circular dichroism (CD) spectroscopy. The thermograms for liposomes containing the OxPl 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PazePC) differed dramatically from those for unmodified liposomes. Moreover, Bax exhibited enhanced thermal stability in the presence of the modified liposomes, indicating that it interacted strongly with PazePC-containing membranes. The presence of PazePC also increased the α-helical character of Bax compared to the protein alone or with PazePC-free vesicles, at 10 °C, 20 °C, and 37 °C. Presumably, the presence of PazePC-like OxPls a) increases the population of membrane-associated Bax and b) facilitates the protein’s insertion into the membrane by distorting the bilayer’s organization, as seen by solid-state high-resolution 1H and 31P magic angle spinning nuclear magnetic resonance (MAS NMR) spectroscopy.

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1. Introduction

Apoptosis is a fundamental biological process which is crucial for embryogenesis and tissue homeostasis including the removal of damaged cells [1,2]. At the mitochondrial level, this process is tightly controlled via the intrinsic (mitochondrial) apoptotic pathway, whose malfunctioning is involved in numerous diseases, ranging from premature cell death in auto-immune/neuro-degenerative disorders to cancer cell survival [1,3,4]. This pathway is controlled by the Bcl-2 protein family, whose pro- and anti-apoptotic members interact at the mitochondrial outer membrane (MOM) system to determine the fate of the cell [5]. Cellular stress signals activate pro-apoptotic proteins such as Bax. Activated Bax is translocated to the MOM and undergoes a series of conformational changes that allow it to insert into the membrane and form homo-oligomeric pores. This mitochondrial outer membrane permeabilization (MOMP) process leads to the release of apoptogenic factors such as cytochrome c, which are lethal for the cell [3,6,7].

Different mechanisms have been proposed to account for the involvement of the Bcl-2 proteins in the formation and regulation of MOMP, in which the mitochondrial membrane systems typically play only a passive role [3]. However, recent studies have suggested that also these membranes are actively and directly involved in MOMP, which are regulated by pro- and anti-apoptotic Bcl-2 proteins [8–10]. The mitochondria-specific negatively charged phospholipid cardiolipin (CL) plays a key role and seems to be important in both recruiting Bax to the membrane and in the subsequent insertion of the protein into the highly flexible membrane system followed by its oligomerization into pores, which is mediated by the BH3-only protein tBid [11–13]. CL is predominantly localized at the inner mitochondrial membrane (IMM), where it binds cytochrome c. However,
it can also be found at the MOM and is even enriched at mitochondrial contact sites that connect the IMM to the MOM [14].

Under conditions of oxidative stress, mitochondrial lipids can also play an active role in triggering cell death. Oxidation of CL has been shown to cause it to dissociate from its cytochrome c complex and to be translocated to the MOM, where it assists in the recruitment of Bax and tBid [15,16]. However, most of the oxidized phospholipids (OxPLs) formed under these conditions are derived from (poly)unsaturated diacyl- and alk(en)ylacyl glycerophospholipids. These lipids possess truncated sn-2 fatty acid chains with aldehyde and carboxyl moieties at the chain’s end [17]. They are involved in the direct activation of acid sphingomyelinase and the subsequent formation of ceramide, a secondary messenger that mediates apoptotic signals [17]. In addition to their role in direct signaling, OxPLs are also integrated into mitochondrial membranes. Once integrated, they can dramatically change the membrane’s properties and structure because of the way they differ from the other membrane constituents in terms of structure, polarity, and shape. In particular, they can favor the formation of polar moieties within the membranes. Their integration is therefore expected to induce functional changes in the membrane that affect both lipid–lipid and lipid–protein interactions and thereby alter the functioning of membrane proteins [18,19]. In this work we studied the impact of OxPLs on the conformation of the pro-apoptotic Bax protein and its binding to lipid vesicles that mimic the properties of MOM. On the basis of MD simulations of the impact of OxPL on lipid bilayers [18] and own preliminary studies, we focused on the OxPL known as PazePC (1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine), which has a carboxyl moiety at the ω-position of its shortened sn-2 fatty acid chain (Fig. 1). Liposomes were prepared consisting of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1,1′,2,2′-tetramyristoyl cardiolipin (TMCL) and PazePC (1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA).

