Tauroursodeoxycholic Acid Prevents Bax-Induced Membrane Perturbation and Cytochrome *c* Release in Isolated Mitochondria[†]

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ABSTRACT: Bax is a potent pro-apoptotic member of the Bcl-2 protein family that localizes to the mitochondrial membrane during apoptosis. Tauroursodeoxycholic acid (TUDCA) modulates the apoptotic threshold, in part, by preventing Bax translocation both in vitro and in vivo. The mechanisms by which Bax induces and TUDCA inhibits release of cytochrome *c* are unclear. We show here that recombinant Bax protein induced cytochrome *c* release in isolated mitochondria without detectable swelling. Co-incubation with TUDCA prevented efflux of mitochondrial factors and proteolytic processing of caspases in cytosolic extracts. Spectroscopic analyses of mitochondria exposed to Bax revealed increased polarity and fluidity of the membrane lipid core as well as altered protein order, indicative of Bax binding, together with loss of spin-label paramagnetism, characteristic of oxidative damage. TUDCA markedly abrogated the Bax-induced membrane perturbation. In conclusion, our results indicate that Bax protein directly induces cytochrome *c* release from mitochondria through a mechanism that does not require the permeability transition. Rather, it is accompanied by changes in the organization of membrane lipids and proteins. TUDCA is a potent inhibitor of Bax association with mitochondria. Thus, TUDCA modulates apoptosis by suppressing mitochondrial membrane perturbation through pathways that are also independent of the mitochondrial permeability transition.

The Bcl-2 family of proteins plays a key role in regulating mitochondrial-mediated programmed cell death. The pro-death members such as Bax regulate the release of apoptogenic proteins from mitochondria, thus inducing apoptosis, whereas the anti-death members such as Bcl-2 and Bcl-x_L promote cell survival (*1*). Pro- and antiapoptotic Bcl-2 proteins can either reside in the cytosol or be associated with other intracellular sites including the outer mitochondrial membrane, the nuclear envelope, and the endoplasmic reticulum. Membrane anchoring is presumably facilitated by a hydrophobic stretch of amino acids present at the COOH terminus (*2*, *3*). Certain apoptotic stimuli are transduced to mitochondria through BH3-only proteins or additional pathways. The signals can either be neutralized by antiapoptotic factors, such as Bcl-2, or further transmitted through Bax and Bak. These proapoptotic molecules undergo conformational changes that allow for their translocation from cytosol to the mitochondrial membrane (*4*). This results in the formation of pores and subsequent release of intermembrane space proteins, and/or destabilization of the mitochondrial outer membrane (*5*). In addition to the activation of apoptotic pathways, the loss of mitochondrial function may itself induce cell death.

Cytochrome *c*, adenylate kinase, Smac/DIABLO, procaspases, and apoptosis-inducing factor have all been reported to enter the cytosol with disruption of the mitochondrial membrane (*6*−*11*). Once released, cytochrome *c* binds to apoptosis-activating factor 1 (Apaf-1)[1] and forms a cytosolic protein complex that also includes dATP and procaspase-9 (*12*). This leads to caspase-9 activation, which in turn activates downstream caspase-mediated events that ultimately lead to the morphologic changes characteristic of apoptosis. Alternatively, since cytochrome *c* release can precede loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) (*13*, *14*), it is conceivable that Bax itself creates pores

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[1] Abbreviations: Apaf-1, apoptosis-activating factor 1; DEVD-pNA, *N*-acetyl-Asp-Glu-Val-Asp-pNA; DSA, doxyl stearic acid; EPR, electron paramagnetic resonance; 4-maleimido-TEMPO, 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl; $\Delta\Psi_m$, mitochondrial transmembrane potential; pNA, *p*-nitroanilide; PARP, poly(ADP-ribose) polymerase; RT-PCR, reverse transcriptase-polymerase chain reaction; SDS, sodium dodecyl sulfate; TUDCA, tauroursodeoxycholic acid; UDCA, ursodeoxycholic acid.

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in the outer membrane allowing escape of intermembrane proteins (*15*−*17*). Bax was indeed shown to act as a pore-forming protein (*16*, *18*, *19*) that can also affect activity of the voltage-dependent anion channel (*20*) and the adenine nucleotide transporter (*21*). Further, Bax-induced release of cytochrome *c* may secondarily result in megapore opening from either uncoupled oxidative phosphorylation or caspase-mediated cleavage of mitochondrial proteins (*22*).

Thus, Bax plays a key role as a central regulator in the release of mitochondrial intermembrane space proteins. However, it remains unclear as to how the translocation of Bax from cytosol to mitochondria mediates the downstream pathways to apoptosis. It appears that the release of proapoptotic factors can occur through at least two major models involving Bax interaction with the mitochondrial membrane (*23*). While formation of the "Bax channel" allows the release of cytochrome *c*, opening of the permeability transition pore results in loss of $\Delta\Psi_m$, swelling of the mitochondrial matrix and nonselective release of intermembrane space proteins. However, it has recently been shown that oligomeric Bax can form rather large pores. This finding challenges the notion that outer membrane rupture is required to release apoptotic proteins larger than cytochrome *c*, such as Smac/DIABLO and the apoptosis inducing factor (*24*). In addition, Bax was demonstrated to permeabilize liposomes without the requirement of the voltage-dependent anion channel and the adenine nucleotide transporter.

The dihydroxy bile acid ursodeoxycholic acid (UDCA) has been in widespread clinical use for the past 20 years, initially for gallstone dissolution and subsequently for the treatment of chronic cholestatic liver diseases (*25*). Despite its clinical efficacy, the precise mechanism(s) by which UDCA improves liver function is still unclear. In recent years, however, it has become evident that UDCA is capable of exerting direct effects at the cellular, subcellular, and molecular levels through multiple mechanisms including the stabilization of cell membranes (*26*). In addition, we have shown that UDCA and its taurine conjugated derivative, tauroursodeoxycholic acid (TUDCA), play a unique role in regulating the apoptotic threshold in both hepatic and nonhepatic cells (*27*). Through both direct and indirect pathways, these bile acids prevent several apoptotic events, such as permeability transition, translocation of the proapoptotic protein Bax to mitochondria, release of cytochrome *c*, activation of cytosolic caspase, and cleavage of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) (*28*, *29*). Thus, as general modulators of cell survival, UDCA and TUDCA are also potential therapeutic agents in the treatment of nonliver diseases, such as neurodegenerative disorders associated with increased levels of apoptosis (*30*, *31*).

In the present study, we investigated the effect of TUDCA on Bax-mediated structural changes in the lipid and protein environment of the mitochondrial membrane. Using spectroscopic analyses of isolated mitochondria exposed to recombinant Bax, we determined that the proapoptotic protein modifies the polarity and fluidity of the membrane lipid core together with altered protein order. These changes are consistent with loose association of Bax with the outer membrane resulting in cytochrome *c* release in the absence of mitochondrial swelling. TUDCA is a potent suppressor of Bax-induced alterations, in part, by preventing the binding of Bax protein to mitochondria. The results suggest that TUDCA can modulate apoptosis by preventing mitochondrial perturbation through pathways that are also independent of the mitochondrial permeability transition.

