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BAX insertion, oligomerization, and outer membrane permeabilization in brain mitochondria: Role of permeability transition and SH-redox regulation

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ABSTRACT

BAX cooperates with truncated BID (tBID) and Ca^{2+} in permeabilizing the outer mitochondrial membrane (OMM) and releasing mitochondrial apoptogenic proteins. The mechanisms of this cooperation are still unclear. Here we show that in isolated brain mitochondria, recombinant BAX readily self-integrates/oligomerizes in the OMM but produces only a minuscule release of cytochrome *c*, indicating that BAX insertion/oligomerization in the OMM does not always lead to massive OMM permeabilization. Ca^{2+} in a mitochondrial permeability transition (mPT)-dependent and recombinant tBID in an mPT-independent manner promoted BAX insertion/ oligomerization in the OMM and augmented cytochrome *c* release. Neither tBID nor Ca^{2+} induced BAX oligomerization in the solution without mitochondria, suggesting that BAX oligomerization required interaction with the organelles and followed rather than preceded BAX insertion in the OMM. Recombinant Bcl-xL failed to prevent BAX insertion/oligomerization in the OMM but strongly attenuated cytochrome *c* release. On the other hand, a reducing agent, dithiothreitol (DTT), inhibited BAX insertion/oligomerization augmented by tBID or Ca^{2+} and suppressed the BAX-mediated release of cytochrome *c* and Smac/DIABLO but failed to inhibit Ca^{2+}-induced swelling. Altogether, these data suggest that in brain mitochondria, BAX insertion/oligomerization can be dissociated from OMM permeabilization and that tBID and Ca^{2+} stimulate BAX insertion/oligomerization and BAX-mediated OMM permeabilization by different mechanisms involving mPT induction and modulation of the SH-redox state.

1. Introduction

Apoptosis is an omnipresent form of cell death involved in various neurodevelopmental [1] as well as neuropathological processes, including age-related neurodegenerations [2–6], stroke [7–10], and secondary brain injury following mechanical brain trauma [11,12]. The release of mitochondrial apoptogenic factors, a key step in executing of apoptosis [13], occurs due to a concert action of pro-apoptotic proteins such as BID and BAX [14]. Under normal conditions, monomeric BAX and full-length BID are located in the cytosol [15]. Caspase-8 activated by apoptotic stimuli cleaves BID, producing activated (truncated) BID (tBID) [16]. In turn, tBID activates BAX either "directly" [15,17,18] or "indirectly" [19,20] leading to oligomerization of BAX, its insertion into the OMM, and OMM permeabilization culminating in the release of mitochondrial apoptogenic proteins [18,21].

In addition to tBID, elevated Ca^{2+} enhances the ability of BAX to integrate into the lipid membranes and permeabilize them [22–24]. Ca^{2+} also amplifies BAX ability to permeabilize the OMM [25], though the mechanism of such amplification is unknown. Since elevated Ca^{2+} induces the mitochondrial permeability transition (mPT), a phenomenon accompanied by mitochondrial depolarization and remodeling [26], it is possible that the mPT is involved in augmentation of BAX-mediated OMM permeabilization. In cerebellar granule neurons, trophic factor withdrawal in low-K^+ medium resulted in the mPT that triggered BAX translocation to mitochondria and release of Cyt *c* [27]. In line with this, in the model of ischemia/reperfusion heart injury, inhibition of the mPT either with Ru_{360}, an inhibitor of the mitochondrial Ca^{2+} uniporter [28], or with cyclosporin A (CsA), an

Abbreviations: tBID, truncated BID; mPT, mitochondrial permeability transition; OMM, outer mitochondrial membrane; ER, endoplasmic reticulum; CsA, cyclosporin A; COX IV, cytochrome oxidase subunit IV; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; NP-40, Nonidet P-40, [octylphenoxy] polyethoxyethanol; OG, octyl glucoside; Tr X-100, Triton X-100; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGS, ethylene glycol bis(succinimidyl succinate), DSS, disuccinimidyl suberate; BMH, bismaleimidohexane

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inhibitor of the mPT [29], precluded BAX insertion in the OMM and OMM permeabilization [30]. Thus, there is evidence suggesting a synergistic relationship between the Ca^{2+}-induced mPT and BAX in OMM permeabilization.

In the present study, we demonstrated that BAX could readily self-integrate and oligomerize in the OMM, but these events were not accompanied by massive Cyt *c* release. We also found that Ca^{2+} in an mPT-dependent and tBID in an mPT-independent manner augmented BAX insertion and oligomerization in the OMM that correlated with the increased OMM permeabilization. Moreover, we showed that the Ca^{2+}- and tBID-stimulated BAX insertion/ oligomerization depended on SH-redox state and could be inhibited by a reducing agent, dithiothreitol (DTT). DTT also attenuated BAX-mediated OMM permeabilization stimulated by Ca^{2+} or tBID, revealing an important role of SH-redox regulation in the release of mitochondrial apoptogenic proteins.

2. Materials and methods

2.1. Recombinant proteins

Full-length human monomeric BAX with a tag of six histidine residues at the N-terminus was expressed in the pBAD plasmid in *Escherichia coli* [31]. Mouse tBID (BID cut with caspase-8 and separated from N-terminal and the caspase) was obtained from full-length BID as described previously [32]. Recombinant Bcl-xL was produced as described previously [17]. Recombinant BAX, tBID, and Bcl-xL were stored in dialysis buffer containing 25 mM HEPES-NaOH, pH 7.5, 0.2 mM dithiothreitol, 30% glycerol (v/v) at −86 °C.