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1,1′,2,2′-tetramyristoyl cardiolipin (TMCL) and PazePC (1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA).

2.2. Expression and purification of recombinant full-length human Bax

The protein was expressed and purified according to the previously published protocol of Suzuki et al. [20]. Briefly, pTYB1-Bax was expressed in BL21(DE3) and the cells were disrupted using a French press prior to affinity chromatography with a chitin column. Intein self-cleavage was then induced by treatment with 40 mM DTT for 48 h and Bax was further purified using a mono-Q column (GE Healthcare) followed by size exclusion chromatography using a HiPrep 16/60 Sephacryl S-100 HR column (GE Healthcare). The pure protein was stored at −80 °C in 10 mM Hepes (pH 7.4), 100 mM KCl and 20% (v/v) glycerol. Protein purity was assessed by SDS–PAGE and protein concentration was determined on the basis of samples’ absorption at 280 nm.

Fig. 1. The chemical structures of POPC, POPE, TMCL and PazePC.
2.3. Sample preparation for DSC and CD experiments

Mixtures of POPC, POPE and TMCL with or without PazePC (POPC:POPE:TMCL:PazePC, 33:36:21:10; POPC:POPE:TMCL, 43:36:21) were dissolved in chloroform:methanol (2:1 v/v), after which the solvent was removed by rotary evaporation. The lipid film was then suspended in pure water (Millipore) under gentle sonication and the aqueous lipid dispersion was lyophilized overnight. The collected dry white powder was stored at −20 °C until further use.

For the DSC measurements, 3 mM MLVs were prepared by dispersing the lipid powder in an aqueous solution containing 10 mM sodium phosphate (pH 7.4), 100 mM KCl, and 0.5 mM EDTA, followed by three freeze-thaw cycles and vigorous vortexing. For the samples containing liposomes with added Bax, the lipid powder was resuspended in a 15 μM protein solution, yielding a protein:lipid molar ratio of 1:200. Cooling-heating cycles involving cooling on ice followed by immersion in a 45 °C water bath were used instead of freeze-thaw cycles to maintain protein integrity; 45 °C is above the liposomes’ transition temperature but not warm enough to denature Bax. Cycles were repeated at least three times with vortexing after each cycle. To reduce light scattering in the CD measurements, the MLVs were sonicated using a probe-type sonicator (Soniprep 150, MSE, USA) to produce SUVs.

2.4. Differential scanning calorimetry

DSC measurements were carried out using a VP-DSC calorimeter (MicroCal, Inc., Northampton, MA, USA) with degassed samples under a pressure of 20 psi. To ensure that thermal equilibrium was reached, three thermograms were recorded. The first involved heating the sample from 5 °C to 45 °C at a scan rate of 60 °C/h, the second involved cooling from 45 °C to 5 °C at a scan rate of 60 °C/h and the third involved heating from 5 °C to 45 °C at a scan rate of 2 °C/h. Only the result from the last up-scan was used. Bax melting was analyzed using a 15 μM protein solution in the absence and presence of POPC:POPE:TMCL:PazePC at a scan rate of 90 °C/h; the analyses were based on data from the first up-scan.

2.5. Circular dichroism spectroscopy

The secondary structure of Bax was analyzed at 10 °C, 20 °C and 37 °C, in the absence and presence of liposomes, using a JASCO J-810 Spectropolarimeter (Japan). The protein and lipid concentrations used in these studies were 5 μM and 1 mM respectively, giving a protein:lipid molar ratio of 1:200, in an aqueous solution containing 10 mM sodium phosphate (pH 7.4), 100 mM KCl, and 0.5 mM EDTA. Spectra were acquired using a cuvette with a path length of 0.1 cm at a bandwidth of 2 nm and a scan rate of 50 nm/min, and generated by averaging 8 scans for each sample. All samples were incubated for 15 min at each temperature. Buffer and lipid backgrounds were subtracted from the protein signal.

