BaxΔC Induces Increased Membrane Lipid Order in Isolated Rat Liver Mitochondria

NATALIA TSESIN*, ELAI DAVICIONI*, RIVKA COHEN-LURIA*, ILANA NATHAN† AND ABRAHAM H. PAROLA*
Departments of *Chemistry, Faculty of Natural Sciences, and †Clinical Biochemistry, Faculty of Health Sciences and the Hematology Institute, Soroka University Medical Center
Ben-Gurion University of the Negev
P.O. Box 635, Beer-Sheva, 84105
ISRAEL
aparola@bgu.ac.il

Abstract: - The interaction of the pro-apoptotic BaxΔC with the excimer-forming lipid, 12-(pyrene-1-yl)-dodecanoic acid (PDA) labeled isolated rat liver mitochondria, caused a decrease in PDA excimer-to-monomer fluorescence emission ratio (E/M). This suggested that BaxΔC caused mitochondrial membrane lipid reorganization. These changes were dependent on the BaxΔC protein-to-mitochondrial lipid ratio. The changes in the fluorescence emission spectra were not attributed to overall changes in the bulk properties of the mitochondrial suspensions or to fluorescence resonance energy transfer between the heme group of cytochrome c and pyrene. These data suggested that BaxΔC increases mitochondrial membrane "microviscosity". Fluorescence anisotropy studies with DPH and TMA-DPH labeled mitochondria independently show reduced rotational dynamics, further supporting this presumed increased membrane "microviscosity". The lifetimes of all probes did not change upon BaxΔC interaction with the mitochondrial membrane, verifying the changes in the dynamics of the probe mobility in the membrane. Another pro-apoptotic protein tBid, a more potent inducer of cytochrome c release, also caused an increase in DPH anisotropy, yet to a lesser extent as compare with equal concentration of BaxΔC. The observed increase in DPH anisotropy caused by BaxΔC-membrane interaction was inhibited by the anti-apoptotic protein Bcl-2ΔTM. The effects of BaxΔC on the mitochondrial membranes were correlated to cytochrome c release. These findings reflect BaxΔC interaction with the mitochondrial membrane, where the binding of BaxΔC lowers probe mobility and induces major reorganization of mitochondria membrane lipids, possibly a phase transition associated with non-lamellar bilayer structures.

Key-Words:- apoptosis, tBid, Bcl-2ΔTM, cytochrome c, pyrene excimers, fluorescence anisotropy & lifetime, lipid probes, "microviscosity", lipid pores

1 Introduction

The pro-apoptotic protein Bax recruits mitochondria into the apoptotic pathway by partitioning exclusively into the mitochondrial outer membrane, releasing cytochrome c and other small globular proteins from the inter membrane space [1, 2]. Cytochrome c assembles into the apoptosome, and this complex is responsible for caspase-9 activation and the apoptotic proteolytic cascade resulting in cell death. The precise mechanism of cytochrome c efflux from apoptotic mitochondria remains unclear although numerous models have been proposed [3-5], including the activation of the permeability transition pore (PTP) or the formation of lipidic pores [6-9].

More recent work describes apoptotic pore formation as a process facilitated by Bax which induce lipid bilayer destabilization, through reduction of the linear tension of the membrane

[10]. The induction of lipidic pores by the so-called pore forming proteins such as the bee venom melittin and the bacterial colicin toxins, is thought to arise from a complex series of interactions between the positively charged amino acid residues on transmembrane inserting alpha helices with negatively charged phospholipid headgroups (i.e., cardiolipin) as well as hydrophobic amino acid residues and hydrophobic fatty acid acyl chains of the phospholipids [11, 12]. Similar mechanisms have been proposed for pro-apoptotic proteins, although all of this work has been conducted on model lipid bilayers [7-9, 13-15].

The present study follows the interaction of the proapoptotic proteins, Bax with isolated rat liver mitochondria labeled with a fatty-acid conjugate of pyrene, 12-(pyrene-1-yl)-dodecanoic acid (PDA), l, 6-diphenyl-l, 3, 5-hexatriene (DPH), and 1-[4(trimethylamino)phenyl]-6- phenyl-1,3,5-hexatriene (TMA-DPH). The pyrene moiety covalently bound to the end of the acyl chain in PDA forms a very hydrophobic fluorophore that readily partitions into the bilayer. A particularly useful feature of pyrene is the ability of the excited monomeric molecule to form a complex with a ground-state pyrene, which results in an excited dimer, i.e., excimer [16]. Since, the ratio of excimer to monomer fluorescence intensities (E/M) for pyrene-containing lipid derivatives depends on the local concentration of the pyrene moiety as well as the rate of lateral diffusion [17-19], the rate of excimer formation is greatly dependent on the viscosity of the membrane lipid environment [20-22]. The properties of these fluorophores are therefore useful for measuring changes in mitochondrial membrane microenvironment due to interactions between apoptotic proteins and mitochondrial membrane lipids. In order to independently verify the observed changes in mitochondrial membrane "microviscosity" obtained with PDA, fluorescence polarization measurements employing DPH and TMA-DPH were carried out [18-20]. DPH was found to be located in the hydrocarbon core oriented mostly (95%) parallel to the lipid acyl chain axis and 5% in the center of the lipid bilayer parallel to the surface [23], while TMA –DPH is anchored at the interface between the membrane phospholipid-head groups and the aqueous phase, due to its positively charged amino groups. The results obtained with these fluorescent membrane probes are consistent with a model in which BaxΔC induces reorganization of the mitochondrial membrane lipids, resulting in the rigidification of the membrane.

2 Methods

2.1 Materials

Cytochrome c (horse heart type VI), bovine serum albumin (BSA), and other chemical reagents were from Sigma, St. Louis, Missouri. PDA, DPH and TMA-DPH were from Molecular Probes, Eugene Oregon. The pro- and anti-apoptotic proteins BaxΔC20 [7] and tBid was a generous gift of Dr. Bruno Antonsson, Serono Pharmaceutical Research Institute, Geneva, Switzerland.

2.2 Isolation of Rat Liver Mitochondria

Mitochondria were isolated from livers of male Witstar rats (200-300 gr) by standard differential centrifugation according to the procedure used [24] and described previously [25]. Livers (12-16 gr) were homogenized in 40 mL of ice-cold mitochondria isolation buffer (MIB, 250 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% BSA and 10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES)/KOH, pH 7.4). Homogenates were centrifuged at 600 g for 5 min and mitochondria were recovered from the supernatant by centrifugation at 10300 g for 10 min, and washed at 8500 g for 10 min. Finally, the pellet was resuspended to the original volume in buffer without BSA, centrifuged at 12000 g for 10 min and re-suspended in a minimal volume (1-2 mL) of MIB.

Protein concentration was determined using the Biuret assay, with BSA as a standard. Mitochondrial suspensions were stored on ice up to 4 hours.

2.3 PDA Labeling Procedure

Mitochondrial aliquots were diluted to a concentration of 10 mg/mL with labeling buffer (LB, 220 mM mannitol, 70 mM sucrose, 0.5 mM EDTA, 5 mM succinate, 1 mM rotenone (to avoid fluctuations in mitochondrial pyridine background autofluorescence [26]), 2 mM HEPES/KOH, pH 7.4) [27, 28]. PDA was added at a final concentration of 0.5% (v/v) dimethyl sulfoxide

(DMSO) to minimize disruptions to the mitochondria, and the initial molar ratio of PDA relative to mitochondrial lipids was 1:100 [17, 29]. Following 15 min incubation on ice, excess probe was removed by centrifugation (6500 g) for 10 min in LB. The final lipid-to-probe ratio was calculated by the use of a calibration curve. The molar concentrations of the probe, 0.1-0.5 mol %, are assumed not to perturb the mitochondria membrane [17]. Mitochondrial labeling under these conditions results in preferential incorporation of the probe into the outer leaflet of the outer mitochondrial membrane [30, 31]. Phospholipid concentration was based on a literature value of a 200-nmol phospholipid/mg mitochondrial protein [32].