2.2. Isolation and purification of brain mitochondria

Mitochondria from the brains of male Sprague-Dawley rats, 200–250 g (Harlan, Indianapolis, IN, USA) were isolated in mannitol-sucrose medium according to an Institutional Animal Care and Use Committee approved protocol and purified on a discontinuous Percoll gradient as described previously [33]. Mitochondrial protein was measured by the Bradford method [34], using BSA as a standard.

2.3. Measurements of mitochondrial light scattering

Mitochondrial swelling was evaluated in the standard incubation medium at 37 °C by monitoring the scattering of light directed on mitochondrial suspension under 90° to the axis of the photodetector at 525 nm in a 0.4-ml cuvette under continuous stirring using a PerkinElmer LS-55 luminescence spectrometer. The standard incubation medium used in these and other experiments contained 125 mM KCl, 10 mM HEPES, pH 7.4, 0.5 mM $MgCl_2$, 3 mM KH_2PO_4, 10 μM EGTA, 0.1% bovine serum albumin (free from fatty acids), 3 mM glutamate, and 3 mM succinate.

2.4. Transmission electron microscopy

Electron microscopy of isolated brain mitochondria was performed as described previously [35]. Mitochondria were incubated in the standard 125 mM KCl-based medium at 37 °C prior to fixation in 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M phosphate buffer in the same incubation medium at room temperature for 15 min. Transmission electron microscopy (TEM) images were taken using a Tecnai G12 BioTwin electron microscope (FEI, Hillsboro, OR) equipped with an AMT 2.6×2.6 K digital CCD camera.

2.5. Alkali-resistant BAX insertion

The alkali treatment of mitochondria removes loosely attached proteins but leaves proteins inserted into the OMM [18]. We determined the alkali-resistant fraction of BAX inserted into the OMM using the earlier described method [36]. Briefly, mitochondria treated with BAX (50 or 150 nM) at 37 °C for 30 min were pelleted at 15,800*g* for 5 min, and supernatant was used for the Cyt *c* release measurements. Mitochondrial pellets were re-suspended in 0.2 ml of 0.1 M Na_2CO_3, pH 11.5, then incubated for 30 min on ice. Samples were centrifuged for 30 min at 100,000*g* in an Optima L-100 K Beckman ultracentrifuge. The pellets were solubilized using 1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) or 1% [octylphenoxy] polyethoxyethanol (Nonidet P-40, Amresco, Solon, OH) and analyzed by western blotting against BAX and cytochrome oxidase subunit IV (COX IV, loading control).

2.6. Immunoblotting

The release of Cyt *c* and Smac/DIABLO from isolated brain mitochondria was assessed in supernatants obtained through incubation of mitochondria in the standard 125 mM KCl-based incubation medium with or without additions for 30 min at 37 °C. For SDS-PAGE, we used 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA). Western blotting was performed as previously described [37]. In some experiments, alamethicin (30 μg/ml) was used to produce the maximal Cyt *c* release. Mitochondrial cytochrome oxidase subunit IV (COX IV) was used as a loading control for the pellet samples. COX IV was detected with mouse monoclonal anti-COX IV antibody, dilution 1:5000 (Invitrogen, Carlsbad, CA). Following SDS-PAGE, proteins were transferred to Hybond™-ECL™ nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ), and blots were incubated with mouse anti-cytochrome *c* antibody (7H8.2C12, PharMingen, San Diego, CA) at 1:3000 dilution or with rabbit anti-Smac/DIABLO antibody (Alexis Biochemicals, San Diego, CA) at 1:1500 dilution for an hour at room temperature in 5% non-fat milk, phosphate-buffered saline, pH 7.2, and 0.15% Triton X-100. Prior to analysis of Smac/DIABLO release, the supernatants were concentrated threefold in the Microcon YM-10 filtering devices (Millipore Corporation, Bedford, MA). In the alkali-resistant BAX insertion experiments, BAX was detected by western blotting with rabbit polyclonal anti-BAX antibody (Upstate, Lake Placid, NY). Recently, it was shown that oxidation of BAX's cysteines favored formation of disulfide bridges and BAX oligomerization [38,39], so it is possible that formation of disulfide bridges might contribute to BAX oligomerization in our experiments. Correspondingly, to prevent disruption of disulfide bridges and disassembly of BAX oligomers, SDS-PAGE was performed under non-reducing conditions. Anti-BAX antibody was used at 1:2000 dilution for an hour at room temperature in 5% BSA (Jackson ImmunoResearch Laboratories, West Grove, PA), phosphate-buffered saline, pH 7.2, and 0.15% Triton X-100. Blots were developed using goat anti-rabbit or anti-mouse IgG (1:20000) coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories) and Supersignal West chemiluminescent reagents (Pierce, Rockford, IL). Molecular weight marker SeeBlue® Plus 2 Standards (5 μl), (Invitrogen, Carlsbad, CA) were used to determine the molecular weights of the bands. NIH ImageJ 1.40 g software (http://rsb.info.nih.gov/ij/) was used to quantify band densities. All immunoblots are representative of at least three independent experiments.

2.7. Analytical gel-filtration

Analytical gel-filtration was carried out on a Superdex 200 HR 10/30 column using FPLC. Prior to injecting into the column, BAX (500 nM) was pre-incubated at 4 °C for 24 h in the solution containing 125 mM KCl, 10 mM HEPES, pH 7.4, and 1% CHAPS. The same solution was used to equilibrate the column. After injecting the column with 150 μl sample, fractions of 0.4 ml were collected and protein was concentrated with trichloroacetic acid/acetone precipitation prior to analysis by western blotting. The column was calibrated using gel-