MATERIAL AND METHODS

Expression and Purification of Recombinant Bax. Full-length recombinant human Bax was expressed by subcloning the cDNA of human Bax into the NdeI/SapI site of pTYB1 vector as described (*32*). The Bax Ptyb construct was transformed into the BL21DE3 strain of *Escherichia coli*. The expression of the Bax protein was induced with IPTG at 37 °C. Bax was incubated on the chitin bead affinity column (New England BioLabs, Inc., Beverly, MA) and eluted using 30 mM dithiothreitol. The protein was further purified by ion-exchange chromatography to a single band on a sodium dodecyl sulfate (SDS)−polyacrylamide silver stained gel. Bax was then dissolved in 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 0.5 mM NaCl for use in all experiments.

Isolation of Rat Liver Mitochondria. Low calcium liver mitochondria were isolated from adult male 200−250 g Wistar rats as previously described (*28*, *33*, *34*). Following isolation, mitochondria were kept on ice for up to 3 h. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). All animal procedures were in accordance with the *NIH Guide for the Care and Use of Laboratory Animals*, and were approved by our Institutional Animal Care and Use Committee.

Mitochondrial Membrane Permeability Transition, Cytochrome c Release and Bax Binding. Mitochondrial membrane permeability was determined using a spectrophotometric assay measuring high amplitude rapid changes in mitochondrial volume as described (*35*−*36*) with modifications. Briefly, mitochondria (0.75−1 mg of protein) were incubated in 1 mL of Chelex-100-treated respiration buffer containing 10 mM HEPES, 10 mM succinate, 215 mM mannitol, 71 mM sucrose, pH 7.4 at 25 °C, and swelling was monitored at 540 nm in a Beckman DU 64 spectrophotometer. Basal levels of absorbance were measured for 5 min, and the optical density was monitored for 1, 5, 20, and 60 min after the addition of 0.5, 1, or 2 μM Bax. In the coincubation studies, mitochondria were preincubated with either 500 μM TUDCA, or 5 μM cyclosporine A (Sigma Chemical Co., St. Louis, MO) for 5 min at 25 °C prior to adding Bax for another 5 min.

Following the permeability assays, mitochondria were centrifuged at 12000*g* for 3 min at 4 °C. Release of cytochrome *c*, loss of cytochrome *c* oxidase subunit II, and binding of Bax were determined by Western blot analysis of both supernatant and mitochondrial pellets. In brief, proteins were separated on a 15% SDS−polyacrylamide gel. Following electrophoretic transfer onto nitrocellulose membranes, the immunoblots were incubated with 15% H_2O_2 for 15 min at room temperature. Blots were sequentially incubated with 5% milk blocking solution, primary monoclonal antibody to cytochrome *c* (PharMingen, San Diego, CA) at a dilution of 1:5000 overnight at 4 °C, and finally with secondary goat anti-mouse IgG antibody conjugated with horseradish peroxidase for 2 h at room temperature. In addition, blots were probed with primary polyclonal antibody to Bax (Santa Cruz Biotechnology, Santa Cruz, CA) at a

dilution of 1:500, and then with secondary anti-rabbit antibody conjugated with horseradish peroxidase. Finally, the isolated mitochondria were incubated with primary monoclonal antibody to cytochrome *c* oxidase subunit II (Molecular Probes, Eugene, OR). They were then processed for detection of cytochrome *c*, Bax, and cytochrome *c* oxidase using the enhanced chemiluminescence light (ECL) system from Amersham Life Science, Inc. (Arlington Heights, IL).

Carbonate Extraction of Bax from Mitochondria. To distinguish between Bax binding and insertion into the mitochondrial membrane, we performed carbonate extraction of Bax in the presence and absence of either TUDCA or cyclosporine A. In brief, after the incubation assays, mitochondria were pelleted at 12000*g* for 3 min at 4 °C and then resuspended in 10 mM HEPES, pH 7.6. Following addition of 1 mM phenylmethylsulfonyl fluoride and freshly made 0.2 M sodium carbonate, samples were kept on ice for 30 min and then centrifuged at 225000*g* for 30 min at 4 °C. The supernatant was removed and precipitated using trichloroacetic acid. Only loosely associated Bax was extracted with carbonate and identified by Western blot analysis.

Glutamate Dehydrogenase and Citrate Synthase Release. Following incubation with Bax, mitochondria were pelleted at 12000*g* for 3 min at 4 °C. Glutamate dehydrogenase and citrate synthase activities were determined by NADH production in supernatant by spectrophotometric analysis at 340 nm and 25 °C, according to the manufacturer's recommendation. Total enzyme activity in mitochondria was determined by disruption of the mitochondria with 0.5% Triton X-100 (Roche Applied Science, Mannheim, Germany).

Mitochondria-Dependent Caspase Activation. Cytosolic protein extracts were prepared from primary rat hepatocytes. Cells were isolated from male Sprague−Dawley rats (200−250 g) by collagenase perfusion as described previously (*37*). Briefly, rats were anesthesized with phenobarbitol and their livers were perfused with 0.05% collagenase. Hepatocyte suspensions were obtained by passing digested livers through 125 μm gauze and washing the cells in modified Eagle's medium (MEM; Atlanta Biologicals, Inc., Norcross, GA). Cell viability was determined by trypan blue exclusion and was typically 85−90%. After isolation, hepatocytes were resuspended in William's E medium supplemented with 26 mM sodium bicarbonate, 23 mM HEPES, 0.01 U/mL insulin, 2 mM L-glutamine, 10 nM dexamethasone, 5.5 mM glucose, and then plated on Primaria tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ) at 1.0×10^6 cells/mL. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 for 3 h. Plates were then washed with medium to remove dead cells, and then incubated in William's E medium containing 10% heat-inactivated FBS for 24 h. Hepatocytes were harvested by centrifugation at 600*g* for 5 min at 4 °C. The cell pellet was washed once in ice-cold PBS and resuspended with extraction buffer containing 10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM KAc, 2 mM dithiothreitol, supplemented with the Complete protease inhibitor cocktail (Roche Applied Science). After disruption by 20 strokes of a glass homogenizer, the homogenates were centrifuged twice at 600*g* for 10 min at 4 °C to remove unbroken cells and nuclei. The supernatant was removed and centrifuged at 3000*g* for 10 min at 4 °C. The resulting cytosolic fraction was used for assaying mitochondria-dependent caspase activation.

Supernatants (10 μL) were recovered from mitochondria treated with either Bax, TUDCA, Bax + TUDCA or no addition (control) and incubated with cytosolic protein extract (10 μL) at 30 °C for 1 h. Aliquots were used for caspase-3-like activity and for Western blot analysis of caspase-3 processing. Caspase processing and activation was initiated by addition of 1 mM dATP (*38*).