2.4 Steady-State Fluorescence

Aliquots of labeled mitochondria were mixed in high ionic strength buffer (HIB, 145 mM KCl, 2.5 mM potassium phosphate, 2.5 mM $MgCl_2$, 5 mM succinate, 1 μM rotenone, 2 mM HEPES/KOH, pH 7.4) [27], to a final volume of 1 mL. The concentration of mitochondrial protein could be varied to as high as 500 μg/mL without ill effects on fluorescence measurements, since the optical density (OD) was kept below 0.2, preventing inner filter effects [26]. Under these conditions labeled mitochondria samples yielded a good fluorescence signal, with signal-to-noise ratio above 10:1. Samples (1 mL) were measured on the Spex Fluorolog (Jobin Yvon Horiba, Edison, NJ) and ISS-K2 (Urbana-Champagne, IL) spectrofluorometer, in 4 mm internal light-path quartz cuvette, which was thermostated at 30.0±0.1°C with a Neslab RTE-111 bath (Newington, NH) [33]. Label fluorescence was obtained at excitation wavelength fixed at 347 nm. For time dependent measurements of PDA fluorescence, excitation and emission wavelengths were fixed at 347 and 377 nm, respectively. Changes in fluorescence were followed for 10 min after the addition of the various agents. Then, 1% (v/v) Triton-X 100 was added to obtain the total fluorescence signal of the sample [34].

To enable a quantitative comparison between fluorescence of samples from different preparations the following equation was used [35]:

$$\%\Delta F = ((F_t-F_0)/(F_\infty-F_0))*100\% \quad (1)$$

where, % ΔF is the normalized percent change in fluorescence, F_t is the fluorescence at time t, F_0 is the initial fluorescence, and F_∞ is the fluorescence after the addition of Triton-X 100.

Samples of rat liver mitochondria (RLM, 50μg/ml) labeled with PDA were incubated in high ionic buffer (HIB) at presence and absence of BaxΔC at different concentrations and IgG (1μM).The samples were centrifuged at 10000g for 10 min and pellet was resuspended in fresh HIB. PDA spectra were measured before centrifugation and in pellets and supernatants.

2.5 Time resolved Fluorescence

PDA lifetime measurements were carried out with a single-photon counting spectrofluorometer (FLS 920; Edinburgh Instruments). The concentration of mitochondrial protein in the samples was 150 μg/ml. Samples were excited at 347 nm, the excitation maximum and their emission was collected at 480 nm for excimer and 377 nm for monomer.

2.6 DPH Labeling Procedure

Mitochondria (0.05 mg protein/mL in HIB) were incubated for 40 min at RT with 6-8 μM DPH at stirring. These labeling concentrations are within the predetermined range of concentrations, 2-8 μM, for which a linear correlation with fluorescence intensity was verified. DPH was added at a maximal concentration of 0.1% (v/v) tetrahydrofuran (THF) to minimize disruptions to the mitochondria. Under these labeling conditions the noise to peak ratio was not above 5 % and no self quenching was observed.

2.7 TMA-DPH Labeling Procedure

Mitochondria (1.0 mg protein/mL in the same buffer used for PDA labeling) were incubated for 40 min on ice with 4-6 μM TMA-DPH. These labeling concentrations are within the predetermined range of concentrations, 2-8 μM TMA-DPH, for which a linear correlation with fluorescence intensity was verified. TMA-DPH was added at a maximal concentration of 0.5% (v/v) dimethyl sulfoxide (DMSO) to minimize disruptions to the mitochondria [36]. Under these labeling conditions the noise to peak ratio amounted to 3-4 % and no self quenching was observed.

2.8 Steady-State Fluorescence Anisotropy of DPH or TMA-DPH Labeled Mitochondria

1ml samples of RLM were incubated at absence and presence of various proteins. Samples with BaxΔC and Bcl-2 proteins together were pre-incubated with Bcl-2 for 10 minutes and then BaxΔC at 0.1 μM concentrations was added.

The fluorescence anisotropy of either DPH or TMA-DPH labeled mitochondria at 50 μg protein/mL in HIB was measured on the Perkin Elmer LS55 fluorometer at 30°C with excitation at λmax = 355 or 350 nm, respectively, by vertically

polarized light, and the emission followed at 430 nm, in parallel and perpendicular orientation relative to the exciting light [36, 37]. The measured steady state fluorescent anisotropy was calculated using the following equation:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (2)$$

where, r is the anisotropy, I_{VV} and I_{VH} are the intensity measured in parallel and perpendicular directions to the polarized excitation light, respectively, and G was calculated as I_{HV}/I_{HH} [38, 39].

The DPH anisotropy of the samples was calculated by following equation [40]:

$\mathbf{a_{DPH}} = (AF - \mathbf{a_b f_b})/\mathbf{f_{DPH}}$ (3),

when A and $\mathbf{a_b}$ are the total and blank anisotropy respectively and the fluorescence of the DPH ($\mathbf{f_{DPH}}$) is the difference between the total fluorescence (F) and the blank fluorescence ($\mathbf{f_b}$).

2.9 Lifetime Measurements of DPH and TMA-DPH Labeled Mitochondria

TMA-DPH and DPH lifetime measurements were performed by using the single-photon-counting setup [41]. The experimental setup consisted of a Ti–sapphire laser (Spectra-Physics, Mountain View, CA), Tsunami laser pumped by Millennia X, which was operated in its picosecond lasing mode (1 ps pulses at 82 MHz). The fundamental train of pulses was pulsed picked (Spectra-Physics, model 3980) to reduce its repetition rate typically to 0.8–4.0 MHz and then passed through a doubling lithium triborate crystal to preserve the quality of the laser mode. The laser was tuned on 360 nm using a Spectra-Physics optics set. The detection system consisted of a Hamamatsu-3809U 6m multi-channel plate (MCP). The fluorescence light was focused onto the entrance slit of the MCP after passing through a 1/8 meter double monochromator (CVI model CM 112, using a bandwidth of 2 nm). The samples of TMA-DPH labeled mitochondria in HIB, with S/N ratio ≈20, in the presence and absence of BaxΔC were excited. Fluorescence lifetime decays were acquired at 25 ns full scale range with 6.28 ps per channel. Typical counting rates were below 1 kHz. The number of counts was 4 K at the peak channel, collected with the Tennelec PCA3 card.

2.10 Light scattering measurements

Swelling of mitochondria causes light scattering to decrease (due to a decreased refractive index) [42, 43]. Light scattering was measured at 90° to the incident beam by setting the spectrofluorometers' excitation and emission monochromators both to 600 nm. With this setup we were able to measure fluorescence and swelling changes of the same sample alternately. A linear dependence of the scattered light on mitochondria concentration was verified, suggesting a linear response of the PMT. A linear dependence of mitochondrial swelling was verified as a function of Ca^{2+} concentration (0.05-1 mM) using light transmittance spectroscopy at 520 nm (Hewlett Packard 8452A Diode Array spectrophotometer) [44].

2.11 Cytochrome c release assay

Samples of mitochondria (12.5 mg) were incubated in HIB at a final volume of 1 mL with various vectors for 15 min at RT. Following centrifugation at 14000 g, the absorption spectrum of the supernatant was recorded between 350-600 nm (HP 8452A spectrophotometer and Varioscan plate reader (Thermo Electron Corporation)). This assay makes use of the intense heme Soret peak (γ band) in the spectrum of cytochrome c ($\varepsilon = 100\ mM^{-1}cm^{-1}$, 410 nm) [45]. To compare data between experiments and preparates, the absorption curves were normalized by subtracting the OD at 600 nm.

3 Results

3.1 BaxΔC interaction with PDA-labeled mitochondria results in increased monomer emission

We utilized the excimer forming properties of the lipid probe PDA to show dynamic changes in the properties of mitochondrial membrane induced by BaxΔC.

We used BaxΔC concentrations to include the physiologically relevant range. IgG was taken as a control that does not interact neither with the mitochondrial membrane nor with the fluorophore [46]. Fig. 1 shows that BaxΔC causes an increase in monomer emission intensity indicative of reduced probe mobility and increased membrane "microviscosity", which is significant at 0.1 μM concentration.

