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Differential Efflux of Mitochondrial Endonuclease G by hNoxa and tBid

Young Woo Seo[1,2], Sun-Young Park[1], Cheol-Won Yun[3] and Tae-Hyoung Kim[1,*]

[1]Department of Biochemistry and Molecular Biology, Chosun University School of Medicine, 375 Seosuk-Dong, Dong-Gu, Gwangju, 501-759, Korea

[2]Korea Basic Science Institute Gwang-Ju branch, Chonnam National University, 300 Yongbong-Dong, Buk-Gu, Gwang-Ju, 500-757, Korea

[3]School of Life Science and Biotechnology, Korea University, Seoul 136-701, Korea

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The Bcl-2 family of proteins regulates mitochondrial functions during cell death by modulating the efflux of death-promoting proteins such as cytochrome *c* and endonuclease G. Upon the binding of death ligands to their receptors, caspase-8 cleaves Bid, a BH3-only protein, into tBid that causes the mitochondrial damages resulting in the release of cytochrome *c* and endonuclease G. Also, another BH3-only protein, hNoxa, has been shown to induce the efflux of cytochrome *c* from the mitochondria. Whether the efflux proteins from the mitochondria in response to tBid or hNoxa are the same or different, however, has not been addressed. We have demonstrated that endonuclease G activities are not detectable among the proteins released from isolated mitochondria by hNoxa but are detectable in that by tBid. These results suggest that the efflux of proteins from the mitochondria are differentially modulated by tBid and hNoxa.

Keywords: Bid, Cytochrome *c* release, Endonuclease G, hNoxa

Introduction

The Bcl-2 family of proteins has been documented as regulators of cell death by modulating mitochondrial functions. These proteins can be categorized into two groups, anti-death and pro-death, depending on the ability of the proteins to inhibit or to promote the death of the cell. Both Bid and hNoxa belong to a BH3-only subgroup of pro-death Bcl-2 family of proteins. The C-terminal region of Bid (tBid) is generated by various proteinases including caspase-8, caspase-3, granzyme B, lysosomal enzymes, and calpains during cell death, and locates to the mitochondrial outer membrane leading to the efflux of the death-promoting proteins such as cytochrome c, Smac/DIABLO, and endonuclease G (Yin, 2000; Wang, 2001).

hNoxa was originally identified as a target gene transactivated by the tumor suppressor gene p53 (Oda *et al.*, 2000). Several reports have demonstrated a close correlation between the p53 level and hNoxa induction in response to genotoxic agents, thereby suggesting that hNoxa is a crucial player in p53-induced cell death. In addition, recent reports show that hNoxa expression can be induced by hypoxia or the proteasome inhibitor Bortezomib (PS-341, Velcade®) in a p53-independent manner (Kim *et al.*, 2004a; Qin *et al.*, 2005; Fernandez *et al.*, 2005; Perez-Galan *et al.*, 2006). Various deletion mutants of hNoxa demonstrate that hNoxa contains the BH3 domain and mitochondrial targeting domain (MTD), which are responsible for the induction of the cell death and for the translocation of hNoxa to mitochondria, respectively (Seo *et al.*, 2003; Sun and Leaman, 2005). Although the release of cytochrome *c* from the mitochondrion is a critical event in hNoxa-mediated cell death, the molecular mechanism whereby hNoxa effects the permeability of the mitochondrial outer membrane remains to be characterized.

We have examined the efflux proteins from the isolated mitochondria in response to tBid and hNoxa. Our results indicate that tBid can release the cytochrome *c* and endonuclease G, whereas hNoxa can release cytochrome *c*, but not endonuclease G.

Abbreviations: NAO (10-*N*-nonyl acridine orange); CsA (cyclosporine A); mitochondrial permeability transition (mPT); mitochondrial targeting domain (MTD).

*To whom correspondence should be addressed.
Tel: 82-62-230-6294; Fax: 82-62-226-4165
E-mail: thkim65@mail.chosun.ac.kr

Materials and Methods

Purification of recombinant proteins and assays of cytochrome *c* release and endonuclease G activity. Purification of recombinant hNoxa, wild type tBid, and mutant tBid (G94E) proteins was described elsewhere (Kim *et al.*, 2000; Seo *et al.*, 2003) and the isolation of mitochondria from mouse liver has been described (Kim *et al.*, 2000; Kim *et al.*, 2004b). Recombinant hNoxa or tBid protein was added to isolated mitochondria in buffer B (250 mM sucrose, 10 mM HEPES-NaOH, pH 7.5, 2 mM KH_2PO_4, 5 mM sodium succinate, and 25 μM EGTA). The mitochondria were incubated at 30°C for 1 h, and then centrifuged at 10,000 × *g* for 10 min. For the assay of the cytochrome *c* release, supernatants were collected and subjected to Western blot analysis using anti-cytochrome *c* antibody. To detect the endonuclease G activity, naked plasmid DNA (2 μg) was added to the supernatants in a buffer containing 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.9. Digested plasmid DNA was detected using 1% agarose gel electrophoresis.