2.6. Membrane binding assay

To assess the fraction of Bax bound to PazePC-containing membranes 2.5 μM protein was incubated with 500 μM MLVs (after three cooling-heating cycles) for 3 h at 37 °C, in 10 mM sodium phosphate (pH 7.4), 100 mM KCl, and 0.5 mM EDTA. Following ultracentrifugation at 70,000 rpm and 4 °C for 1 h, the pellet containing membrane-bound Bax, and the supernatant containing free protein, were separated. The supernatant was treated with acetone and the precipitated Bax was resuspended in the same volume of SDS-cocktail as the pellet, and run on a 15% SDS-gel. To quantify the fraction of membrane-bound Bax, the intensities of the protein bands were analyzed by densitometry using Multi Gauge v3.1 software.

2.7. Solid-state 1H and 31P MAS NMR

For MAS NMR experiments PazePC-containing MLVs were prepared prior and upon addition of Bax at over 200:1 lipid:protein ratio using 10 mM sodium phosphate (pH 7.4), 100 mM KCl, and 0.5 mM EDTA with D2O as solvent. Treatment of vesicles was carried out as described for DSC and after centrifugation the vesicle pellets were transferred into 4 mm MAS NMR rotor (Chemagnetics, USA). 1H and 31P MAS NMR spectra were acquired at 37 °C using a 400 MHz Infinity spectrometer (Chemagnetics, USA) as described previously [21]. 1H NMR spectra were acquired with a single π/2-pulse with 5.5 μs (1H) duration, repetition delays between 2 s and 10 s at a spinning rate of 6 kHz. 31P MAS NMR experiments were acquired under proton decoupling at 6 kHz spinning speed with a single 5.5 μs long π/2-pulse and a 3 s repetition rate. 31P NMR spectra were referenced externally to −0.9 ppm, using DMPC vesicles at 308 K [22].

3. Results

3.1. DSC profiles: Bax interacts with OxPl-containing mitochondria-like membranes

DSC measurements were conducted to investigate the interactions between Bax and the lipid vesicles used to model the mitochondrial membrane and to determine how these interactions are affected by the presence of OxPis. Two different liposome preparations were used: POPC:POPE:TMCL (in a molar ratio of 43:36:21) and POPC:POPE:TMCL:PazePC (in a molar ratio of 33:36:21:10). TMCL, which is the saturated analogue of cardiolipin, was used to detect phase transition in the physiologically-relevant temperature range. The phase behavior of these two different lipidome systems was measured before and immediately after the addition of Bax. The DSC profile of pure POPC:POPE:TMCL MLVs shows a single, fairly symmetrical transition (Fig. 2A (−)), with an approximate melting temperature (Tm) of 21 °C. Replacing 10 mol% of the POPC with an equivalent (molar) quantity of PazePC caused pronounced changes in the thermogram (Fig. 2B (−)). The rugged, non-symmetrical heat profile that was observed following PazePC incorporation is consistent with the dynamic impact of this OxPl on the membrane bilayers in MLVs that was observed in related MD simulations [18]. The peak broadening and multi-component thermogram in the presence of PazePC reflects a
much less cooperative phase transition, presumably due to the formation of PazePC-rich and PazePC-poor lipid domains as occurs with sterols in membranes [23,24].

The heat profile did not change significantly after the addition of 15 μM Bax to OxPl-free lipid vesicles in a lipid:protein molar ratio of 200:1 (Fig. 2A (−−)), indicating that the protein was only loosely associated with the membrane. In contrast, when Bax was added to PazePC-containing MLVs under identical conditions, the DSC profile changed dramatically (Fig. 2B (···)). The multi-component heat profile observed for the Bax-free case was replaced by a single transition peak with an enhanced intensity, whose onset occurred at higher temperatures. This behavior indicates that Bax is binding to and interacting with PazePC containing membranes, probably by partial penetration into the membrane at lower temperatures. Such behavior would indicate an increased degree of order in the membrane’s fatty acid region.

