Characterization of tBid-induced cytochrome *c* release from mitochondria and liposomes

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Abstract tBid, the cleaved form of Bid, can induce cytochrome *c* (Cyt. *c*) release from rat heart mitochondria more efficiently and reproducibly than that from liver or brain mitochondria. Unlike Bax, such release was not prevented by cyclosphorin A, an inhibitor of the opening of permeability transition pore. Carbonyl-cyanide *m*-chlorophenyl-hydrazone or oligomycin also have no obvious effect on the release of Cyt. *c*. In contrast to ceramide, tBid-mediated Cyt. *c* release from mitochondria is independent of the redox state of Cyt. *c*. Furthermore, Bid or tBid can directly trigger the efflux of encapsulated Cyt. *c* or trypsin within liposomes without involvement of other protein factors.

Key words: Apoptosis; Bid; Cytochrome *c* release; Mitochondrion; Permeability transition pore; Liposome

1. Introduction

Apoptosis is an evolutionarily conserved process critical in various biological events, such as embryonic development, maintenance of tissue homeostasis, removal of non-instructed, misinstructed, as well as damaged cells, and immunological defense [1]. Various stimuli including developmental and environmental deliver complex signals to promote apoptosis or survival. A large number of pro- and anti-apoptotic molecules were identified to play principal roles in apoptosis, in which Bcl-2 superfamily proteins act as regulators [2] and caspase family proteases act as signal transducers and executors [3].

Recently, mitochondria have been recognized to play a central role in the regulation of apoptosis by disrupting electron transport and energy metabolism, by releasing proteins such as cytochrome *c* (Cyt. *c*) and apoptosis-inducing factor, and by altering cellular redox potential [4]. The Bcl-2 superfamily proteins, mainly located in mitochondrial outer membrane, constitute a pivotal checkpoint within the common portion of the apoptotic pathway. The ratio of pro- (Bax, Bak, Bid, etc.) versus anti-apoptotic (Bcl-2, Bcl-x_L, Bcl-W, etc.) determines in part how cells respond to proximal death and survival signals [5].

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Abbreviations: Cyt. *c*, cytochrome *c*; PTP, permeability transition pore; CsA, cyclosporin A; $\Delta\Psi$, transmembrane electric potential difference; CCCP, carbonyl-cyanide *m*-chlorophenyl-hydrazone; AA, antimycin A; BAEE, *N*α-benzoyl-l-arginine ethyl ester; LUV, large unilamellar vesicle; VDAC, voltage-dependent anion channel

Cyt. *c* release from mitochondria has been proved to play a crucial role in apoptosis [6,7]. It may have an antioxidant effect after being released to the intermembrane space [8]. Once it is in the cytoplasm, it can form 'apoptosome' together with apoptosis-activating factor 1 and caspase 9 as a critical activator to amplify the apoptotic signals to ensure fast and irreversible apoptosis [9]. But it is still enigmatic how the Cyt. *c* transports across the mitochondrial outer membrane. Recent evidence has shown that Bax and Bak induce mitochondria changes such as Cyt. *c* release and transmembrane electric potential difference ($\Delta\Psi$) loss by directly interacting with the permeability transition pore (PTP) [10]. Overexpression of Bax produces apoptosis upon induction of PT [11]. F_0F_1-ATPase in the inner membrane of mitochondria is necessary for the optimal function of Bax [10], which means that mitochondria energization affects Bax-induced Cyt. *c* release. In the present paper, characterization of Bid-induced Cyt. *c* release from mitochondria has been studied. Obtained results showed that, unlike Bax or ceramide, Bid may trigger Cyt. *c* release from mitochondria by an alternative mechanism. Additionally, Bid can directly induce the release of encapsulated Cyt. *c* or trypsin within the large unilamellar vesicles (LUVs) without involvement of other protein factors.

2. Materials and methods

2.1. Materials

Anti-Cyt. *c* monoclonal antibody (7H8.2C12) was from Pharmingen. Human Bid expression plasmid, pET-15b, and caspase 8 expression plasmids, pEt28, were kindly provided by Dr. Xiaodong Wang. Carbonyl-cyanide *m*-chlorophenyl-hydrazone (CCCP), cyclosporin A (CsA), oligomycin, antimycin A (AA), trypsin, lubrol PX, dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) were obtained from Sigma. *N*α-Benzoyl-l-arginine ethyl ester (BAEE) was from the Shanghai Institute of Biochemistry, Chinese Academy of Sciences.