3.2 The effect of BaxΔC did not result from the redistribution of PDA

In order to ascertain that the observed change in membrane dynamics does not result from the redistribution of the probe between the membrane pellet and the supernatant, we separated the membrane pellet from the supernatant. Fig.2 shows that at the examined Bax concentrations of 0.05 and 0.1 mM there is no redistribution of PDA caused by

the addition of Bax. IgG does not interact with the membrane [46] and also does not cause PDA redistribution.

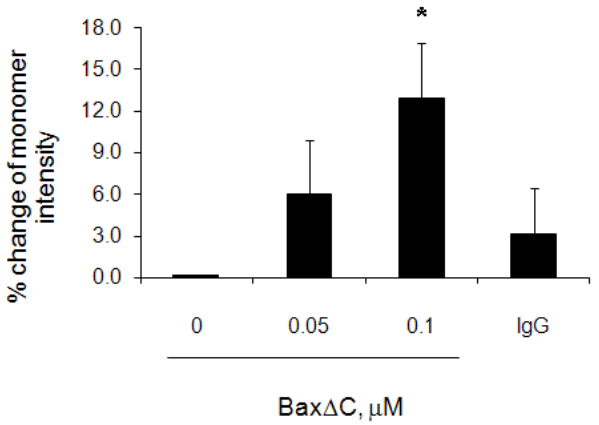

Fig.1: **Percent change of monomer intensity of PDA after IgG or BaxΔC addition to the mitochondrial membrane.** Samples of rat liver mitochondria (RLM, 50μg/ml) labeled with PDA were incubated in high ionic buffer (HIB) in the presence and absence of BaxΔC (0.05 and 0.1 μM) and IgG (1μM) and the intensity of PDA monomer fluorescence emission at λmax = 377nm are plotted. * P-value <0.05 vs control sample. Error bars indicate the standard error of at least three measurements.

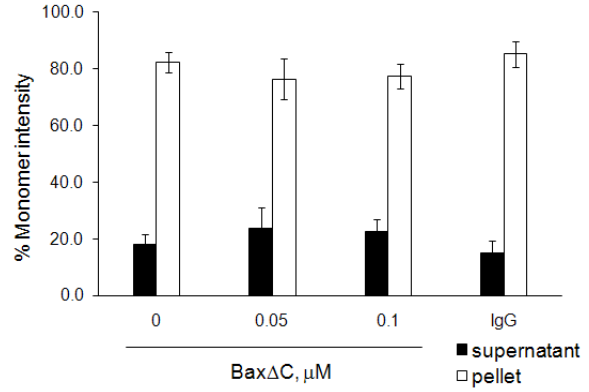

Fig.2: **No redistribution of PDA between the pellet and the supernatant following incubation of the mitochondrial membrane with either IgG or BaxΔC.** The samples from the previous section were centrifuged at 10000g for 10 min and pellet was resuspended in fresh HIB. PDA spectra were measured in pellets and supernatants following 10 min incubation with either BaxΔC or IgG. The percentage was calculated as a fraction of each component from the sum of both supernatant (black) and pellet(white). Error bars indicate the standard error of at least three measurements.

3.3 BaxΔC reduced E/M values of PDA labeled mitochondrial membrane.

As shown in Fig. 1 low concentrations of BaxΔC (0.05; 0.1 mM) significantly increased monomer emission, which is not associated with probe redistribution (Fig.2).

BaxΔC interaction with PDA-labeled mitochondria results in decreased E/M values indicating extensive lipid reorganization and altered membrane dynamics (Fig.3), suggesting that BaxΔC induced membrane rigidification. induced membrane rigidification

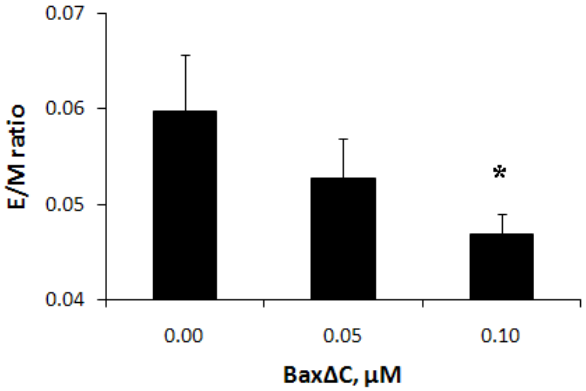

Fig.3: **E/M ratio alterations induced by BaxΔC.** E/M values were calculated from measurements of PDA monomer (at 377 nm) and eximer (at 480 nm) intensities in the pellet. Error bars indicate the standard error of three measurements. * p=0.05.

3.4 E/M reduction is specific for BaxΔC induced changes in membrane dynamics

Processes such as changes in light scattering properties of biological membrane preparations are known to cause shifts in the fluorescent emission profiles of probes such as PDA [47]. In order to rule out such a possibility, we preformed time course studies measuring in parallel the percentage changes in monomer and excimer emissions, and the change in light scattering (Fig.4A). Indeed, the 50% increase in monomer fluorescence and the concomitant decrease in excimer fluorescence were accompanied with only a slight decrease in light scattering. Control experiments with 62.5 and 200 μM of $CaCl_2$, known to induce both cytochrome c release and mitochondrial swelling [48, 49], confirmed that although the treatment with calcium resulted in a rapid decrease in light scattering (i.e., increased mitochondrial swelling) it did so without changing the emission spectra of the probe.

Similar results of light scattering as a function of Ca^{2+} concentration were obtained by absorbance/transmittance spectroscopy (data not

shown). Therefore, the changes in the fluorescence emission spectra are not attributed to overall changes in the bulk properties of the mitochondrial

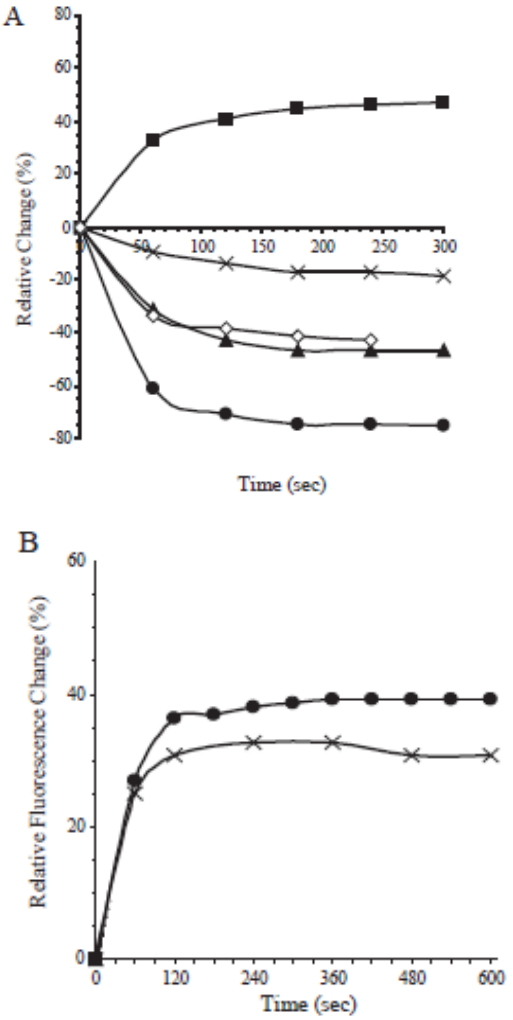

Fig.4. **BaxΔC induced changes in the fluorescence emission profile of PDA-labeled mitochondria are neither an artifact of mitochondrial swelling nor a de-quenching mechanism due to FRET from cytochrome c release.** (**A**) The profiles show the time dependence changes in monomer (■) and excimer (▲) fluorescence emission kinetic scans, the monomer to excimer E/M fluorescence ratio (●) and 90° light scattering (**x**). BaxΔC (2 μM) was added to PDA-labeled mitochondria (50 μg protein/mL) at t = 0 sec. Control experiments of light scattering induced by 200 μM $CaCl_2$ (◊). (**B**) Fluorescence of PDA-labeled mitochondria (50 μg protein/mL) was monitored in intact (●) or hypotonically lysed cytochrome c-free mitochondria (x) as described in Methods section, treated with 1 μM BaxΔC.

suspensions but rather to specific changes in the properties of the membrane, and hence, in the probe microenvironment [26, 47].