3.2. CD spectroscopy: OxPl containing membranes induce conformational changes in Bax

Bax alone exhibited a predominantly α-helical CD profile (Fig. 3 (−−)), displaying the typical minima at 208 and 222 nm as reported previously [8,25]. To monitor conformational changes induced in Bax by interaction with a membrane, a solution of the protein was added to a suspension of SUVs whose lipid composition was identical to that used in the DSC experiments. In the presence of PazePC-free vesicles (Fig. 3 (−−)), the protein was slightly more structured, as demonstrated by a small increase in its α-helical content. In the presence of PazePC-containing vesicles (Fig. 3 (···)), the α-helical characteristic of the protein was further increased (by 7% compared to the protein alone). Interestingly, temperature dependent CD studies (Fig. 4) revealed that while the structural changes in Bax induced by PazePC vesicles were strongly temperature dependent, those for Bax alone or Bax in the presence of PazePC-free vesicles exhibited negligible temperature dependence (Fig. 5A and B). The overall CD profile obtained at 37 °C (Fig. 4 (···)) differed substantially from those obtained at lower temperatures, particularly in terms of the relative depths of the 208 and 222 nm minima. At this temperature, the PazePC membranes have completed their transition into the liquid-crystalline phase (Fig. 2B). The onset of the membrane phase transition occurs at 10 °C (Fig. 4 (−−)), but even at 20 °C (Fig. 4 (−−−)) the CD profile for Bax in the presence of PazePC vesicles was similar to those for Bax alone and for Bax incubated with PazePC-free vesicles (Fig. 5A and B). The intensity of the CD signal was higher in all cases in the presence of PazePC membranes. Analysis of the CD spectra revealed that incubation with PazePC-containing vesicles at 10 °C caused an 11% increase in the α-helical character of Bax (whereas no such increase was observed in the absence of PazePC). However, at 37 °C, the α-helical content of Bax dropped by 8% in the presence of PazePC-containing membranes, but the decrease for Bax alone was much more moderate. The α-helical character of Bax also decreased slightly on going from 10 °C to 37 °C in the presence of PazePC-free membranes.

Fig. 3. Far-UV CD spectra of 5 μM Bax in the absence of lipids (−−), in the presence of 1 mM POPC:POPE:TMCL (−−−) or 1 mM POPC:POPE:TMCL:PazePC (···), in 10 mM sodium phosphate (pH 7.4), 100 mM KCl, and 0.5 mM EDTA, at 37 °C.

Fig. 4. Far-UV CD spectra of 5 μM Bax, in the presence of 1 mM POPC:POPE:TMCL:PazePC at 10 °C (−−), 20 °C (−−−) and 37 °C (···), in 10 mM sodium phosphate (pH 7.4), 100 mM KCl, and 0.5 mM EDTA.

Fig. 5. Far-UV CD spectra of 5 μM Bax alone (A), and in the presence of 1 mM POPC:POPE:TMCL (B), at 10 °C (−−), 20 °C (−−−) and 37 °C (···), in 10 mM sodium phosphate (pH 7.4), 100 mM KCl, and 0.5 mM EDTA.
3.3. Increased thermostability of Bax in the presence of PazePC-containing MLVs

The strong interaction between Bax and PazePC-containing membranes indicated by the membranes’ heat profiles and the CD measurements was investigated in more detail using DSC at higher temperatures (far above the transition of the vesicles) that caused heat-induced unfolding of Bax [8]. The thermograms indicate that in the absence of membranes, Bax undergoes a broad transition with a $T_{m}$ of approximately 82–83 °C (Fig. 6 (−)). In the presence of PazePC liposomes there was no evidence of protein unfolding in this temperature range, as the onset of melting occurred sharply at above 90 °C (Fig. 6 (−)). This result further reinforce that Bax extensively interacts with PazePC-containing membranes.