Caspase Activity and Proteolytic Processing. Caspase activity was determined by the ability of the enzyme to cleave the chromophore *p*-nitroanilide (pNA) from the enzyme substrate *N*-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA; Sigma Chemical Co.). The proteolytic reaction was carried out in isolation buffer, containing 10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 1.5 mM KAc, 2 mM dithiothreitol, supplemented with the Complete protease inhibitor cocktail, after adding 20 μg of cytosolic protein and 50 μM DEVD-pNA. The reaction mixtures were incubated at 37 °C for 2 h, and the formation of pNA was measured at 405 nm using a 96-well plate reader. Caspase-3 processing was determined from cytosolic proteins separated by 15% SDS−polyacrylamide gel electrophoresis. After membrane transfer, the blots were probed with primary rabbit polyclonal antibody to caspase-3 (Santa Cruz Biotechnology) at a dilution of 1:1000, and subsequently incubated with secondary anti-rabbit antibody conjugated with horseradish peroxidase. Finally, membranes were processed for active caspase-3 detection using the ECL system (Amersham Life Science, Inc.).

RNA Isolation and RT-PCR. TUDCA-induced changes in Bax expression from isolated hepatocytes were determined by RT-PCR. Total RNA was extracted from rat primary hepatocytes exposed to 50 μM TUDCA for 36 h using the TRIZOL reagent from Invitrogen (Grand Island, NY). For RT-PCR, 5 μg of total RNA was reverse-transcribed using oligo(dT) (IDT, Inc., Coralville, IA) and SuperScript II reverse transcriptase (Invitrogen). Specific oligonucleotide primer pairs were incubated with cDNA template for PCR amplification using the Expand High Fidelity PCR system from Roche Applied Science. The following sequences were used as primers: Bax sense primer, 5'-TGGTTGCCCTTTTC-TACTTTG-3', and Bax antisense primer, 5'-GAAGTAG-GAAAGGAGGCCATC-3'; β-actin sense primer, 5'-TGC-CCATCTATGAGGGTTACG-3', and β-actin antisense primer, 5'-TAGAAGCATTTGCGGTGCACG-3'. The product of constitutively expressed β-actin mRNA served as control.

Electron Paramagnetic Resonance (*EPR*) *Spectroscopy and Spin-Labeling.* Changes in membrane structure were measured by EPR spectroscopy using paramagnetic reporter groups incorporated into membranes. The polarity of mitochondrial membrane lipids was examined at varying depths with 5- and 16-doxyl stearic acid (5-DSA and 16-DSA) spin labels (Sigma Chemical Co.), containing a nitroxide group at different positions along the hydrocarbon chain of the stearic acid molecule (*39*−*41*). The 5-DSA probe resides near the lipid−water interface, while the 16-DSA localizes deeper in the lipid bilayer. The isotropic splitting factor a_0 is a reliable parameter of the environment of these probes, and calculated from direct measurements of the parallel and perpendicular components of the hyperfine tensor of the spin label. A high a_0 reflects increased polarity of the membrane.

In addition, alterations in membrane dynamic properties of the 5-DSA probe, showing restricted motion in the membrane, were evaluated by measuring the outer half-width at half-height of the low-field extremum (Δl). The larger the Δl, the more motion and less order in the local microenvironment reported by the nitroxide group. For the 16-DSA spin label, showing a higher degree of motional freedom, the ratio of the height of the low-field and the center-field line (h_{+1}/h_0) was used as an empirical measurement of membrane lipid organization (*42*, *43*). Finally, we also tested 4-maleimido-2,2,6,6-tetramethylpiperidinooxyl (4-maleimido-TEMPO) label (Sigma Chemical Co.), which binds to the sulfhydryl groups of proteins. It provides information on the mobility of protein reactive groups by measuring changes in structure of protein-rich membranes at the surface level (*41*, *44*, *45*). The ratio of the height of the midline to the height of the high-field line of the spectrum (h_0/h_{-1}) reflects the freedom of motion of this probe at its binding site. A high ratio indicates a low freedom of motion.

All spin labels were dissolved in chloroform, separated into 1 μg aliquots, evaporated under nitrogen, and left under vacuum for 2 h. Freshly isolated intact mitochondria (10−50 μg of protein) were incubated in spin label-coated tubes with gentle shaking for 90 min at 37 °C (5- and 16-DSA) or for 1 min at 22 °C (4-maleimido-TEMPO).

Mitochondrial membrane lipid peroxidation was also assessed using EPR as described previously (*46*). In brief, freshly isolated mitochondria (5−10 μg of protein) were incubated with a membrane-associated, oxidation-sensitive, paramagnetic probe 5-DSA (1 μg aliquots), with gentle agitation for 20 min at 22 °C. Increased production of reactive oxygen species results in loss of paramagnetism of the probe, and hence loss of signal amplitude, measured as the height of the center field-line of the spectrum.

Labeled mitochondrial membranes were then exposed to Bax (0.5 μM) in Chelex-100-treated respiration buffer for 5 min at 22 °C. In co-incubation studies, labeled mitochondria were pretreated with either TUDCA (500 μM) or cyclosporine A (5 μM) for 5 min, which remained in the incubation medium after adding Bax. However, in washout experiments, TUDCA was removed from the media by centrifugation of the mitochondrial suspension and only then Bax was added to the pellet for an additional 5 min. After incubations, the mitochondrial suspension was spun at 12000*g* for 3 min, and the pellet resuspended in respiration buffer (60 μL). Samples were then aspirated into glass capillaries, sealed at both ends, and introduced in standard 4-mm quartz tubes containing silicone oil for thermal stability. All spectra were acquired at 9.8 GHz (X-band) on a Bruker EMX EPR spectrometer (Bruker, Karlsruhe, Germany) using a rectangular cavity (model ER 4102ST) and 100 kHz field modulation frequency, 1.05 G modulation amplitude, and 20 mW microwave power, at 22 °C.

RESULTS

Bax Directly Induces Cytochrome c Release from Isolated Mitochondria without Mitochondrial Swelling. We examined the effect of Bax on cytochrome *c* release by adding full-length recombinant Bax to mitochondria isolated from rat liver and energized through complex II with succinate. Cytochrome *c* release was then determined by Western blot analysis of both mitochondrial pellets and supernatants. Bax protein alone was sufficient to trigger a marked efflux of cytochrome *c*, since supernatants from Bax-treated mitochondria contained greater amounts of cytochrome *c* compared to untreated controls (Figure 1A). The release of cytochrome *c* was dependent on the concentrations of Bax protein. In fact, mitochondria exposed to 2 μM Bax released approximately 90% of their total cytochrome *c* after 5-min incubation. Moreover, kinetic experiments performed with the lowest concentration of Bax showed that cytochrome *c* release was already detectable after 1 min incubation and continued to increase thereafter. Interestingly, the concentration of 0.5 μM Bax used for most of the studies was within a physiological range. Similar and even greater levels of endogenous Bax have been detected in human tumor cells (*15*, *16*) and in cells following exposure to DNA-damaging agents (*47*).