Another explanation for the changes induced by BaxΔC to the emission profile of PDA-labeled mitochondria could be through fluorescence resonance energy transfer (FRET). The heme group of cytochrome c is a good quencher of pyrene fluorescence emission through FRET [50]. In order to rule out the possibility that efflux of cytochrome c induced by BaxΔC contributed to the observed fluorescence changes through a dequenching mechanism, PDA-labeled mitochondria were lysed by a hypotonic buffer pretreatment. Under these conditions, cytochrome c is removed from these mitochondria membrane preparations prior to the addition of BaxΔC [28, 51]. The observed PDA monomer emission profile induced by the addition of BaxΔC to cytochrome c-free PDA-labeled mitochondria is shown in Fig.4B. Compared to that observed in intact mitochondria, the addition of BaxΔC to cytochrome c-free PDA-labeled mitochondria resulted in increased monomer fluorescence with similar overall kinetics to that observed with intact mitochondria, but with slightly decreased saturation fluorescence intensity. Therefore, the BaxΔC-induced increase in the monomer fluorescence appears to be independent of the presence or absence of cytochrome c, excluding dequenching effects (i.e., FRET-dependent emission profile) by Bax-induced cytochrome c release as the cause to PDA-monomer fluorescence increase. Instead, these results suggest that the lysis treatment disrupted the continuity of the membrane, and as such, changed the diffusion properties of the lipids (and of the lipid-conjugated probe) within the bilayer plane [29]. This attenuated the increase in the fluorescence intensity following BaxΔC treatment, but did not change the kinetics of the interaction between the protein and the mitochondria membrane (Fig.4B).

Changes in the fluorescence lifetime of the mitochondrial membrane embedded probe can potentially cause changes in the steady-state fluorescence emission profile. Changes in the lifetime of the probe have been observed in mitochondrial membranes due to interactions with molecular oxygen from the mitochondrial respiratory chain which is known to decrease the fluorescence lifetime of pyrene and its derivatives [52]. The average (±SE) of three independent measurements of excimer lifetime for PDA labeled mitochondria was 42 ± 5 ns both in the presence and absence of BaxΔC (p=0.9). Similarly, the monomer species lifetime was 109 ± 15 ns both in the absence and the presence of BaxΔC with no statistically significant difference between the two (p = 0.8). Therefore, it is suggested that the BaxΔC-induced

changes in the monomer and excimer fluorescence are not attributed to changes in the fluorescent properties of the probe but rather reflect changes in the dynamics of the probe mobility in the membrane.

3.5 BaxΔC induced an increase in DPH-labeled mitochondrial membrane-"microviscosity" that is inhibited by the anti-apoptotic protein Bcl-2ΔTM

In order to further verify the observed increase in membrane –"microviscosity" based on increase PDA monomer emission we independently studied the rotational dynamics of DPH labeled membranes which is another known fluorescence probe for membrane dynamics. Changes in mitochondrial membrane "microviscosity" can be followed by the lipophilic DPH probe [53, 54], which is embedded mostly parallel to the membrane phospholipids chains [23] of both the inner and outer leaflets. Fig.5A depicts the dose dependent increase in anisotropy of DPH labeled RLM upon the addition of BaxΔC. Another pro-apoptotic protein tBid, also caused an increase in anisotropy at 50 nM, yet to a lesser extent. IgG at 1 μM, used as a control protein, showed no increase in anisotropy. The anti-apoptotic protein Bcl-2ΔTM caused only about 40% increase in DPH labeled membrane-"microviscosity" compared with BaxΔC but inhibited BaxΔC-induced increase in mitochondrial membrane- "microviscosity" (Fig.5B).

The observed increase in the steady state fluorescence anisotropy values, following BaxΔC treatment, occurs with no concomitant changes in DPH's fluorescence lifetimes (Table 1). This indicates restricted rotational motion of DPH in BaxΔC treated vs. untreated mitochondrial membrane. It is thus concluded the observed increase in anisotropy reflect a corresponding increase in membrane "microviscosity".

3.6 BaxΔC induced an increase in TMA-DPH-labeled mitochondrial membrane-"microviscosity"

Changes in mitochondrial membrane "microviscosity" can be additionally followed by the lipophilic TMA-DPH probe. It is an amphipathic molecule, anchored into the membrane phospholipids' head groups-water interface (i.e., at the outer leaflet of the outer mitochondrial membrane) with the aliphatic chain parallel to the lipid chains [36].

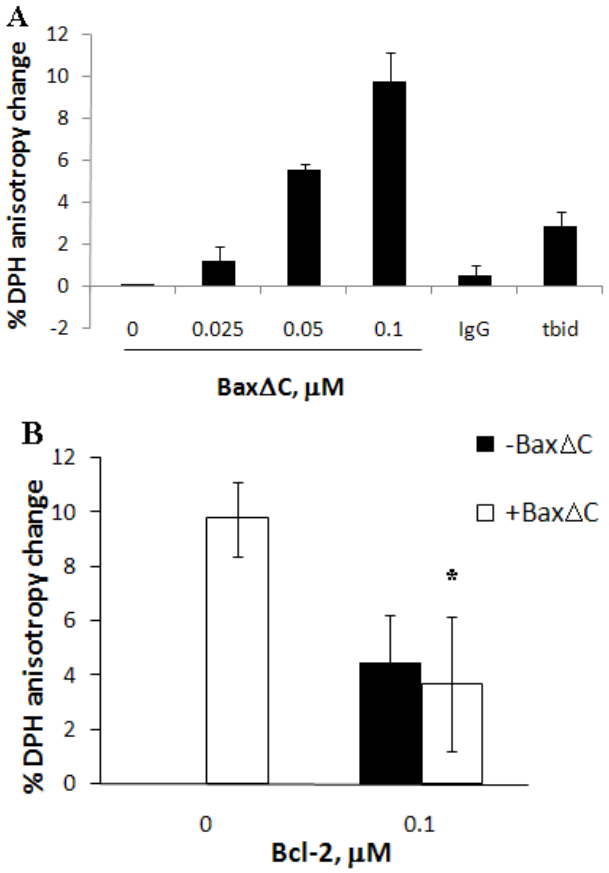

Fig.5: **The effect of pro-and anti-apoptotic proteins on the fluorescence anisotropy of DPH labeled RLM. A**. BaxΔC was added to mitochondria suspension at indicated concentrations and IgG and tbid proteins were added at 1 μM and 50 nM respectively. **B**. Bcl-2ΔTM inhibits anisotropy changes of DPH labeled RLM. Samples were pre-incubated with 0.1 μM Bcl-2ΔTM for 10 min before BaxΔC addition. Anisotropy change was calculated as a difference between initial and final anisotropy value shown as a percent of initial value. * P-value <0.05 vs 0 μM Bcl-2ΔTM with BaxΔC. Error bars indicate the standard error of three measurements.

Control experiments showed that BaxΔC did not lead to an increase in TMA-DPH fluorescence in the buffer. The results in Table 2 showed ~6% increase in the measured steady state fluorescence anisotropy values, following BaxΔC treatment, with no concomitant changes in TMA-DPH's fluorescence lifetime. Those results imply restricted rotational motion of TMA-DPH in BaxΔC treated vs. untreated mitochondrial membranes. Such a change reflects about a 20% increase in membrane "microviscosity" [53].

	- BaxΔC	+ BaxΔC
Lifetime (nsec)		
τ_1	7.6±0.1	7.5±0.1
α_1	0.7	0.7
τ_2	0.7±0.1	0.7±0.1
α_2	0.3	0.3

Table 1: **Lifetime values of DPH in mitochondria membrane.** Samples of DPH labeled mitochondria (50 μg/ml) in HIB, in the absence and the presence of BaxΔC (0.1 μM) were used. A two-lifetime component analysis resulted in χ^2 values of 1.09 & 1.05, for BaxΔC untreated and treated samples, respectively. The results of a representative experiment are shown in the table.