3.4. Membrane binding assay

The capability of Bax to associate with PazePC-containing liposomes was further examined by assessing the membrane-bound protein fraction after incubation with MLVs containing PazePC OxPl. After ultracentrifugation the pellet and the acetone precipitated supernatant were analyzed with SDS-PAGE. As seen in Fig. 7 the protein band corresponding to the pellet (P) is larger and much more pronounced than for the supernatant (S) counterpart, indicating that the equilibrium of Bax is shifted towards a membrane-bound population in the presence of PazePC-vesicles. Quantification of the intensities by densitometry using Multi Gauge v3.1 software revealed that approximately 70% of the 2.5 μM Bax was bound the membranes, supporting the putative strong membrane–protein interactions as seen e.g. in the DSC profiles. In the DSC measurement, no free Bax was detected for the 15 μM protein sample used; only a membrane-associated Bax population was observed, which melted at higher temperatures compared to membrane-free Bax (see Fig. 6).

3.5. NMR experiments: insight into the interaction of Bax with OxPl-containing vesicles

While $^{31}$P NMR provides detailed insight into lipid specific interactions with protein at the hydrophilic membrane headgroup region, $^1$H MAS NMR gives information about Bax induced perturbation across the whole membrane [21,22]. In Fig. 8 (−), high resolution $^{31}$P MAS NMR spectra of the pure lipid vesicles are shown, where quite broad PC, PE and CL phosphate signals are observed, separated by their different isotropic chemical shift ($\delta_i$) values (−1.07 ppm (PC), −0.6 ppm (PE) and −0.1 ppm (CL)). The small peak at 0.4 ppm corresponds to phosphate buffer. Upon the presence of Bax (Fig. 8 (−−)) major changes occurred. Not only did the lines become narrower as a result of increased fluidity due to an increase in molecular disorder and dynamics, but also the lipid specific resonances moved downfield to −0.816 ppm, −0.19 ppm and 0.203 ppm, respectively. These changes in shift indicate the presence of membrane-associated Bax, whose total negative charge at pH 7.4 (pI− 5) induced a downfield shift as expected for membranes with an increase in their negative surface potential [22]. Not surprisingly, the relevant $^1$H MAS NMR spectra for the hydrophobic fatty acid chain region are shown in Fig. 9B. The presence of Bax clearly induced two distinct features into the NMR spectra of the lipid vesicles. Firstly, adding Bax caused a new lipid component to appear at 1.45 ppm (marked by an arrow). Multicomponent analysis of this spectral region which is typical for fatty acid CH$_2$ segments in a liquid-crystalline lipid phase, indicates that the typical NMR peak of pure lipid vesicles (1.32 ppm) is composed of two overlapping resonances at 1.36 ppm and 1.31 ppm, an integral ratio of 53:47 and linewidths of 48 Hz and 27 Hz respectively. Secondly, while not affecting the frequencies of the two pre-existing peaks, the presence of Bax and the appearance of the third component at 1.45 ppm and 38 Hz linewidth, changed the linewidth of those other two peaks to become narrower (34 Hz and 22 Hz, respectively) and the total intensity pattern became 15:38:47, respectively. Clearly, Bax penetrates into the hydrocarbon chain region and increases the local fatty acid disorder as reflected in reduced NMR linewidths. Also, as the new peak indicates, part of the fatty acid chains feels directly the presence of Bax.
It is believed that the action of tBid causes Bax to be concentrated at sites where mitochondrial contact sites form in the MOM during the early stages of the activation process [28,29]. Consequently, model liposomes whose properties resemble those of the MOM at these locations were used in this work [14]. Our results show that even in the absence of tBid, Bax could associate with these liposomes in a temperature-dependent way when PazePC (a typical OxPl product) was present. In addition, Bax interacted much less strongly with PazePC-free mitochondrial membranes, as previously described for cases where tBid was not present [10,30]. The DSC profiles revealed that the presence of PazePC induced major changes in the biophysical properties of the lipid membranes, which presumably enhanced their ability to interact with Bax. These drastic changes in membrane properties are not surprising because there are significant structural differences between oxidized lipids such as PazePC and the usual PC lipids. In particular, the truncated sn-2 chain of PazePC has a terminal polar carboxylic acid moiety that severely perturbs the structure of the membrane [18]. This chain can easily be reoriented in the bilayer such that the polar terminal group points towards the membrane interface or even the aqueous phase. This tendency is enhanced by deprotonation, which converts the carboxylic acid to an anionic carboxylate group at the α-position. Preliminary studies using a PazePC analogue in which the terminal carboxylic acid is replaced by an aldehyde (PoxnoPC) revealed the latter to cause a much weaker association between Bax and the membrane.