2.2. Expression and purification of recombinant proteins

Human Bid and caspase 8 were expressed recombinantly as described [12]. The plasmids for expression of Bid and caspase 8 were separately transformed into bacteria BL21 (DE3) cells. Then the recombinant proteins were purified from cell lysate by nickel affinity column (Qiagen), and bio-scale Q5 (Bio-Rad) was used for further purification.

2.3. Isolation of mitochondria

Female rats were killed by decapitation, and liver, heart and brain mitochondria were isolated as described [12,13]. In brief, tissues were doucely homogenized in ice-cold buffer containing 250 mM mannitol, 0.5 mM EGTA, 5 mM HEPES (pH 7.2) and 0.1% bovine serum albumin (BSA) supplemented with protease inhibitors. A continuous Percoll gradient consisted of 30% (v/v) Percoll, 225 mM mannitol, 25 mM HEPES, 0.5 mM EGTA, and 0.1% BSA (pH 7.2) was used for further purification. Mitochondria were washed and then resuspended gently in MT buffer (400 mM mannitol, 10 mM KH_2PO_4, 50 mM

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Tris–HCl, pH 7.2) and stored on ice for up to 4 h, or stored in MSB buffer (220 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.2, 1 mg/ml BSA) with 10% DMSO in liquid nitrogen.

2.4. Cyt. c release assays

Mitochondria (1 mg protein/ml) were incubated with Bid (10 μg/ml) or Bid in the presence of caspase 8 (2 μg/ml) in 50 μl MT buffer plus 5 mM potassium succinate. After 30 min incubation at 30°C, the samples were centrifuged at 12 000×*g* for 5 min at 4°C. The resulting supernatants were subjected to 15% SDS–PAGE and transferred to a nitrocellulose filter, which was probed with a monoclonal anti-Cyt. *c* antibody followed by alkaline phosphatase detection as described [12].

2.5. Encapsulation of Cyt. c or trypsin within LUVs and detection of their release triggered by Bid

Cyt. *c* or trypsin-containing LUVs were prepared as described in [14]. 2.5 ml diethyl ether and 0.5 ml PIPES buffer (10 mM PIPES, pH 7.0, 50 mM NaCl, 0.2 mM EDTA) containing trypsin (2 mg/ml) or Cyt. *c* (0.5 mg/ml) were added to a dry lipid film of 5 μm phospholipid composed of 80% DOPC and 20% DOPG. After sonication for 20 min at 4°C with a bath sonicator, and evaporation of ether under reduced pressure, the formed vesicles were dialyzed overnight at 4°C in PIPES buffer. Then, the vesicles were fractionated by centrifugation in a Beckman Optima TLX table-top ultracentrifuge (TLA-100.3 rotor) at low speed (15 min, 12 000 rpm, 4°C) to discard the multilamellar vesicles, and high speed centrifugation (30 min, 33 000 rpm, 4°C) to collect the LUVs. The LUVs were washed at least three times to remove the non-enclosed Cyt. *c* or trypsin.

For detection of Cyt. *c* release from LUVs, Cyt. *c*-entrapped vesicles were treated with trypsin in order to remove the bound Cyt. *c* on the outer surface. After this, soybean trypsin inhibitor was added to inhibit the excess trypsin, then Bid was introduced to 100 μl Cyt. *c*-containing LUVs. After incubation for 15 min at 30°C, centrifugation was carried out at 50 000 rpm for 30 min at 4°C, the supernatant was subjected to SDS–PAGE and immunoblotted with Cyt. *c* antibody as described in [12].

For monitoring the trypsin release, Bid was added to 100 μl trypsin-containing LUVs preincubated at 30°C for 15 min. After centrifugation, BAEE as substrate was introduced to the supernatant to measure the degradation rate by the released trypsin at 253 nm by using a spectrophotometer (Shimadzu UV-2101PC) [14].

3. Results

3.1. Comparison of Bid-induced Cyt. c release from rat heart mitochondria with that from liver or brain

Recombinant Bid was expressed and purified as reported [12], and the purity of the protein was above 95% as determined by SDS–PAGE analysis with Coomassie staining (Fig. 1). The full-length Bid can be cleaved by caspase 8 at 30°C for 30 min, and truncated Bid (tBid) was formed (Fig. 1), which can induce Cyt. *c* release from rat liver, heart and brain mitochondria separately. Usually, liver mitochondria are used for Cyt. *c* release experiments, however, it can be seen from Fig. 2A that Cyt. *c* release by Bid from heart mitochondria is more efficient than that from liver or brain mitochondria. Moreover, the results are reproducible and thus it may be used as a better model system for studying the molecular mechanism of Cyt. *c* release in vitro.