Mitochondrial swelling assay. Mitochondrial swelling was measured as previously described (Kim *et al.*, 2000). In brief, the mitochondria (1 mg/mL; 800 mg in total) suspended in Buffer B were treated with reagents as indicated in the figure legends. Light absorbance at 540 nm was measured at the designated time points at 30°C using a spectrophotometer (Amersham Pharmacia Biotech, Ultrospec Plus). A decrease in light absorbance is an indication of mitochondrial swelling (Petronilli *et al.*, 1993).

Results and Discussion

hNoxa has been reported to be localized to mitochondria and to release cytochrome *c* from mitochondria (Seo *et al.*, 2003). The cytochrome *c* release induced by hNoxa has been shown to be inhibited by cyclosporine A (CsA) or Mg^{2+}, inhibitors of the mitochondrial permeability transition the (mPT) pore (Seo *et al.*, 2003). hNoxa was proposed to activate the opening of mPT pore and release cytochrome *c* through the mPT opening. Thus, we investigated the possibility that hNoxa may open mPT pore and cause large amplitude swelling of the mitochondria that can be measured by a reduced O.D. value at 540 nm. The calcium ion, a well-defined mPT pore activator, also induced large amplitude mitochondrial swelling, which was inhibited by CsA (Fig. 1). In this condition, however, neither hNoxa nor tBid evidenced any change of O.D. at 540 nm, which indicates that hNoxa does not induce the large amplitude swelling of the mitochondria.

Cardiolipin, a lipid exclusively present in the mitochondria, has been shown to provide a binding site of tBid and to play a key role in the release of cytochrome *c* induced by tBid (Lutter *et al.*, 2000; Lutter *et al.*, 2001; Kim *et al.*, 2004b). The targeting of tBid to the mitochondria was inhibited by NAO, which specifically binds to cardiolipin (Kim *et al.*, 2004b). To investigate whether hNoxa localizes to the same site, cardiolipin, as tBid, the levels of cytochrome *c* release induced

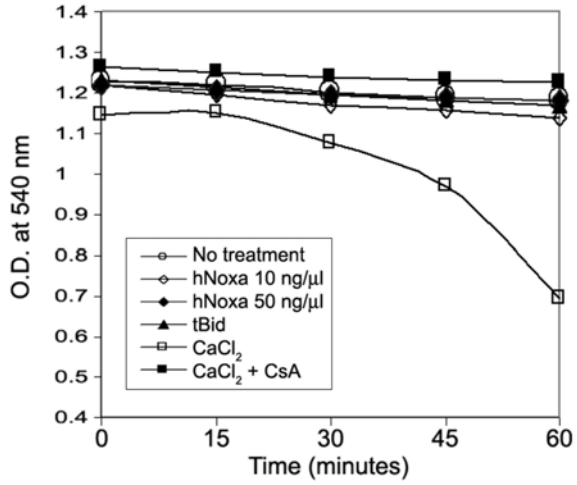

Fig. 1. hNoxa does not induce large amplitude mitochondrial swelling. The isolated mitochondria were suspended in Buffer B at 1 mg/mL (800 mg in total) to which 0 (○), 10 ng/μL (◇), 50 ng/μL (◆) of hNoxa, 0.1 ng/μL of tBid (▲), 50 μM of $CaCl_2$ (□), or 50 μM of $CaCl_2$ plus 50 μM of CsA (■) were added. Light absorbance at 540 nm was measured at the indicated time points at 30°C using a spectrophotometer (Amersham Pharmacia Biotech Ultraspec Plus). Data are representative of five individual experiments.

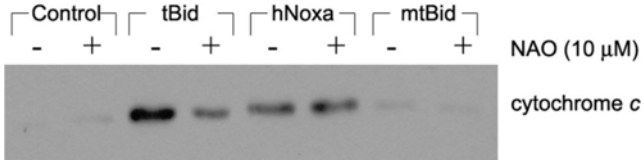

Fig. 2. NAO does not affect hNoxa-induced cytochrome *c* release. The isolated mitochondria were pretreated with NAO (10 μM) for 15 min, and then treated with recombinant wild type tBid (0.1 μg/mL), mutant tBid (G94E) (0.1 μg/mL) or hNoxa (50 μg/mL) at 30°C for 1 h. After centrifugation, cytochrome *c* in the supernatants was analyzed by Western blot analyses with anti-cytochrome *c* antibody.

by tBid or hNoxa were examined in the presence of NAO. As shown in Fig. 2, the tBid-induced cytochrome *c* release was significantly reduced by NAO in agreement with a previous report (Kim *et al.*, 2004b); however, NAO did not interfere in the hNoxa-induced cytochrome *c* release, indicating that cardiolipin is not involved in hNoxa-induced cytochrome *c* release. This result strongly suggests that the mitochondrial damage induced by hNoxa differs from that induced by tBid. This suggestion is supported by the fact that hNoxa does not induce Bak-oligomerization for cytochrome *c* release, whereas Bak-oligomerization is a necessary event for tBid-induced cytochrome *c* release (Wei *et al.*, 2000; Wei *et al.*, 2001).

