

Inhibition of Bax protects neuronal cells from oligomeric A β neurotoxicity

W Kudo¹, H-P Lee¹, MA Smith¹, X Zhu^{1,2}, S Matsuyama^{*,1,3} and H-g Lee^{*,1,2}

Although oligomeric β -amyloid (A β) has been suggested to have an important role in Alzheimer disease (AD), the mechanism(s) of how A β induces neuronal cell death has not been fully identified. The balance of pro- and anti-apoptotic Bcl-2 family proteins (e.g., Bcl-2 and Bcl-w versus Bad, Bim and Bax) has been known to have a role in neuronal cell death and, importantly, expression levels of these proteins are reportedly altered in the vulnerable neurons in AD. However, the roles of apoptotic proteins in oligomeric A β -induced cell death remain unclear *in vivo* or in more physiologically relevant models. In addition, no study to date has examined whether Bax is required for the toxicity of oligomeric A β . Here, we found that treatment with oligomeric A β increased Bim levels but decreased Bcl-2 levels, leading to the activation of Bax and neuronal cell death in hippocampal slice culture and *in vivo*. Furthermore, the inhibition of Bax activity either by Bax-inhibiting peptide or *bax* gene knockout significantly prevented oligomeric A β -induced neuronal cell death. These findings are first to demonstrate that Bax has an essential role in oligomeric A β -induced neuronal cell death, and that the targeting of Bax may be a therapeutic approach for AD.

Cell Death and Disease (2012) 3, e309; doi:10.1038/cddis.2012.43; published online 17 May 2012

Subject Category: Neuroscience

Alzheimer disease (AD) is the most common neurodegenerative disorder, and β -amyloid (A β) has been suggested to have a critical role in the pathogenesis of AD. It has been shown that oligomeric A β is a main A β species inducing neurodegeneration in AD, yet the molecular mechanism(s) of its neurotoxicity remains elusive.¹ At present, it is reported that oligomeric A β induces apoptotic neuronal death in the rat and mouse neurons *in vitro* and *in vivo*.^{2,3}

Previous studies found that expression levels of Bcl-2 family proteins, such as Bax, Bak, Bad, Bcl-2, Bim, Bcl-w and Bcl-x are altered in the vulnerable neurons in AD.⁴ Bcl-2 family is structurally defined by the presence of up to four conserved 'BCL-2 homology' (BH) domains. The family proteins are the key regulators of evolutionally conserved pathway of apoptosis.^{5,6} Bax and Bak belong to the multi-BH domains pro-apoptotic subfamily, which promotes apoptosis by translocating into the mitochondrial membrane and facilitating cytochrome c release whereas Bcl-2 and Bcl-XL belong to the prosurvival subfamily, which prevents apoptotic death in multiple cell types including neuron.⁷ It is hypothesized that the BH3-only proteins, such as Bim, Bid, Puma, Noxa and Bad induce the activation of Bax and Bak, either directly or indirectly by inactivating the prosurvival Bcl-2 proteins. In the presence of apoptotic stimuli, Bax is known to change its conformation. Specifically, as an early step of Bax activation, the N-terminus exposure is considered a prerequisite for membrane insertion of Bax at mitochondria and multimerization of Bax.^{8,9} Ku70, a DNA repair factor, can prevent

the conformational change by binding with Bax in the cytosol, which leads to the inhibition of Bax-mediated cell death.¹⁰ Previously, a cell permeable Bax-inhibiting peptide (BIP), designed to induce the Bax-binding domain of Ku70, was found to rescue cells from Bax-mediated cell death.^{10–12}

Notably, overexpression of anti-apoptotic proteins, Bcl-w, or genetic ablation of a proapoptotic effector, Bim, significantly protected neurons against fibrillar A β -induced apoptosis in neuroblastoma cell lines and primary neuron culture.^{13,14} Giovanni *et al.*¹⁵ also reported that fibrillar A β -induced cell death is dependent on Bax in primary neuron culture. However, these results have never been confirmed *in vivo* or in a more physiologically relevant model, and all the previous studies examined the toxicity of fibrillar A β , not oligomeric A β . Therefore, to advance our understanding of the involvement of Bcl-2 protein family as the major mechanism of oligomeric A β -induced neuronal cell death, in this study, we examined the effect of oligomeric A β on the regulation of Bcl-2/Bim/Bax and its functional importance in neuronal cell death in the organotypic hippocampal culture and mouse model for A β toxicity.

Results

Oligomeric A β induces Bim upregulation and Bcl-2 downregulation in hippocampal slice culture. To characterize oligomeric A β in this study, we synthesized A β

¹Department of Pathology, Case Western Reserve University School of Medicine, Cleveland, OH, USA; ²Department of Neurology, Case Western Reserve University School of Medicine, Cleveland, OH, USA and ³Departments of Medicine and Pharmacology, Case Western Reserve University School of Medicine, Cleveland, OH, USA
*Corresponding authors: H-g Lee or S Matsuyama, Department of Pathology, Case Western Reserve University, 2103 Cornell Road, Cleveland, OH 44106, USA. Tel: 216 368 6887; Fax: 216 368 8964; E-mail: hyoung-gon.lee@case.edu (H-gL) or shigemi.matsuyama@case.edu (SM)

Keywords: Alzheimer disease; neuronal cell death; Bax; Bim; Bcl-2; β -amyloid

Abbreviations: A β , β -amyloid; AD, Alzheimer disease; BH, BCL-2 homology; BIP, Bax-inhibiting peptide; PI, propidium iodide; TUNEL, terminal dUTP nick-end labeling; FITC, fluorescein isothiocyanate; JNK, c-Jun N-terminal kinase; cdk4, cyclin-dependent kinase 4

Received 6.1.12; revised 7.3.12; accepted 26.3.12; Edited by D Bano

peptide, and then denatured the peptide and allowed oligomers to form, as described for A β -derived diffusible ligands.¹ Consistent with previous findings for oligomeric A β ,^{1,16} our oligomeric A β preparations contain spherical particles visible by negative staining with transmission electron microscopy, and western blots show that the preparations contain various size of oligomeric proteins (Figure 1).