	- BaxΔC	+ BaxΔC
Fluorescence Anisotropy*	0.232±0.004	0.245±0.003
Lifetime**(nsec)		
τ_1	0.13	0.13
α_1	0.40	0.40
τ_2	4.40	4.40
α_2	0.42	0.45
τ_3	1.20	1.10
α_3	0.18	0.15

Table 2: **Steady state fluorescence anisotropy of TMA-DPH labeled mitochondria membrane.** Samples of TMA-DPH labeled mitochondria (50 μg/ml) in HIB, in the absence and the present of BaxΔC (1 μM) were used. * ± standard error, n = 3; ** A three-lifetime component analysis resulted in χ^2 values of 1.10 & 1.15, for BaxΔC untreated and treated samples, respectively.

3.7 BaxΔC-induced increase in membrane "microviscosity" is correlated to cytochrome c release

In order to correlate the changes in the emission spectra of the DPH labeled mitochondria to the biological activity of BaxΔC (i.e., cytochrome c release) we utilized a spectrophotometric method to determine the extent of cytochrome c release (Fig. 6). Spontaneous release of cytochrome c was detected in untreated mitochondria and this basal level of cytochrome c release was subtracted from measurements data [28]. Calcium ($CaCl_2$, 150 μM) was used as a positive control which induces release of most of the cytochrome c [49, 55]. Treatments with the pro-apoptotic proteins BaxΔC and tBid resulted in cytochrome c release as well (Fig. 6A). The anti-apoptotic protein Bcl-2ΔTM inhibited BaxΔC-induced cytochrome c release (Fig. 6B) [56]. Thus, under these experimental conditions, isolated rat liver mitochondria exhibit a characteristic normal response to the pro- and the anti-apoptotic stimuli [57].

The anti-apoptotic protein Bcl-2ΔTM was suggested to inhibit BaxΔC pro-apoptotic activity through heterodimerization and thus to prevent BaxΔC binding to the mitochondria membrane [52].

4 Conclusion

The present study was aimed at elucidating the changes in lipid dynamics associated with the interaction of Bax with intact mammalian mitochondria. The observations made here in functional rat liver mitochondria could shed further light on the mechanism of cytochrome c release from apoptotic mitochondria and further expand observations made before with model lipid bilayer systems [6, 7, 14, 58, 59, 60]. In our study we utilized the excimer forming properties of the lipid probe PDA and the rotational relaxation of both DPH and TMA-DPH to report on dynamic changes in the properties of mitochondrial membrane induced by BaxΔC. The use of these three fluorophores enables the sensing of three different membrane regions: the hydrophobic inter leaflet region of the outer mitochondria membrane, in which the pyrene end of PDA is situated, the phospholipids head group-water interface anchored TMA-DPH which senses the outer leaflet region (parallel to the lipidic chains) as well as the inner leaflet region sensed by DPH.

BaxΔC was previously shown to penetrate mitochondrial membranes through its α5 and α6 helixes, causing the release of cytochrome c [7]. With the fatty acid pyrene derivative, PDA, we suggest that BaxΔC lowers the mobility of the probe in the membrane (Fig.1 & 3).

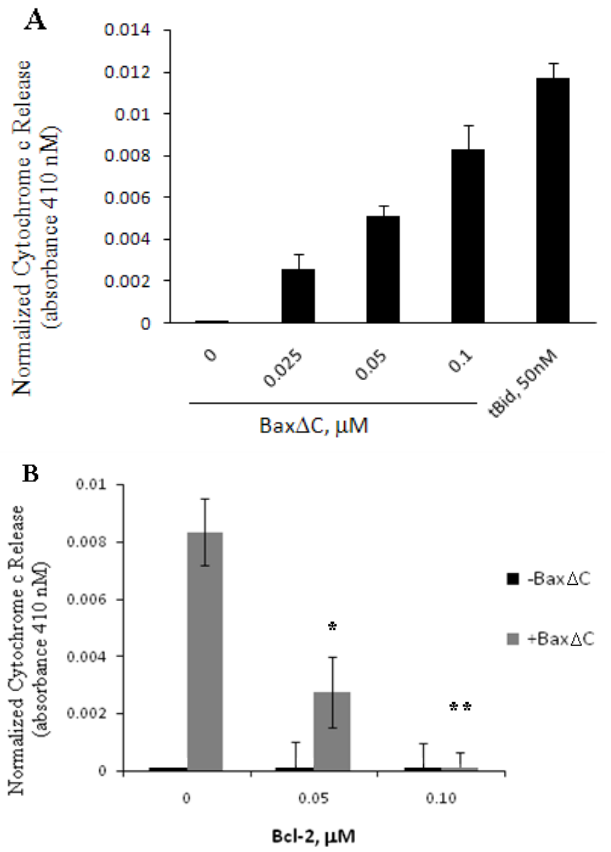

Fig.6: **BaxΔC induced fluorescence changes of mitochondria are correlated to cytochrome c release. A**. Normalized cytochrome c Soret peak absorbance (410 nm) of supernatants after centrifugation of the isolated rat liver mitochondria (12.5 mg protein/mL) following 10 min incubation with 0.025 μM, 0.05 μM or 0.1 μM BaxΔC; 50 nM tBid. **B**. Bcl-2ΔTM inhibits cytochrome c release from mitochondria. Samples were pre-incubated with 0.05 μM or 0.1 μM Bcl-2ΔTM for 10 min before BaxΔC addition. *P-value <0.02; ** p-value <0.001 from control cells treated with BaxΔC. Error bars indicate the standard error of three measurements.

In order to show that BaxΔC interact with the mitochondria through specific interactions, control experiments were performed with the non-apoptotic protein IgG which caused no increase in monomer emission nor PDA redistribution. No direct interaction between PDA and BaxΔC was evident at BaxΔC concentration up to 0.1 μM (Fig. 2). The extent of mobility was affected by BaxΔC-to-mitochondrial phospholipid ratio (Fig.3) and reflects increased local rigidification or "microviscosity" when Bax binds to the mitochondrial membrane. Control experiments showed that the observed decline in E/M ratio was not associated with significant changes in light scattering which could affect such fluorescence measurements [47, 61]. These results are consistent with previous reports that Bax does not cause large amplitude swelling of isolated mitochondria [49, 61]. Moreover, further possible explanations for the observed changes in E/M induced by BaxΔC were excluded. Those include changes in the lifetime of the probe caused by molecular oxygen quenching [52] and probe quenching due to resonance energy transfer between the pyrene probe and the heme moiety of cytochrome c [62]. Thus, time resolved fluorescence measurements of PDA lifetime in labeled mitochondria showed that the lifetime of the monomer and excimer species were not altered under the tested assay conditions. In addition, the results obtained from control experiments with cytochrome c-free mitochondrial membrane preparations did not show any apparent fluorescence resonance energy transfer by the release of cytochrome c through a putative BaxΔC induced pore [27, 50, 62, 63]. Therefore, direct PDA-cytochrome c interactions, were either excluded or masked by the overwhelming BaxΔC-induced changes in membrane dynamics. But, our fluorescence anisotropy studies combined with lifetime measurements of DPH and TMA-DPH labeled mitochondria (Fig.5A and Tables 1 & 2) independently showed reduced rotational dynamics, which further verified the claimed increased membrane "microviscosity" upon interaction with BaxΔC.

In order to show that BaxΔC interact with the mitochondria through specific interactions, control experiments were performed with the non-apoptotic protein IgG, which, as indicated above did not induce an increase in DPH anisiotropy, implying again no change in membrane "microviscosity". The pro-apoptotic protein, tBid, which at 50 nM effectively induced cytochrome c release, caused membrane "microviscosity" increase, yet to a lesser extent as compare with equal concentration of BaxΔC (Fig.6A). The membrane-insertion properties of different segments from proapoptotic Bax and Bid correspond to defined α helices in the structure of their soluble forms. It was shown that BaxΔC has an additional putative transmembrane fragment made of helix α1 in comparison with tBid [64]. tBid is known to promote the redistribution of membrane lipids in model systems, inducing changes in membrane curvature [7, 65, 66], but does not undergo transmembrane insertion [67, 14], which could account for the smaller effect observed when compared with BaxΔC. Furthermore, tBID oligomerizes BAK which is largely associated with outer mitochondrial membrane and with ER

membranes in healthy cells [3] to release cytochrome c [68]. The addition of Bcl2-ΔTM caused a slight increase in DPH anisotropy, presumably due to its hydrophobicity, but its addition together with BaxΔC, results in inhibition of the mitochondrial lipids reorganization caused by BaxΔC (Fig.5B).