We assume that the mitochondrial damages caused by hNoxa or tBid may be mediated through different mechanisms. Previous reports showed that tBid can release endonuclease G

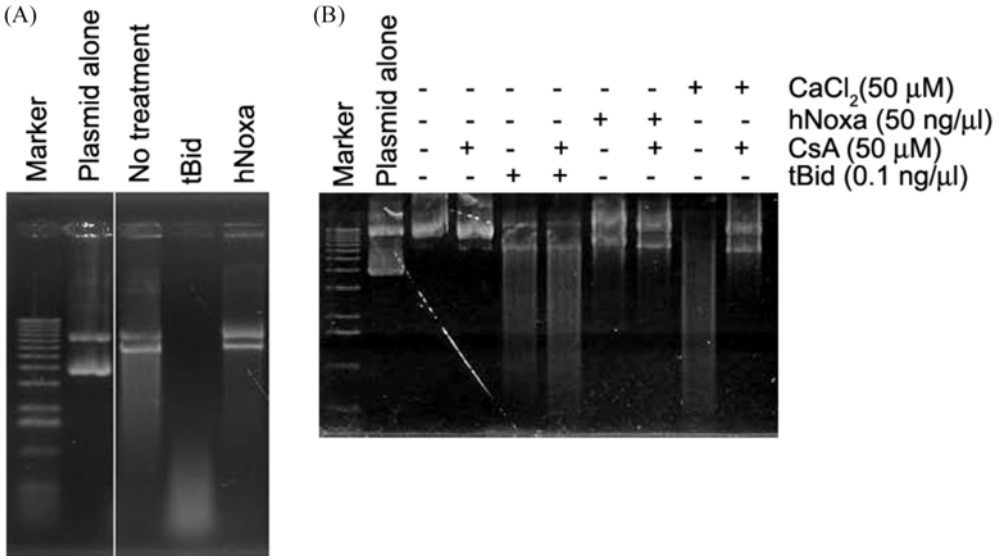

Fig. 3. Endonuclease G activities in the supernatants released by tBid and hNoxa. The isolated mitochondria were treated with tBid (0.1 μg/mL) or hNoxa (50 μg/mL) for 1 h, and then the supernatants were collected after centrifugation. Naked plasmid DNA (2 μg) was added to the indicated supernatants and incubated for 2 h, and then the reactions were subjected to 1% agarose gel electrophoresis.

from the mitochondria (Li *et al.*, 2001). To examine whether endonuclease G can also be released by hNoxa, the isolated mitochondria were incubated with tBid or hNoxa, and then the supernatants containing the released proteins were harvested and incubated with naked plasmid DNA. The endonuclease G activities were determined by the degradation of naked plasmid DNA. As seen in Fig. 3A, the supernatants harvested from the mitochondria treated with tBid contained DNA degrading activities. Interestingly, hNoxa-treated supernatants did not contain the endonuclease activity. Because CsA has been reported to inhibit the cytochrome *c* release induced by hNoxa or calcium, but not by tBid, we investigated whether the released endonuclease activities in response to tBid or calcium can be affected by CsA. The endonuclease activities released by tBid were not affected by CsA, whereas the calcium-induced endonuclease activities were inhibited by CsA (Fig. 3B).

Several Bcl-2 family proteins are reported to induce cytochrome *c* release *in vivo* and *in vitro*, assuming that these proteins might cause the efflux of the same pro-apoptotic mitochondrial proteins, such as cytochrome *c* and endonuclease G. Comparing the released proteins from mitochondria *in vitro* induced by tBid and hNoxa as a model system, we demonstrated that this assumption might not be supported. Although tBid can release both cytochrome *c* and endonuclease G from the mitochondria, hNoxa can release cytochrome *c* but not endonuclease G. These results support the hypothesis that there are two different pathways activated by tBid or hNoxa that selectively release the mitochondrial proteins to the intracellular space. That CsA can inhibit hNoxa-induced cytochrome *c* release but not tBid-induced cytochrome *c* release (Kim *et al.*, 2000); (Seo *et al.*, 2003), and that Bak-oligmerization can be formed by tBid but not by hNoxa, could provide an additional aspect to the differentially regulated pathways for tBid- and hNoxa-induced mitochondrial damages.

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