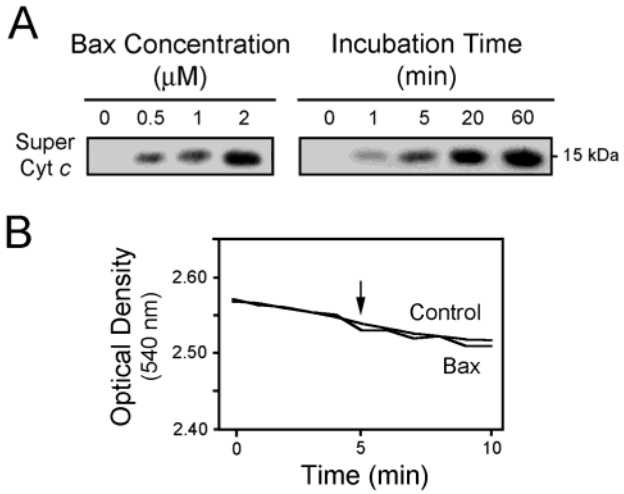

FIGURE 1: Purified recombinant Bax directly induces cytochrome *c* release from isolated mitochondria in the absence of mitochondrial swelling. (A) Mitochondria were isolated from rat liver and incubated with no addition (control), 0.5, 1, or 2 μM Bax for 5 min (left). In addition, mitochondria were incubated with 0.5 μM Bax for 0 (control), 1, 5, 20, or 60 min (right). Cytochrome *c* release was assayed in supernatants by Western blot analysis. Following SDS−polyacrylamide gel electrophoresis and transfer, the nitrocellulose membranes were incubated with a monoclonal antibody to cytochrome *c*, and the 15-kDa protein was detected using ECL chemiluminescence. (B) Basal levels of absorbance of mitochondrial suspensions were measured at 540 nm for 5 min. After the addition of 0.5 μM Bax (arrow), the optical density was monitored for an additional 5 min.

Mitochondrial swelling, a colloidosmotic process that is observed during permeability transition induction (*48*, *49*) was undetectable after 5 min exposure to Bax protein and remained insignificant to at least 60 min of incubation (Figure 1B). In contrast, the addition of Ca^{2+} or deoxycholic acid to isolated mitochondria induced a rapid, cyclosporine A-sensitive swelling under otherwise identical conditions (data not shown). Thus, the release of cytochrome *c* induced by Bax occurs through a mechanism different from that of Ca^{2+} and deoxycholic acid-induced toxicity. In the isolated mitochondria, Bax did not trigger opening of the mitochondrial megapore leading to permeability transition and the characteristic swelling.

TUDCA Prevents Bax-induced Release of Pro-apoptotic Factors that Trigger Activation and Processing of Cytosolic Caspases. We have shown that TUDCA can abrogate the

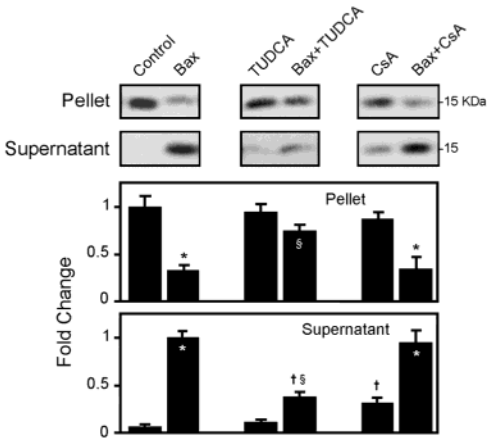

FIGURE 2: TUDCA inhibits Bax-induced release of cytochrome *c* in isolated mitochondria. Mitochondria were isolated from rat liver and incubated with either no addition (control), 0.5 μM Bax, 500 μM TUDCA, 5 μM cyclosporine A (CsA), Bax + TUDCA, or Bax + CsA for 5 min. In coincubation experiments, mitochondria were pretreated with TUDCA or CsA for 5 min. Mitochondrial pellets and supernatants were examined for cytochrome *c* levels by Western blot analysis (top). Following SDS−polyacrylamide gel electrophoresis and transfer, the nitrocellulose membranes were incubated with the monoclonal antibody to cytochrome *c* and the 15-kDa protein was detected using ECL chemiluminescence. The accompanying histograms are the densitometric means ± SEM relative to control (pellet) or Bax (supernatant) for at least three independent experiments (bottom). $^{\dagger}p < 0.05$ and $^{*}p < 0.01$ from control, and $^{\S}p < 0.01$ from Bax.

effect of several activators of the permeability transition in isolated mitochondria (*28*, *29*). Although Bax did not affect mitochondrial swelling, we examined whether TUDCA influenced Bax-induced cytochrome *c* release. Cytochrome *c* protein levels were determined by Western blot analysis in mitochondrial pellets and supernatants following incubation with Bax, in the presence or absence of TUDCA. Very low levels of cytochrome *c* were detected in mitochondria after incubation with Bax alone, while supernatant levels were markedly increased (Figure 2). Moreover, cytochrome *c* oxidase was not significantly changed in the mitochondrial pellet and, therefore, remained undetectable in supernatants, consistent with the absence of mitochondrial disruption (data not shown). Similarly, less than 2% of the matrix proteins glutamate dehydrogenase and citrate synthase were released after 5-min exposure to Bax. Together, these results suggest that cytochrome *c* release occurs because of perturbation of the outer mitochondrial membrane and not simply organelle bursting. Addition of TUDCA to the incubation media significantly reduced Bax-associated release of cytochrome *c* ($p < 0.01$). In fact, after 1, 5, 20, and 60 min of incubation with Bax plus TUDCA, cytochrome *c* release was reduced by >50% at 1 min to almost 75% at 60 min, compared with Bax alone (Table 1). Thus, in less than 1 min TUDCA was able to reduce the efflux of cytochrome *c* to half the maximum amount released by Bax alone. In contrast, cyclosporine A did not protect against Bax-induced cytochrome *c* efflux, again suggesting that the effect was independent of the permeability transition. In addition, hydrophobic deoxycholic acid aggravated and hydrophilic taurocholic acid did not alter Bax-induced changes in supernatant cytochrome *c* levels (data not shown).

Cytochrome *c* has been shown repeatedly to induce processing and activation of caspase-3, which in turn cleaves

Table 1: Time-Dependent Inhibition of Cytochrome *c* Release and Caspase Activity by TUDCA[a]

	inhibition (%)	
incubation time (min)	cytochrome *c* release	caspase activity
1	56.1 ± 6.9	40.3 ± 4.9
5	60.5 ± 5.2	43.1 ± 3.5
20	63.3 ± 6.5	49.4 ± 2.9
60	74.2 ± 11.1	59.3 ± 5.2

[a] Mitochondria were isolated from rat liver and incubated with either no addition (control), 0.5 μM Bax, 500 μM TUDCA, or Bax + TUDCA for 1, 5, 20, and 60 min. Mitochondrial supernatants were examined for cytochrome *c* levels by Western blot analysis. In addition, cytosolic protein extracts were prepared from primary rat hepatocytes and incubated with supernatants from Bax-treated mitochondria. Caspase activity was measured using the colorimetric substrate DEVD-pNA in cytososlic fractions after incubation with supernatants from the treated mitochondria. In coincubation experiments, mitochondria were pretreated with TUDCA for 5 min. Data were calculated as means ± SEM relative to controls for at least three separate experiments.