Thus, the present decreased rate of excimerization is most likely due to physical constraints induced by BaxΔC on probe mobility acting as protein barriers to lipid diffusion [22, 69]. The increased membrane "microviscosity" could represent the induction of local phase transitions, leading to non-lamellar bilayer structures which are known to promote lipid poration [6, 7, 70]. It is proposed that this Bax-induced local rigidification could lower the energetic barrier for lipid pore formation as proposed for other lipid-pore promoting proteins [11, 12].

The present study utilizing isolated rat liver mitochondria supports studies on model membrane systems that have found an important role for membrane lipid dynamics in the mechanism of cytochrome c release [10]. Accordingly, the destabilizing effects of Bax on the mitochondrial membranes, which lead to cytochrome c release, could be the result of Bax insertion and reorganization of the bilayer phospholipids as was previously documented [14, 71]. Cisplatin an anti-cancer drug causing apotosis, was recently reported to induce similar mitochondria membrane rigidification [72]. The formation of a large pore complex with endogenous mitochondrial proteins in addition to Bax itself, cannot be excluded [73]. To conclude, Bax-induced reorganization of the bilayer phospholipids in the intact mitochondria, preceding cytochrome c release, suggests a mechanism based on protein-lipid interactions forming 'apoptotic pores' as crucial components of the commitment to cell death.

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References:

[1] R.M. Kluck, E. Bossy-Wetzel, D.R. Green and D.D. Newmeyer, The Release of Cytochrome C from Mitochondria: a Primary Site for Bcl-2 Regulation of Apoptosis, *Science,* Vol.275, Vol.5303, 1997, pp. 1132-1136.

[2] G. Kroemer and J.C. Reed, Mitochondrial Control of Cell Death, *Nature Medicine*, Vol.6, No.5, 2000, pp. 513-519.

[3] M. Degli Esposti and C. Dive, Mitochondrial Membrane Permeabilisation by Bax/Bak, *Biochemical and Biophysical Research Communications*, Vol.304, No.3, 2003, pp. 455-461.

[4] L. Scorrano and S.J. Korsmeyer, Mechanisms of Cytochrome C Release by Proapoptotic BCL-2 Family Members, *Biochemical and Biophysical Research Communications*, Vol.304, No.3, 2003, pp. 437-444.

[5] O. Von Ahsen, NJ. Waterhouse, T. Kuwana, DD. Newmeyer and DR. Green, The 'Harmless' Release of Cytochrome C. *Cell Death and Differentiation*, Vol.7, No.12, 2000, pp. 1192-1199.

[6] G. Basanez, J.C. Sharpe, J. Galanis, T.B. Brandt, J.M. Hardwick and J. Zimmerberg, Bax-Type Apoptotic Proteins Porate Pure Lipid Bilayers Through a Mechanism Sensitive to Intrinsic Monolayer Curvature, *The Journal of Biological Chemistry*, Vol.277, No.51, 2002, pp. 49360-49365.

[7] O. Terrones, B. Antonsson, H. Yamaguchi, H.G. Wang, J. Liu, R.M. Lee, A. Herrmann and G. Basanez. Lipidic Pore Eormation by the Concerted Action of Proapoptotic BAX and tBid, *The Journal of Biological Chemistry*, Vol.279, No.29, 2004, pp. 30081-30091.

[8] A.J. Garcia-Saez, M. Coraiola, M.D, Serra, I. Mingarro, P. Muller and J. Salgado, Peptides Corresponding to Helices 5 and 6 of Bax can Independently form Large Lipid Pores, *The FEBS Journal*, Vol.273, No.5, 2006, pp. 971-981.

[9] A.J. Garcia-Saez, S. Chiantia, J. Salgado, P. Schwille, Pore Formation by a Bax-Derived Peptide: Effect on the Line Tension of the Membrane Probed by AFM, *Biophysical Journal*, Vol.93, No.1, 2007, pp. 103-112.

[10] G. Basanez, A. Nechushtan, O. Drozhinin, A. Chanturiya, E. Choe, S. Tutt, K.A.Wood, Y. Hsu, J. Zimmerberg and R.J. Youle, Bax, but not Bcl-xL, Decreases the Lifetime of Planar

Phospholipid Bilayer Membranes at Subnanomolar Concentrations, *Proceedings of the National Academy of Sciences of the United States of America*, Vol.96, No.10, 1999, pp. 5492-5497.

[11] Y. Shai, Mechanism of the Binding, Insertion and Destabilization of Phospholipid Bilayer Membranes by Alpha-Helical Antimicrobial and Cell Non-Selective Membrane-Lytic Peptides, *Biochimica et Biophysica Acta*, Vol.1462No.1-2, 1999, pp. 55-70.

[12] S.D. Zakharov, E.A. Kotova, Y.N. Antonenko and WA. Cramer, On the Role of Lipid in Colicin Pore Formation, *Biochimica et Biophysica Acta*, Vol.1666, No.1-2, 2004, pp. 239-249.

[13] G. Basanez, J. Zhang, B.N. Chau, G.I. Maksaev, V.A. Frolov, T.A. Brandt, J. Burch, J.M. Hardwick and J. Zimmerberg, Pro-Apoptotic Cleavage Products of Bcl-xL form Cytochrome c-Conducting Pores in Pure Lipid Membranes, *The Journal of Biological Chemistry*, Vol.276, No.33, 2001, pp. 31083-31091.

[14] R.F. Epand, J.C. Martinou, S. Montessuit and R.M. Epand, Transbilayer Lipid Diffusion Promoted by Bax: Implications for Apoptosis, *Biochemistry*, Vol.42, No.49, 2003, pp. 14576-14582.

[15] N. Zamzami and G. Kroemer, Apoptosis: Mitochondrial Membrane Permeabilization-the (W)hole Story? *Current Biology*, Vol.13, No.2, 2003, pp. R71-73.

[16] H.J. Galla and E. Sackmann, Lateral Diffusion in the Hydrophobic Region of Membranes: Use of Pyrene Excimers as Optical Probes, *Biochimica et Biophysica Acta*, Vol.339, No.1, 1974, pp. 103-115.

[17] H.J. Galla and W. Hartmann, Excimer-Forming Lipids in Membrane Research, *Chemistry and Physics of Lipids*, Vol.27, No.3, 1980, pp. 199-219.

[18] S.S. Gupte, L.K. Lane, J.D. Johnson, E.T. Wallick and A. Schwartz, The Interaction of Divalent Cations with (Na,K)-ATPase. A Lipid-Bound Fluorescence Probe Study, *The Journal of Biological Chemistry*, Vol.254, No.12, 1979, pp. 5099-5103.

[19] O.T. Jones and A.G. Lee, Interactions of Pyrene Derivatives with Lipid Bilayers and with (Ca^{2+}-Mg^{2+})-ATPase, *Biochemistry*, Vol.24, No.9, 1985, pp. 2195-2202.

[20] Z. Binenbaum, A.H. Parola, A. Zaritsky and I. Fishov, Transcription- and Translation-Dependent Changes in Membrane Dynamics in Bacteria: Testing the Transertion Model for Domain Formation, *Molecular Microbiology*, Vol.32, No.6, 1999, pp. 1173-1182.

[21] S. Hazan, I. Fishov, M. Zamai, V.R. Caiolfa and A.H. Parola. Fluorescence Polarization Assay for Endothelin-Converting Enzymes, *Peptides*, Vol.16, No.5, 1995, pp. 833-836.

[22] S. Vanounou, A.H. Parola and I. Fishov, Phosphatidylethanolamine and phosphatidylglycerol are segregated into different domains in bacterial membrane. A study with pyrene-labelled phospholipids, *Molecular microbiology*, Vol.49, No.4, 2003, pp. 1067-1079.