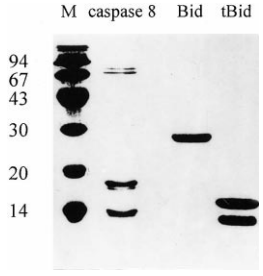

Fig. 1. Bid is cleaved by caspase 8. Aliquots of 5 μg of recombinant Bid were incubated with 0.2 μg caspase 8 for 30 min at 30°C. After incubation, the samples were subjected to 15% SDS–PAGE and the gel was subsequently stained by Coomassie brilliant blue. 'M' was protein marker.

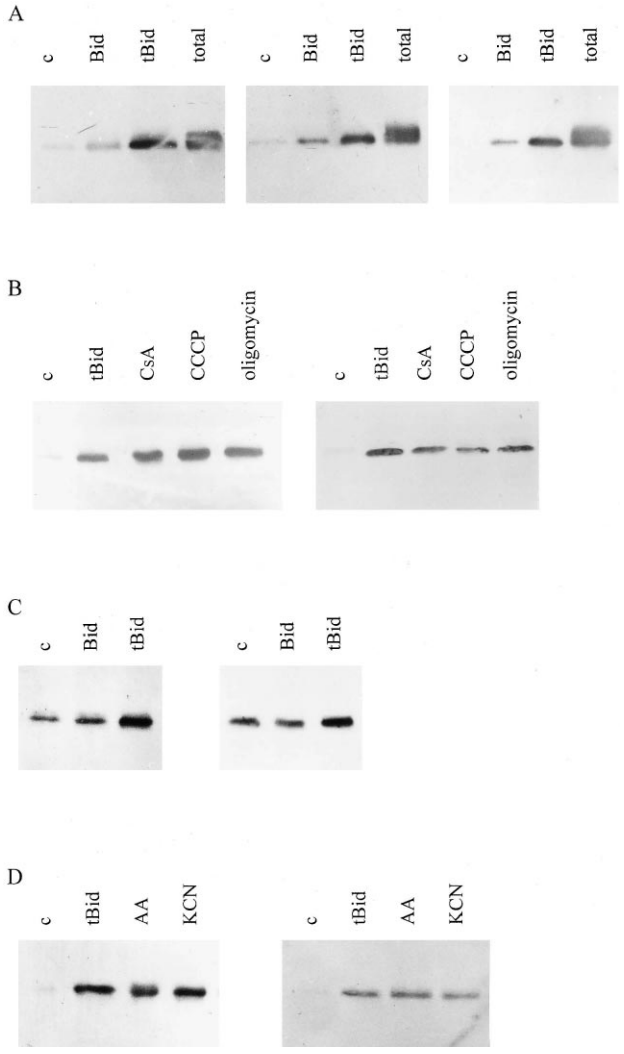

Fig. 2. Characterization of Bid-induced Cyt. *c* release from isolated mitochondria. A: Heart (left), liver (middle) and brain (right) mitochondria were incubated with Bid or Bid plus caspase 8 for 30 min at 30°C. 'Total' represents the whole Cyt. *c* contained in an equivalent aliquot of mitochondria. B: Heart (left) and liver (right) mitochondria preincubated for 5 min with 1 μM CsA, 1 μM CCCP or 1 μM oligomycin were followed by treatment with Bid plus caspase 8. C: Frozen-thawed heart (left) or liver (right) mitochondria were incubated with caspase 8 (c), Bid or Bid plus caspase 8 (tBid) separately. D: 50 nM AA or 1 mM KCN was added to heart (left) and liver (right) mitochondria separately. After 2 min, Bid plus caspase 8 was introduced. After 30 min incubation at 30°C, all of the above mitochondria were then pelleted by centrifugation, and the resulting supernatants were subjected to SDS–PAGE immunoblot analysis. Mitochondria incubated in the presence of caspase 8 were used as control.