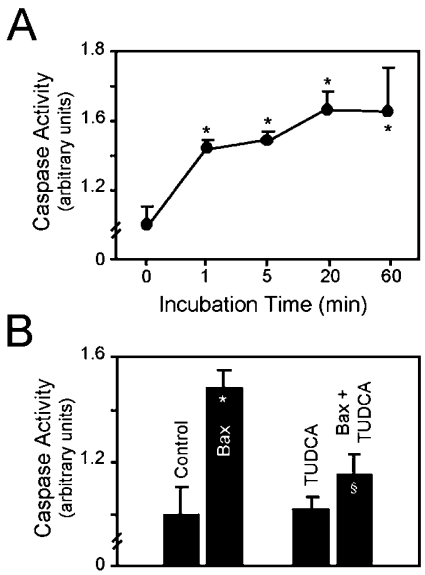

FIGURE 3: TUDCA inhibits Bax-induced activation of DEVD-specific caspases. Cytosolic protein extracts were prepared from primary rat hepatocytes and incubated with supernatants from Bax-treated mitochondria. Caspase activity was measured using the colorimetric substrate DEVD-pNA as described in Materials and Methods. (A) DEVD-specific caspase activity in cytososlic fractions after incubation with supernatants from mitochondria treated with 0.5 μM Bax for either 0 (control), 1, 5, 20, and 60 min. (B) TUDCA inhibition of DEVD-specific caspase activation in cytosolic fractions after incubation with supernatants from mitochondria treated with either no addition (control), 0.5 μM Bax, 500 μM TUDCA, or Bax + TUDCA for 5 min. In coincubation experiments, mitochondria were pretreated with TUDCA for 5 min. Data are means ± SEM values for at least three different experiments. $^{*}p < 0.01$ from control; $^{\S}p < 0.01$ from Bax.

the endogenous substrate PARP. Therefore, we investigated whether TUDCA could prevent the release of mitochondrial factors that induce DEVD-specific caspase activation. Mitochondrial supernatants from the various test groups were incubated with cytosolic fractions from primary rat hepatocytes, and caspase-3 like activity measured using DEVD-pNA as a substrate. Supernatants from Bax-treated mitochondria resulted in a marked elevation in DEVD-cleaving activity that increased with prolonged exposure (Figure 3A). In contrast, pretreatment of mitochondria with TUDCA consistently prevented caspase activation, and reduced

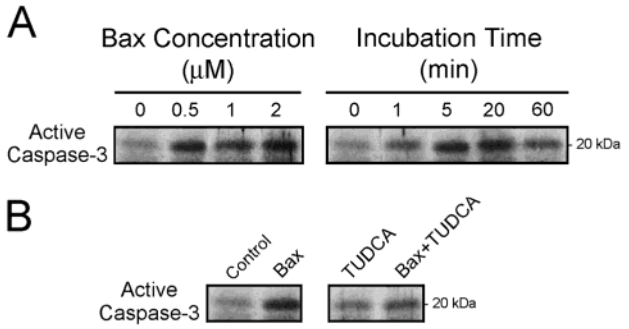

FIGURE 4: TUDCA inhibits Bax-induced proteolytic processing of caspase-3. Cytosolic protein extracts were prepared from primary rat hepatocytes and incubated with supernatants from Bax-treated mitochondria. Caspase-3 processing was examined by Western blot. Following SDS−polyacrylamide gel electrophoresis and transfer, the nitrocellulose membranes were incubated with the polyclonal antibody to caspase-3 and the active 20-kDa protein was detected by ECL chemiluminescence. (A) Caspase-3 processing in cytosolic fractions after incubation with supernatants from mitochondria treated with no addition (control), 0.5, 1, or 2 μM Bax for 5 min (left). In addition, mitochondria were incubated with 0.5 μM Bax for 0 (control), 1, 5, 20, or 60 min (right). (B) TUDCA inhibition of caspase-3 processing after incubation with supernatants from mitochondria treated with either no addition (control), 0.5 μM Bax, 500 μM TUDCA, or Bax + TUDCA for 5 min. In coincubation experiments, mitochondria were pretreated with TUDCA for 5 min.

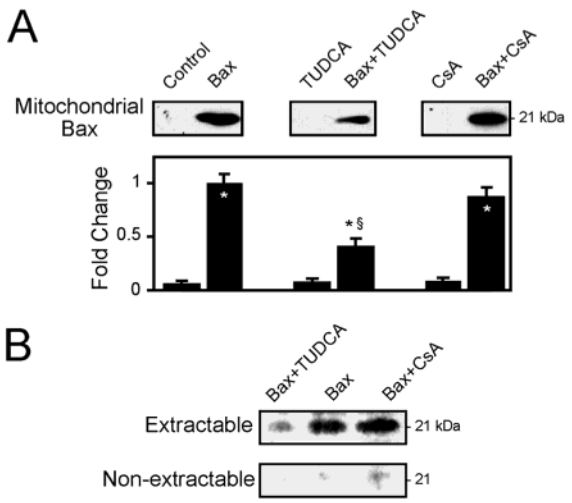

FIGURE 5: TUDCA inhibits binding of Bax to isolated mitochondria. Mitochondria were isolated from rat liver and incubated with no addition (control), 0.5 μM Bax, 500 μM TUDCA, 5 μM cyclosporine A (CsA), Bax + TUDCA, or Bax + CsA for 5 min. In coincubation experiments, mitochondria were pretreated with TUDCA or CsA for 5 min. (A) Mitochondrial levels of Bax protein were determined by Western blot analysis (top). Following SDS−polyacrylamide gel electrophoresis and transfer, the nitrocellulose membranes were incubated with the polyclonal antibody to Bax and the 21-kDa protein was detected using ECL chemiluminescence. The accompanying histogram is the densitometric means ± SEM relative to Bax for at least three independent experiments (bottom). *$p < 0.01$ from control; §$p < 0.01$ from Bax. (B) Carbonate extraction was performed on mitocondrial Bax and the supernatant and pellet fractions were visualized by Western blot analysis for extractable and nonextractable Bax protein, respectively.

enzyme activity to significantly lower levels ($p < 0.01$). Incubation with Bax plus TUDCA for 1, 5, 20, and 60 min reduced caspase activation ~40, 45, 50, and 60%, respectively, compared to Bax alone (Table 1). Thus, it required about 20 min of incubation with TUDCA to reduce caspase activity to half of the maximum activation induced by Bax alone. This suggests that apoptotic factors in addition to cytochrome *c* may be involved in caspase activation. Neither Bax nor isolated mitochondria alone were activators of DEVD-cleaving caspases (data not shown). Western blot analysis of the same cytosolic extracts revealed that supernatants derived from Bax-treated mitochondria induced a concentration- and time-dependent proteolytic processing of caspase-3 (Figure 4A). The active caspase-3 fragment was increased 1.6-fold after 1 min incubation with Bax, almost 3-fold at 20 min, and remained the same at 60 min. In contrast, TUDCA co-incubation resulted in more than a 40% reduction in active caspase-3 after 5 min of incubation (Figure 4B).