[23] A.H. Parola, P.W. Robbins and E.R. Blout, Membrane Dynamic Alterations Associated with Viral Transformation and Reversion: Decay of Fluorescence Emission and Anisotropy Studies of 3T3 Cells, *Experimental cell research*, Vol.118, No.1, 1979, pp.205-214.

[24] U. De Marchi, S. Campello, I. Szabo, F. Tombola, J.C. Marinou and M. Zoratti, Bax does not Directly Participate in Ca(2+)-Induced Permeability Transition of Isolated Mitochondria, *The Journal of Biological Chemistry*, Vol.279, No.36, 2004, pp. 37415-37422.

[25] D. Gincel, V. Shoshan-Barmatz, Glutamate Interacts with VDAC and Modulates Opening of the Mitochondrial Permeability Transition Pore, *Journal of Bioenergetics and Biomembranes*, Vol.36, No.2, 2004, pp. 179-186.

[26] S.A. French, P.R. Territo and R.S. Balaban, Correction for Inner Filter Effects in Turbid Samples: Fluorescence Assays of Mitochondrial NADH, *The American Journal of Physiology*, Vol.275, No.3, 1998, pp. C900-909.

[27] J.D. Cortese and C.R. Hackenbrock, Motional Dynamics of Functional Cytochrome C Delivered by Low pH Fusion Into the Intermembrane Space of Intact Mitochondria, *Biochimica et Biophysica Acta*, Vol.1142, No.1-2, 1993, pp. 194-202.

[28] E. Doran and A.P. Halestrap, Cytochrome C Release from Isolated Rat Liver Mitochondria Can Occur Independently of Outer-Membrane Rupture: Possible Role of Contact Sites, *The Biochemical Journal*, Vol.348 No.Pt2, 2000, pp. 343-350.

[29] R. Jasinska, J. Zborowski and P. Somerharju, Intramitochondrial Distribution and Transport of Phosphatidylserine and its Decarboxylation

Product, Phosphatidylethanolamine. Application of Pyrene-Labeled Species, *Biochimica et Biophysica Acta*, Vol.1152, No.1, 1993, pp. 161-170.

[30] P. Jezek, M. Modriansky, K.D. Garlid, Inactive Fatty Acids are Unable to Flip-Flop Across the Lipid Bilayer, *FEBS Letters*, Vol.408, No.2, 1997, pp. 161-165.

[31] P. Jezek, M. Modriansky, K.D. Garlid. A Structure-Activity Study of Fatty Acid Interaction with Mitochondrial Uncoupling Protein, *FEBS Letters*, Vol.408, No.2, 1997, pp. 166-170.

[32] R. Hovius, H. Lambrechts, K. Nicolay and B. de Kruijff, Improved Methods to Isolate and Subfractionate Rat Liver Mitochondria. Lipid Composition of the Inner and Outer Membrane. *Biochimica et Biophysica Acta*, Vol.1021, No.2, 1990, pp. 217-226.

[33] M. Zamai, C. Hariharan, D. Pines, M. Safran, A. Yayon, V.R. Caiolfa, R. Cohen-Luria, E. Pines and A.H. Parola, Nature of Interaction Between Basic Fibroblast Growth Factor and the Antiangiogenic Drug 7,7-(carbonyl-bis[imino-N-methyl-4,2-pyrrolecarbonylimino[N-methyl-4,2-pyr role]-carbonylimino])-bis-(1,3-naphtalene disulfonate). II. Removal of Polar Interactions Affects Protein Folding, *Biophysical Journal*, Vol.82, No.5, 2002, pp. 2652-2664.

[34] J.I. Gurtubay, F.M. Goni, J.C. Gomez-Fernandez, J.J. Otamendi and J.M. Macarulla, Triton X-100 Solubilization of Mitochondrial Inner and Outer Membranes, *Journal of Bioenergetics and Biomembranes,* Vol.12, No.1-2, 1980, pp. 47-70.

[35] G. Schwarz and A. Arbuzova, Pore Kinetics Reflected in the Dequenching of a Lipid Vesicle Entrapped Fluorescent Dye, *Biochimica et Biophysica Acta*, Vol.1239, No.1, 1995, pp. 51-57.

[36] I. Nathan, I. Ben-Valid, R. Henzel, H. Masalha, S.N. Baram, A. Dvilansky, A.H. Parola, Alterations in Membrane Lipid Dynamics of Leukemic Cells Undergoing Growth Arrest and Differentiation: Dependency on the Inducing Agent, *Experimental Cell Research*, Vol.239, No.2, 1998, pp. 442-446.

[37] G. Deliconstantinos, V. Villiotou, J.C. Stavrides, Nitric Oxide and Peroxynitrite Released by Untraviolet B-irradiated Human Endothelial Cells are Possibly Involved in Skin Erythema and Inflammation, *Experimental Physiology*, Vol.81, No.6, 1996, pp. 1021-1033.

[38] P. Fuchs, A.H. Parola, PW. Robbins and ER. Blout, Fluorescence Polarization and Viscosities of Membrane Lipids of 3T3 Cells, *Proceedings of the National Academy of Sciences of the United States of America*, Vol.72, No.9, 1975, pp. 3351-3354.

[39] J.R. Lakowicz, F.G. Prendergast, Detection of Hindered Rotations of 1,6-Diphenyl-1,3,5-Hexatriene in Lipid Bilayers by Differential Polarized Phase Fluorometry, *Biophysical Journal*, Vol.24, No.1, 1978, pp. 213-227.

[40] K.H. Cox, J.B. Ross, A.P. Peterson, T.J. Benedetti, Fetal Lung Maturity Assessed by Fluorescence Polarization: Evaluation of Predictive Value, Correction for Endogenous Fluorescence, and Comparison with L/S Ratio, *Clinical Chemistry*, Vol.29, No.2, 1983, pp. 346-349.

[41] E.P. Pines, D. Pines, T. Barak, B.Z. Magnes, L.M. Tolbert, J.E. Haubrich, Isotope and Temperature Effects in Ultrafast Proton-Transfer from a Strong Excited-State Acid, *Berichte der Bunsen-Gesellschaft fur Physikalische Chemie*, Vol.102, No.3, 1998, pp. 511-517.

[42] F. Ricchelli, S. Gobbo, G. Moreno and C. Salet, Changes of the Fluidity of Mitochondrial Membranes Induced by the Permeability Transition, *Biochemistry*, Vol.38, No.29, 1999, pp. 9295-9300.

[43] J.L. Scarlett and M.P. Murphy, Release of Apoptogenic Proteins from the Mitochondrial Intermembrane Space During the Mitochondrial Permeability Transition, *FEBS Letters*, Vol.418, No.3, 1997, pp. 282-286.

[44] P.X. Petit, M. Goubern, P. Diolez, S.A. Susin, N. Zamzami and G. Kroemer, Disruption of the Outer Mitochondrial Membrane as a Result of Large Amplitude Swelling: the Impact of Irreversible Permeability Transition, *FEBS Letters*, Vol.426, No.1, 1998, pp. 111-116.

[45] F. Appaix, M. Minatchy, C. Riva-Lavieille, J. Olivares, B. Antonsson and V.A. Saks, Rapid Spectrophotometric Method for Quantitation of Cytochrome C Release from Isolated Mitochondria or Permeabilized Cells Revisited, *Biochimica et Biophysica Acta*, Vol.1457, No.3, 2000, pp. 175-181.

[46] D. Tang, D. Borchman, N. Harris and S. Pierangeli, Lipid Interactions with Human Antiphospholipid Antibody, β2-Glycoprotein 1, and Normal Human IgG Using the Fluorescent Probes NBD-PE and DPH, *Biochimica et Biophysica Acta-Biomembranes,* Vol.1372, No.1, 1998, pp. 45-54.

[47] A.H. Parola, *Biomembranes, Physical Aspects*, New York: Balaban Press VCH, 1993.