3.2. Bid-induced Cyt. c release is independent of PTP opening

The precise molecular mechanism of how Bid induces Cyt. *c* release has not yet been elucidated. It has been reported that another Bcl-2 family member, Bax, can trigger Cyt. *c* release through the PT pore [10]. However, in the case of Bid, Cyt.

c release was not inhibited in the presence of PTP opening poison CsA (Fig. 2A). Moreover, Bid-induced Cyt. *c* release was not sensitive to the F_0F_1-ATPase inhibitor oligomycin (Fig. 2B), while in the case of Bax, the induced passage of Cyt. *c* was inhibited [10]. From Fig. 2B, it can also be seen that CCCP, which can eliminate the mitochondrial membrane potential, exerts no effect on Bid-induced Cyt. *c* release from rat liver or heart mitochondria, indicating that Bid-triggered Cyt. *c* release is independent of $\Delta\Psi$ and mitochondrial energization.

3.3. Bid can still induce Cyt. c release from frozen-thawed mitochondria

To further investigate if the mitochondria energization is related to the Bid-induced Cyt. *c* release, frozen-thawed mitochondria were used. After frozen in liquid nitrogen and followed by quickly thawed, the $\Delta\Psi$ and respiratory control ratio of mitochondria were almost completely dissipated and the PT pore was no more to open as normal, albeit that the intactness of the mitochondrial outer membrane, detected by the measurements of cytochrome oxidase activity, was not obviously changed (data not shown). However, Cyt. *c* can still be released by tBid even from such uncoupled mitochondria (Fig. 2C).

3.4. Bid-induced Cyt. c release is independent of the redox state of Cyt. c

Various stimuli can lead to Cyt. *c* release from mitochondria. Ghafourifar, P. et al. have reported that ceramide can also trigger Cyt. *c* release but only when Cyt. *c* is oxidized [15]. But the results shown in Fig. 2D clearly indicate that there is no obvious difference in the Bid-induced Cyt. *c* release in the presence of AA (Cyt. *c* in oxidized state) or KCN (Cyt. *c* in reduced state).

3.5. Bid can directly induce Cyt. c or trypsin release from liposomes

Schendel, S.L. et al. recently showed that tBid could form channels in synthetic membranes [16]. Shimizu, S. et al. reported that Bax and Bak could induce Cyt. *c* release from the liposomes, incorporated with mitochondria voltage-dependent anion channel (VDAC) [17]. Thus, it is interesting to see whether Bid or tBid would trigger Cyt. *c* release directly from the lipid vesicles. It can be seen from Fig. 3, when Bid was added to Cyt. *c*-entrapped LUVs composed of 20% DOPG and 80% DOPC, Cyt. *c* could be obviously released and the efflux was dependent on the concentration of Bid used.

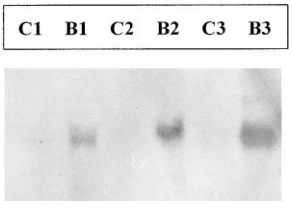

Fig. 3. Bid induced Cyt. *c* release from Cyt. *c*-entrapped LUVs. LUVs (1 mM lipid) were incubated at 30°C in the presence of different amounts of Bid in a 100 μl reaction mixture for 15 min. After centrifugation at 50 000 rpm for 30 min at 4°C, the supernatants were subjected to SDS–PAGE and immunoblot analysis. Lanes B1, B2 and B3 were Bid/lipid (mol/mol) of 1:4000, 1:2000 and 1:1000, respectively, lanes C1, C2 and C3 were the control.

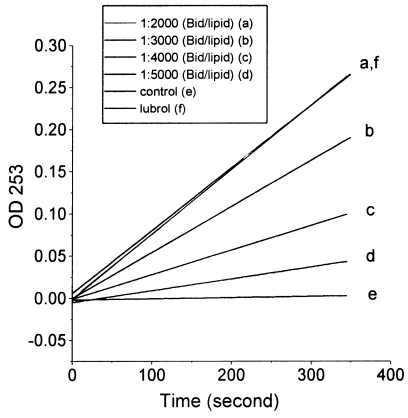

Fig. 4. Bid induces trypsin release from trypsin-enclosed LUVs. LUVs (300 μM lipid) were incubated at 30°C for 15 min in the presence of different amounts of Bid in a 100 μl reaction mixture. After incubation, centrifugation was carried out at 50 000 rpm for 30 min at 4°C, BAEE as substrate was then introduced to the supernatants to measure the degradation rate by the released trypsin at 253 nm. The ratio of the Bid/lipid (mol/mol) is indicated in the frame. The same amount of buffer was used as control.