Although our results indicated that TUDCA prevented Bax-induced release of cytochrome *c* and the subsequent activation and processing of cytosolic caspases, it did not significantly modulate expression of Bax by RT-PCR in isolated hepatocytes (data not shown). Thus, it appears that TUDCA markedly alters the interaction of Bax with mitochondrial membranes without modulating gene expression. It remains to be determined whether transcript and/or protein stability are altered by the hydrophilic bile acid.

TUDCA Prevents Bax Binding and Insertion into Mitochondrial Membranes. Levels of endogenous Bax protein in control rat liver mitochondria were almost undetectable by Western blot analysis (Figure 5A). Incubation with Bax, however, resulted in a rapid increase in the levels of the proapoptogenic protein either inserted and/or associated with the isolated mitochondria. Co-incubation with TUDCA reduced these levels by ~70% ($p < 0.01$), which was similar to the observed decrease in cytochrome *c* release. In contrast, cyclosporine A did not significantly reduce the levels of Bax protein associated with the mitochondria. When the mitochondria were extracted with carbonate, however, essentially all of the remaining Bax protein from incubations with either Bax alone, TUDCA or CsA was recovered in the supernatant (Figure 5B). The results indicated that TUDCA but not cyclosporine A significantly reduced Bax association with the mitochondrial membrane. In addition, neither TUDCA nor cyclosporine A increased Bax levels that were nonextractable from the mitochondrial membrane ($p < 0.01$), consistent with the results from Table 2.

TUDCA Prevents Bax-Induced Alterations in Mitochondrial Membrane Lipid Polarity, Protein Order, and Redox Status. EPR studies were designed to test whether Bax could induce macromolecular membrane structural perturbation and cytochrome *c* release in isolated mitochondria in the absence of permeability transition pore opening. We investigated the effect of Bax on mitochondrial membrane polarity at varying depths using 5- and 16-DSA spin labels and determined several spectroscopic parameters (Figure 6). Bax interaction with mitochondria resulted in an almost 10% increase in membrane polarity at both the 5-DSA superficial and 16-DSA deeper layers of the membrane leaflet ($p < 0.05$) (Table 2). In addition, the motion parameters Δl and h_{+1}/h_0 reflected a 20% increase in membrane fluidity at C-5 ($p < 0.01$), but only a 5% change at C-16 ($p < 0.05$). These results suggest that Bax interacts preferably with superficial regions of the membrane, resulting in loose association with mitochondria. In contrast, TUDCA alone marginally altered dynamic properties of the mitochondrial membranes. However, pretreatment with TUDCA partially prevented the Bax-induced

Table 2: TUDCA Prevents Bax-Induced Alterations in Mitochondrial Membrane Lipid Polarity and Fluidity, and Protein Order[a]

	5-DSA		16-DSA		4-maleimido-TEMPO
	lipid polarity a_0	lipid fluidity Δl	lipid polarity a_0	lipid fluidity h_{+1}/h_0	protein order h_0/h_{-1}
control	1.00 ± 0.02	1.00 ± 0.10	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.01
Bax	1.09 ± 0.03*	1.25 ± 0.07*	1.10 ± 0.04†	1.06 ± 0.02†	1.12 ± 0.02*
TUDCA	1.02 ± 0.01	1.09 ± 0.12	1.03 ± 0.03	0.97 ± 0.01	0.93 ± 0.05
Bax + TUDCA	1.04 ± 0.01‡	1.03 ± 0.12§	1.04 ± 0.01‡	0.93 ± 0.01§	0.96 ± 0.02§
CsA	1.05 ± 0.02	1.03 ± 0.01	1.03 ± 0.02	1.02 ± 0.01	1.00 ± 0.01
Bax + CsA	0.99 ± 0.02‡	1.19 ± 0.16	1.04 ± 0.03	0.92 ± 0.03§	1.03 ± 0.02‡

[a] Mitochondria were labeled with the 5-DSA, 16-DSA, and 4-maleimido-TEMPO spin probes and incubated with either no addition (control), 0.5 μM Bax, 500 μM TUDCA, 5 μM cyclosporine A (CsA), Bax + TUDCA, or Bax + CsA for 5 min. In coincubation experiments, mitochondria were pretreated with TUDCA or CsA for 5 min. Mitochondrial pellets were examined for lipid polarity and fluidity, as well as protein order by EPR spectroscopy analyses. The 5- and 16-DSA spin labels were used to sense alterations in mitochondrial membrane isotropic hyperfine splitting constant (a_0). Changes in the half-width at half-height ($h/2$) of the low-field extremum or Δl and in the ratio of the height of the low-field line (h_{+1}) to the height of the midline (h_0) were calculated for the 5-DSA and the 16-DSA spin labels, respectively. Alterations in the ratio of the height of the midline (h_0) to the height of the high-field line (h_{-1}) were measured by the 4-maleimido-TEMPO spin label. Values are means ± SEM for at least three separate experiments. †$p < 0.05$ and *$p < 0.01$ from control; ‡$p < 0.05$ and §$p < 0.01$ from Bax.

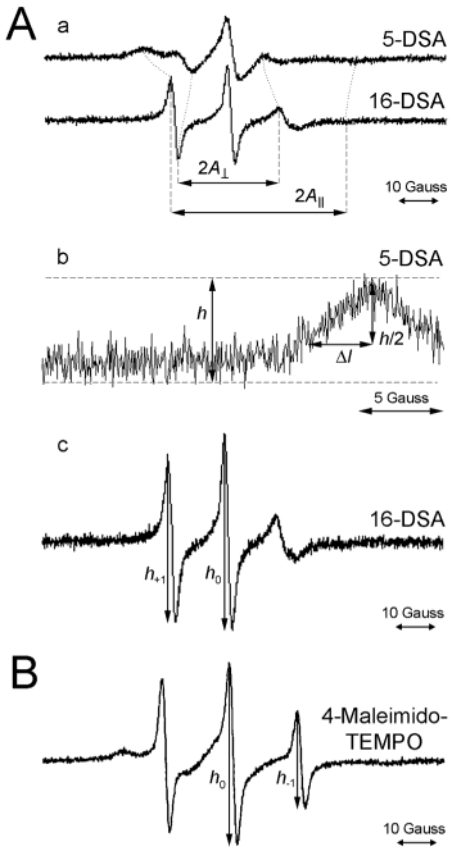

FIGURE 6: Spectral parameters of spin labels incorporated into mitochondrial membranes. (A) Spectra of nitroxide-labeled stearic acids and measurements of maximum ($A_{||}$) and minimum ($A_\perp$) hyperfine splittings to estimate a_0 [$a_0 = 1/3(A_{||} + 2A_\perp)$] for 5- and 16-DSA spin labels (a); outer half-with at half-height ($h/2$) of the low-field extremum or Δl calculated for the 5-DSA spin label (b); ratio of the height of the low-field line (h_{+1}) to the height of the midline (h_0) calculated for the 16-DSA spin label (c). (B) spectrum of 4-maleimido-TEMPO label and measurement of the ratio of the height of the midline (h_0) to the height of the high-field line (h_{-1}). All spectra were acquired at 9.8 GHz (X-band) on a Bruker EMX EPR spectrometer using a rectangular ER 4102ST cavity and 100 kHz field modulation frequency, 1.05 G modulation amplitude, and 20 mW microwave power, at 22 °C.