[48] V. Gogvadze, J.D. Robertson, B. Zhivotovsky, and S. Orrenius, Cytochrome C Release Occurs via Ca^{2+}-Dependent and Ca^{2+}-Independent Mechanisms that are Regulated by Bax, *The Journal of Biological Chemistry*, Vol.276, No.22, 2001, pp. 19066-19071.

[49] J.M. Jurgensmeier, Z. Xie, Q. Deveraux, L. Ellerby, D. Bredesen and J.C. Reed, Bax Directly Induces Release of Cytochrome C from Isolated Mitochondria, *Proceedings of the National Academy of Sciences of the United States of America*, Vol.95, No.9, 1998, pp. 4997-5002.

[50] M. Rytomaa and P.K. Kinnunen, Dissociation of Cytochrome C from Liposomes by Histone H1. Comparison with Basic Peptides, *Biochemistry*, Vol.35, No.14, 1996, pp. 4529-4539.

[51] M.A.Matlib and P.J. O'Brien, Properties of Rat Liver Mitochondria with Intermembrane Cytochrome C, *Archives of Biochemistry and Biophysics*, Vol.173, No.1, 1976, pp. 27-33.

[52] D. Huglin, W. Seiffert and H.W. Zimmermann, Time-Resolved Microfluorometric Study of the Binding Sites of Lipophilic Cationic Pyrene Probes in Mitochondria of Living HeLa Cells, *Journal of Photochemistry and Photobiology. B, Biology*, Vol.31, No.3, 1995, pp. 145-158.

[53] M. Shinitzky, Y. Barenholz, Fluidity Parameters of Lipid Regions Determined by Fluorescence Polarization, *Biochimica et Biophysica Acta*, Vol.515, No.4, 1978, pp. 367-394.

[54] A. Zaritsky, A.H. Parola, M. Abdah, H. Masalha, Homeoviscous Adaptation, Growth Rate, and Morphogenesis in Bacteria, *Biophysical Journal*, Vol.48, No.2, 1985, pp. 337-339.

[55] R. Eskes, B. Antonsson, A. Osen-Sand, S. Montessuit, C. Richter, R. Sadoul, G. Mazzei, A. Nichols and J.C. Martinou, Bax-Induced Cytochrome C Release from Mitochondria is Independent of the Permeability Transition Pore but Highly Dependent on Mg^{2+} Ions, *The Journal of Cell Biology*, Vol.143, No.1, 1998, pp. 217-224.

[56] B. Antonsson, F. Conti, A. Ciavatta, S. Montessuit, S. Lewis, I. Martinou, L. Bernasconi, A. Bernard, J.J. Mermod, G. Mazzei, K. Maundrell, F. Gambale, R. Sadoul and J.C. Martinou, Inhibition of Bax Channel-Forming Activity by Bcl-2, *Science*, Vol.277, No.5324, 1997, pp. 370-372.

[57] Y. Tsujimoto, Role of Bcl-2 Family Proteins in Apoptosis: Apoptosomes or Mitochondria? *Genes to Cells: Devoted to Molecular & Cellular Mechanisms*, Vol.3, No.11, 1998, pp. 697-707.

[58] A. Gross, J. Jockel, M.C. Wei and S.J. Korsmeyer, Enforced Dimerization of BAX Results in Its Translocation, Mitochondrial Dysfunction and Apoptosis, *The EMBO Journal*, Vol.17, No.14, 1998, pp. 3878-3885.

[59] M.G. Annis, E.L. Soucie, P.J. Dlugosz, J.A. Cruz-Aguado, L.Z. Penn, B. Leber and D.W. Andrews, Bax Forms Multispanning Monomers that Oligomerize to Permeabilize Membranes During Apoptosis, *The EMBO journal*, Vol.24, No.12, 2005, pp. 2096-2103.

[60] P.H. Schlesinger and M. Saito, The Bax Pore in Liposomes, Biophysics, *Cell Death and Differentiation*, Vol.13, No.8, 2006, pp. 1403-1408.

[61] D. Zhai, Q. Miao, X. Xin and F. Yang, Leakage and Aggregation of Phospholipid Vesicles Induced by the BH3-only Bcl-2 Family Member, BID, *European Journal of Biochemistry*, 268, No.1, 2001, pp. 48-55.

[62] P. Mustonen, J.A. Virtanen, P.J. Somerharju and P.K. Kinnunen, Binding of Cytochrome C to Liposomes as Revealed by the Quenching of Fluorescence from Pyrene-Labeled Phospholipids, *Biochemistry*, Vol.26, No.11, 1987, pp. 2991-2997.

[63] J. Vanderkooi, M. Erecinska and B. Chance, Cytochrome C Interaction with Membranes. I. Use of a Fluorescent Chromophore in the Study of Cytochrome c Interaction with Artificial and Mitochondrial Membranes, *Archives of Biochemistry and Biophysics*, Vol.154, No.1, 1973, pp. 219-229.

[64] A.J. Garcia-Saez, I. Mingarro, E. Pérez-Payá, J. Salgado, Membrane-Insertion Fragments of Bcl-x_L, Bax, and Bid, *Biochemistry*, Vol.43, No.34, 2004, pp. 10930–10943.

[65] T. Kuwana, M.R. Mackey, G. Perkins, M.H. Ellisman, M. Latterich, R. Schneiter, D.R. Green and D.D. Newmeyer, Bid, Bax, and Lipids Cooperate to Form Supramolecular Openings in the Outer Mitochondrial Membrane, *Cell*, Vol.111, No.3, 2002, pp. 331-342.

[66] R.F. Epand, J.C. Martinou, M. Fornallaz-Mulhauser, D.W. Hughes, R.M. Epand, The Apoptotic Protein tBid Promotes Leakage by Altering Membrane Curvature, *The Journal of Biological Chemistry*, Vol.277, No.36, 2002, pp. 32632-32639.

[67] X.M. Gong, J. Choi, C.M. Franzin, D. Zhai, J.C. Reed and F.M. Marassi, Conformation of Membrane-Associated Proapoptotic tBid, *The Journal of Biological Chemistry*, Vol.279, No.28, 2004, pp. 28954-28960.

[68] M.C. Wei, T. Lindsten, V.K. Mootha, S. Weiler, A. Gross, M. Ashiya, C.B. Thompson, S.J. Korsmeyer, tBID, a Membrane-Targeted Death Ligand, Oligomerizes BAK to Release Cytochrome C, *Genes & development*,Vol.14, No.16, 2000, pp. 2060-71.

[69] Y. Barenholz, T. Cohen, E. Haas and M. Ottolenghi, Lateral Organization of Pyrene-Labeled Lipids in Bilayers as Determined from the Deviation from Equilibrium Between Pyrene Monomers and Excimers, *The Journal of Biological Chemistry*, Vol.271, No.6, 1996, pp. 3085-3090.

[70] K. Matsuzaki, S. Yoneyama and K. Miyajima, Pore Formation and Translocation of Melittin, *Biophysical Journal*, Vol.73, No.2, 1997, pp. 831-838.

[71] C.M.P. Rodrigues, S. Sola, J.C. Sharpe, J.J.G. Moura and J.C. Steer, Tauroursodeoxycholic Acid Prevents Bax-Induced Membrane Perturbation and Cytochrome C Release in Isolated Mitochondria, *Biochemistry,* Vol.42, No.10, 2003, pp. 3070-3080.

[72] N.M. Martins, N.A. Santos, C. Curti, M.L. Bianchi, A.C. Santos, Cisplatin Induces Mitochondrial Oxidative Stress With Resultant Energetic Metabolism Impairment, Membrane Rigidification and Apoptosis in Rat Liver, *Journal of Applied Toxicology,* Vol.28, No.3, 2008, pp. 337-344.

[73] L.M. Dejean, S. Martinez-Caballero, L. Guo, C. Hughes, O. Teijido, T. Ducret, F. Ichas, S.J. Korsmeyer, B. Antonsson, E.A. Jonas and K.W. Kinnally, Oligomeric Bax is a Component of the Putative Cytochrome C Release Channel MAC, Mitochondrial Apoptosis-Induced Channel, *Molecular Biology of the Cell*, Vol.16, No.5, 2005, pp. 2424-2432.