Additionally, Bid can also trigger the trypsin efflux from unilamellar lipid vesicles. The degree of release was determined through enzymatic degradation of BAEE located in the medium by effused trypsin. When Bid was added to trypsin-enclosed liposome composed of 20% DOPG and 80% DOPC, BAEE was obviously hydrolyzed. As shown in Fig. 4, the amount of released trypsin has a linear relationship with the concentration of Bid added. When Bid/lipid is about 1:2000 (mol/mol), the effect is similar to that of lubrol. The tBid has more potency in triggering the Cyt. *c* release than Bid, but the difference is much less than that in mitochondria (data not shown).

4. Discussion

Cyt. *c* release has been documented for apoptosis induced by chemotherapeutic drugs, UV irradiation, growth factor withdrawal, and ligation of FAS and tumor necrosis factor receptors, etc. [18]. It is still obscure how Cyt. *c* translocates from the mitochondria into the cytosol during apoptosis. Different stimuli may use distinct mechanisms to induce Cyt. *c* release, and tissue diversity may also exist as indicated in the present paper, i.e. tBid-induces Cyt. *c* release from rat heart mitochondria is more efficient and reproducible than that of liver or brain.

Bax is a pro-apoptotic member of the Bcl-2 protein family that resides in the outer mitochondrial membrane [10]. It was reported that Bax can induce dissipation of mitochondrial $\Delta\Psi$, swelling and Cyt. *c* release. All of these changes were prevented by CsA, which inhibits the opening of PT pores [10]. In addition, Bax-induced mitochondrial changes were inhibited by oligomycin, suggesting a possible regulatory effect of F_0F_1-ATPase on Cyt. *c* release triggered by Bax.

Bid, another pro-apoptotic member of the Bcl-2 family of proteins, has also been shown to induce Cyt. *c* release from mitochondria [12,19]. It is a much more potent Cyt. *c* releasing factor than Bax, particularly its cleaved form by caspase 8, tBid. Unlike Bax, obtained results shown in the present paper demonstrate that Bid-mediated Cyt. *c* release was not pre-

vented by PT pore opening poison CsA, oxidative phosphorylation uncoupler CCCP or F_0F_1-ATPase inhibitor oligomycin. Such findings imply that Bid initiates the release of Cyt. *c* and is independent of permeability transition and energization of mitochondria. This is further supported by Bid-induced Cyt. *c* release even from the frozen-thawed uncoupled mitochondria.

Ghafourifar, P. et al. have reported that ceramide can also trigger Cyt. *c* release from mitochondria, but only in its oxidized state [15]. Bid, on the other hand, is able to induce Cyt. *c* release from mitochondria both in its oxidized and reduced forms.

Taken together, these findings suggest that there is an alternative mechanism responsible for the triggering Cyt. *c* release from mitochondria.

The mechanisms of Cyt. *c* release in response to apoptotic stimuli and its regulation by the Bcl-2 family of proteins still remain elusive. The three-dimensional structure of Bcl-x_L suggests that many Bcl-2 family proteins share similarity with the pore-forming domains of certain bacterial toxins, including diphtheria toxin and colicins [20]. So, it has been hypothesized that some Bcl-2 family proteins could form ion channels, which was supported by the ion transport activity of the reconstituted Bax and Bid in synthetic lipid membranes [16,17,21].

Schendel, S.L. et al. showed that tBid formed channels in planar bilayer at neutral pH and in liposomes at acidic pH [16]. Shimizu, S. et al. reported that Bax and Bak accelerate the opening of VDAC, which is incorporated into liposomes. So, it was postulated that in consequence of protein–protein interaction, Cyt. *c* might be released from liposomes through incorporated VDAC [17]. Luo, X et al. proposed that Bid may interact with a yet unidentified target on the outer membrane of mitochondria through protein–protein (or protein–lipid) interaction, which would result in triggering Cyt. *c* release [12]. We presented here that Bid or tBid can directly trigger the efflux of encapsulated Cyt. *c* or even trypsin within liposomes at neutral pH (pH 7.0) without involvement of other protein factors. Inasmuch as Bid contains eight helices [22,23], which is generally more indicative of smaller ion channels not large enough to transport proteins, it is tentatively suggested that Bid may induce Cyt. *c* release from unilamellar vesicles in consequence of the perturbation of phospholipid bilayers due to Bid–lipid interaction, which may also play an important role in the triggering of Cyt. *c* release from mitochondria. This postulation deserves further investigation and related studies are still in progress.

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