increase in lipid polarity ($p < 0.05$), while almost completely inhibiting changes in mitochondrial membrane lipid fluidity ($p < 0.01$) both at C-5 and C-16. Cyclosporine A, although protective of deeper membrane changes, did not significantly prevent the superficial interaction of Bax. These results support the carbonate extraction data in that TUDCA, but not cyclosporine A, inhibits superficial binding of Bax to mitochondria.

Using the protein-oriented spin label 4-maleimido-TEMPO, the mobility parameter h_0/h_{-1} was significantly increased after exposure to Bax ($p < 0.01$). The results reflected decreased mobility of the probe and greater structural order in mitochondrial membrane proteins at the surface level, consistent with loose association of Bax with the membrane (Table 2). In contrast, pretreatment with TUDCA completely abrogated Bax-induced effects, and decreased protein order to control levels ($p < 0.01$). Washout experiments were performed in which TUDCA was removed from the incubation medium prior to incubation with Bax. The results were similar and suggested that the inhibitory effect of TUDCA did not occur from a direct interaction between the bile acid and Bax protein in the incubation media. Furthermore, the spectra obtained from mitochondria incubated only with TUDCA revealed no differences between the washout and standard incubation conditions (data not shown).

We then determined if exposure to Bax directly modified levels of mitochondrial membrane lipid oxidation. Isolated mitochondria were probed with 5-DSA spin label and then exposed to Bax (Figure 7). Bax caused a rapid loss of spin-probe intensity, manifested by a >30% decrease in peak amplitude ($p < 0.01$). Using a different methodological approach to measure signal intensity, based on the double integration of spectra, we confirmed the results described above (data not shown). Spin-label signal amplitude in isolated mitochondria was also assessed in the presence of TUDCA. Treatment of 5-DSA-labeled mitochondria with the bile acid alone did not significantly decrease signal amplitude, while incubation with TUDCA for 5 min prior to exposure to Bax markedly inhibited oxidative lipid injury ($p < 0.01$). This protection resulted in signal amplitudes that were no different from controls. The results suggested that TUDCA either directly confers protection against lipid peroxidation in isolated mitochondrial membranes, or indirectly prevents reactive oxygen species production by interfering with Bax interaction with the membrane. Cyclosporine A was less effective at preventing Bax-induced spectral changes.

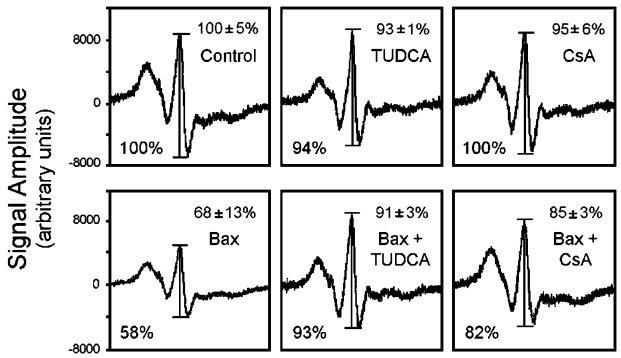

FIGURE 7: TUDCA prevents Bax-induced oxidative injury in isolated mitochondrial membranes. Mitochondria were labeled with the 5-DSA spin probe and incubated with no addition (control), 0.5 μM Bax, 500 μM TUDCA, 5 μM cyclosporine A (CsA), Bax + TUDCA, or Bax + CsA for 5 min. In coincubation experiments, mitochondria were pretreated with TUDCA or CsA for 5 min. Mitochondrial pellets were examined for lipid oxidative damage by EPR spectroscopy analyses. Individual values of signal amplitude and means ± SEM relative to controls are representative of at least three separate experiments. All spectra were acquired at 9.8 GHz (X-band) on a Bruker EMX EPR spectrometer using a rectangular ER 4102ST cavity and 100 kHz field modulation frequency, 1.05 G modulation amplitude, and 20 mW microwave power, at 22 °C.

DISCUSSION

In the present study, we have demonstrated that (a) the addition of recombinant Bax protein to isolated mitochondria can induce cytochrome *c* release; (b) Bax-mediated release of cytochrome *c* occurs in the apparent absence of permeability transition and subsequent swelling that is thought to rupture the outer mitochondrial membrane; (c) altered membrane lipid polarity and fluidity, protein order, and redox status accompany the insertion of Bax into the mitochondrial membrane; and (d) mitochondria treated with TUDCA prior to addition of Bax are resistant to Bax-induced alterations. These observations support the notion that TUDCA alters the ability of Bax to interact with the mitochondrial membrane, thereby preventing the induced perturbation. Thus, at least one mechanism for the antiapoptotic effect of TUDCA is related to its ability to modulate Bax association with mitochondria, and it appears to do so without directly interacting with the protein in solution.

UDCA and its conjugated derivatives play a unique role in modulating the apoptotic threshold in both hepatic and nonhepatic cells (*27*−*29*). The inhibition of cytochrome *c* release and downstream caspase activation in apoptotic cells by these bile acids suggested that the hydrophilic bile acid might act at the mitochondrial level. The interaction of UDCA with the mitochondrial membrane was recently demonstrated in a study where the fluorescent-labeled UDCA colocalized with mitochondria in cultured hepatocytes (*50*). Thus, the protective effects of UDCA and TUDCA in intact cells at micromolar concentrations are thought to result, in part, from modulation of Bax translocation from cytosol to mitochondria, together with inhibition of mitochondrial depolarization and outer membrane rupture (*51*).

To establish a direct effect of TUDCA on mitochondria, we first examined whether recombinant Bax alone was sufficient to trigger membrane perturbation. We then investigated the ability of TUDCA to prevent those changes. Previous studies have demonstrated the importance of the BH3 domain of Bax for homo- and heterodimerization with other antiapoptotic Bcl-2 family proteins (*52*). In contrast to monomeric recombinant Bax, oligomeric Bax forms channels large enough to allow the release of cytochrome *c* from liposomes (*53*, *54*). It is therefore conceivable that Bax directly constitutes the structural entirety of a channel in the outer mitochondrial membrane that permits cytochrome *c* release (*55*). In this model, Bax and Bak are activated by BH3-only factors forming tetrameric channels that allow the release of apoptogenic factors from the mitochondrial intermembrane space. Genetic knockout analysis suggests that Bax and Bak are indeed essential to mitochondrial dysfunction and subsequent cell death by a wide variety of stimuli (*56*). However, it remains controversial as to whether truncated Bid is required to induce Bax oligomerization and cluster formation in the outer mitochondrial membrane. In fact, it was recently reported that Bax can induce cytochrome *c* release and loss of membrane potential in the absence of Bid or Bak translocation to the mitochondria (*57*).