As shown for cytochrome c, the interaction between the PazePC-containing membrane and Bax is pH-dependent; in the membrane–protein complex, PazePC is deprotonated with the sn-2 chain pointed towards the protein, as previously described for cytochrome c [31]. However, we cannot assume that the interactions between Bax and PazePC-containing membranes are primarily dependent on the presence of a deprotonated carboxylic group in the sn-2 acyl chain because the diionic species cardiolipin did not induce any significant association of the protein to POPC:POPE:TMCL membranes. Furthermore, at pH 7.4, Bax has a negative net charge, which is not consistent with the hypothesis that electrostatic factors alone might be responsible for the observed interaction. The 6A7-epitope is considered a marker for the initiation of Bax activation, and both neutral and charged variants have been shown to be exposed in the presence of membranes [8]. Our results do not rule out the possibility that the POPC:POPE:TMCL liposomes may have triggered a conformational change corresponding to 6A7-epitope exposure, but in any case the interaction of Bax with the liposomes lacking PazePC was insufficiently strong to trigger any significant change in the thermogram.

4. Discussion

Our combined data from DSC, CD, membrane binding assay and NMR measurements provide evidence for strong interactions between Bax and OxPl-containing membranes, which could play an important role in the molecular mechanism underpinning the onset and continuation of apoptotic activity under oxidative stress. As shown previously, the action of the pro-apoptotic Bax protein is closely related to the unique features of the mitochondrial membranes. These complex interfaces are crucial for the ability of Bax to associate with the mitochondrial membrane and undergo the conformational changes necessary for membrane penetration and the formation of cytochrome c releasing pores [9]. However, in healthy cells, Bax is mainly found in the cytosol; only a small fraction of the total population is (loosely) associated with the MOM and does not form pores or have any other pro-apoptotic effects. As such, it is perhaps not surprising that the presence in the membrane of lipid oxidation products generated under oxidative stress changes the nature of the interactions between the membrane and Bax. These changes could potentially affect the ability of Bax (and potentially also its activating partner, tBid) to bind to these membranes in vivo and to assemble transmembrane pore structures. Since mitochondria are the primary location of the formation of reactive oxygen species (ROS) in the cell, it is not surprising that locally high concentrations of OxPls can be generated [26,27], a process which can have pronounced effects on membrane protein function due to the high protein-to-lipid ratio in mitochondria [14].
a greater level of α-helical character than was the case for the other conditions examined. As the NMR results obtained at 37 °C show, Bax is still partially penetrating in the membrane in its physiologically active liquid-crystalline phase. The relevant 1H MAS NMR spectra in Fig. 9 reveal still major changes in the fatty acid chain indicating CH2 spectral region. In addition, part of Bax must still be situated at the interface, since the glycerol region in the 1H NMR spectrum and the corresponding 31P NMR spectra undergo changes in a way as expected for the mushroom model. These findings indicate that the PazePC sn-2 chain alters the nature of the membrane interface and can interact intimately with the protein, as previously reported for cytochrome c [31].

Monomeric Bax has recently been shown to bind to lipid membranes, albeit only weakly and transiently in the absence of triggering factors such as tBid [30]. Also, presence of lipids alone has demonstrated Bax at the membrane surface. Both factors would prime the energetic cost, and b) increase the population of loosely associating Bax membranes, which provides a plausible molecular mechanism for the onset of mitochondrially-mediated apoptosis induced by oxidative stress [23].

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