These in situ experiments suggest that Bid may only be required in certain, but not all cell types. Alternatively, Bax might indirectly alter membrane permeability through interaction with other proteins. To this end, Bax, but not Bcl-2 or Bcl-x, was found to copurify with the permeability transition pore (*58*). In addition, it has been suggested that Bax and Bak, but not BH3-only factors, can cause $\Delta\Psi_m$ collapse in isolated mitochondria (*59*). According to this model, Bax binds to the permeability transition pore complex, resulting in its opening, swelling of the mitochondrial matrix and rupture of the outer mitochondrial membrane (*60*). Interaction between the voltage-dependent anion channel and the mitochondrial permeability transition pore remains unclear. In fact, cytochrome *c* release can occur in the absence of mitochondrial swelling and megapore activation (*16*). Further, cross-linking studies failed to demonstrate an interaction between Bax and the voltage-dependent anion channel (*61*), while Bax has been shown to directly release cytochrome *c* from isolated mitochondria (*15*). It was also shown that increased membrane permeability may be attributed to a new channel, the mitochondria-apoptosis-induced channel, located in the outer mitochondrial membrane (*62*). This channel, although similar to the Bax/Bak channel, may contain additional components that allow the release of larger-sized factors, such as Smac/DIABLO, and simultaneously provide a type of gating system. Interestingly, it was recently demonstrated that mitochondrial lipid rather than protein composition is the key determinant in the permeabilization process induced by Bax via a supramolecular complex (*24*).

In this study, we confirm that the addition of purified Bax to isolated mitochondria is sufficient to directly induce release of cytochrome *c* (*15*, *16*) in the absence of permeability changes that cause mitochondrial swelling. This suggests that Bax plays a key role in mediating cytochrome *c* release during apoptosis, perhaps by acting independently of the permeability transition pore opening. Our results, however, differ from those reported in an earlier study in which recombinant Bax caused a sustained opening of the mitochondrial permeability transition pore with subsequent persistent organelle swelling and deenergization (*63*). Nevertheless, their experimental design used truncated recombinant Bax instead of the full-length protein and their purification procedure involved exposures to low pHs and

detergents which induce significant conformational changes in the protein. Further, the finding that cyclosporine A failed to block Bax-induced cytochrome *c* release supports the notion that the release of intermembrane proteins does not necessarily involve disruption of the megapore. Although we have not excluded the possibility that Bax interacts with other components of the permeability transition pore, these results suggest that the pro-apoptotic protein triggers cytochrome *c* release independently of at least cyclophilin D. Different methodological conditions, such as the presence or absence of calcium chelators in the incubation medium, may account for contradictory results published by different groups (*15*, *16*). In fact, a protective effect by cyclosporine A may reflect the inhibition of calcium-activated permeability transition rather than Bax-induced changes.

In addition to cytochrome *c* release and subsequent caspase processing and activation, EPR analysis indicated that Bax also induced marked changes in lipid and protein structure of the mitochondrial membranes. The ability of Bax to increase fluidity and permeability of membrane lipids at superficial membrane regions is consistent with its loose association with mitochondria. Moreover, increased surface protein order in mitochondrial membranes exposed to Bax is compatible with its binding to the membrane, thus reducing the mobility of other structural proteins. Therefore, Bax may become active only after interaction with the mitochondrial membrane. This is consistent with recent results demonstrating that oligomeric Bax forms complexes with mitochondrial membrane proteins in cultured cells exposed to staurosporine or UV radiation (*55*). Interestingly, the direct interaction of Bax with mitochondrial membrane may occur only briefly during apoptosis, coalescing thereafter with other proapoptotic proteins into clusters adjacent to mitochondria (*64*).

When mitochondria were treated with TUDCA, we were unable to detect any major changes in membrane dynamic properties. This was not surprising considering the hydrophilic qualities of the bile acid. However, our results indicate that TUDCA was able to alter the membrane environment and inhibit Bax protein from interacting to form channels and disrupting lipid dynamics. This is consistent with supramolecular openings in the outer mitochondrial membrane induced by the interaction of Bax with membrane lipids (*24*) and inhibited by TUDCA. It remains to be determined how TUDCA associates with the membrane for a prolonged inhibitory effect on Bax. It is also unclear whether specific lipids in mitochondrial membranes, such as cardiolipin, would affect the ability of TUDCA to abrogate Bax-induced changes in membrane lipid fluidity and polarity. Interestingly, the mitochondrial cardiolipin content was shown to be significantly greater in bile duct-ligated rats, with increased levels of hydrophobic bile acids (*65*). Finally, the washout experiments suggest that the effect of TUDCA does not occur by interaction of the bile acid with Bax in solution. Rather, the protective effect appears to be mediated at the outer membrane, resulting in significantly reduced levels of cytochrome *c* in the supernatants of mitochondria incubated with both Bax and TUDCA. Not surprisingly, TUDCA also prevented the generation of reactive oxygen species from failed electron chain transport after cytochrome *c* realease. Under these conditions, most cytochrome *c* remained associated with the inner membrane to sustain electron transport chain. The small proportion of cytochrome *c* released from mitochondria incubated with Bax plus TUDCA only modestly triggered caspase activation as compared to mitochondria exposed to Bax alone.

Interestingly, the partial inhibition of Bax-induced structural changes in membrane motion by cyclosporine A suggests that the effect on Bax function is indirect, and occurs primarily at deeper membrane regions. In such scenario, cyclosporine A appears to inhibit secondary opening of the megapore altering the overall environment of lipids and proteins, which collaborate with Bax to induce membrane permeability and fluidity. This, however, does not prevent loose association of Bax protein with the outer membrane, nor does it influence cytochrome *c* release through the Bax channel, which is very rapid and complete. The results suggest that both TUDCA and cyclosporine A act at the mitochondrial membrane. However, TUDCA stabilizes the lipid and protein structure, thus inhibiting Bax binding. This is in contrast to cyclosporine A that allows association but prevents deeper structural changes and opening of the megapore secondary to uncoupled oxidative phosphorylation.

In conclusion, we have demonstrated that Bax can directly trigger the release of cytochrome *c* in isolated mitochondria, and appears to do so independently of the mitochondrial permeability transition pore. Although not severely affected, the physical properties of membrane lipids and proteins reflect association of Bax with the mitochondrial membrane and the subsequent release of cytochrome *c*. In clear contrast, pretreatment with TUDCA partially inhibited Bax binding and subsequent events, including the release of mitochondrial factors that trigger cytosolic caspase activation. Our results suggest that the antiapoptotic properties of TUDCA occur, in part, by inhibition of Bax through direct stabilization of the mitochondrial membrane by the bile acid.

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