Time-dependent changes of the levels of Bax, Bcl-2 and Bim were examined in oligomeric A β -treated brain slices. Oligomeric A β increased the expression of Bim but decreased the level of Bcl-2 (Figure 2a). However, the level of Bax was not affected by oligomeric A β (Figure 2a). As the N-terminus exposure is an early step of Bax activation that occurs in the cytosol, we analyzed this conformational change of Bax with a monoclonal antibody (6A7) recognizing the epitope in the N-terminus of Bax.⁹ Although the level of Bax expression was not affected by oligomeric A β , the number of 6A7-positive cells were significantly increased in oligomeric A β treated slice cultures (Figure 2b), suggesting that oligomeric A β induces Bax activation.

Ablation of Bax reduces oligomeric A β -induced neuronal cell death in hippocampal slice culture.

To further determine the functional role of Bax in oligomeric A β -induced neuronal cell death, hippocampal slice culture prepared from wild-type and *bax*^{-/-} mice (Figures 3a and b) were used. Slices were treated with oligomeric A β in the presence of propidium iodide (PI), which penetrates cell membranes of dead or dying cells, and is widely used for evaluation of cell death. Although A β ₄₂₋₁ peptide, a reverse sequence of A β ₁₋₄₂, had no effect on PI uptake, the number of PI-positive cells was significantly increased in oligomeric A β -treated slices after 48 h of the treatment, indicating that

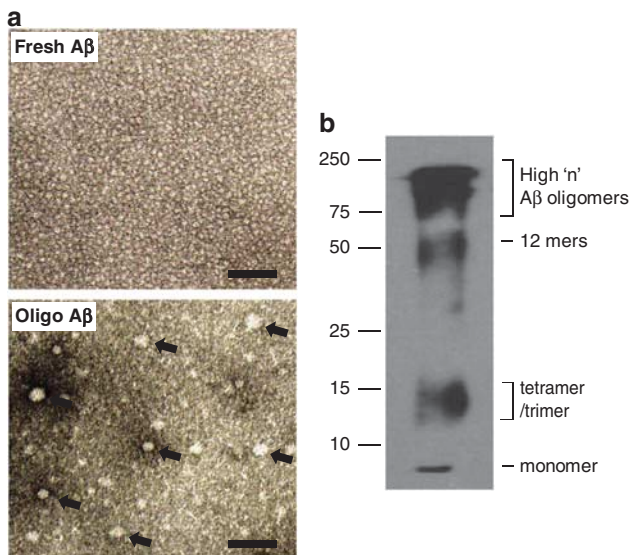


Figure 1 Physicochemical and morphological features of the synthetic A β ₁₋₄₂. (a) Electron micrograph shows the typical pattern of oligomer formation of A β . The arrows indicate oligomers in the lower panel. Scale bar, 100 nm. (b) Synthetic A β ₁₋₄₂ was subjected to SDS-polyacrylamide gel and detected by western blotting with 6E10 antibody. Molecular weight markers in kDa are at left

oligomeric A β induces neuronal cell death in the hippocampal slice culture. However, the neuronal cell death induced by oligomeric A β was dramatically reduced in the slice culture from *bax*^{-/-} mice (Figure 3c). Nissl staining analysis further confirmed neuronal cell death by oligomeric A β in wild-type (WT) mouse slice culture but not in *bax*^{-/-} mouse slice culture (Figure 3d). These results indicate that Bax is a critical mediator for the neurotoxicity induced by oligomeric A β .

Intrahippocampal injection of oligomeric A β increases Bim expression and active forms of Bax.

To further examine the involvement of pro-apoptotic proteins in oligomeric A β -induced cell death, we determined the change of the levels of Bim and the active form of Bax *in vivo*. We injected oligomeric A β into the hippocampus and assessed its neurotoxicity. A β ₄₂₋₁ was used as a control. Oligomeric A β injection into the WT mouse hippocampus induced neuronal cell death in 20 days after injection (Figure 4a). The number of terminal dUTP nick-end labeling (TUNEL)-positive neurons was significantly increased by A β injection (Figure 4b). To see the involvement of Bim and Bax, we measured the level of Bim and active form of Bax, and found that the level of Bim was dramatically increased as early as 10 days after oligomeric A β injection (Figure 4c). The number of neurons containing the active form of Bax recognized by 6A7 antibody was also dramatically increased by A β injection (Figure 4d). These data further support that both Bim and Bax have roles in oligomeric A β -induced neuronal cell death.

BIP suppresses neuronal cell death and Bax activation induced by oligomeric A β in hippocampal slice culture.

In this study, in addition to utilizing Bax knockout (KO) mice, we utilized BIP to examine the role of Bax in oligomeric A β -induced neurotoxicity. BIP used in this study consists of five amino acids, VPTLK, a sequence that is known to inhibit Bax activation.^{10,11,17} A mutated (scrambled) peptide, KLVPT, which does not bind Bax but has the same cell permeability, was used as a negative control. Both peptides were tagged with fluorescein isothiocyanate (FITC) so that intracellular delivery can be tracked by FITC signal. We first treated hippocampal slices with each peptide and analyzed green fluorescence to confirm their cell permeability, and confirmed that BIP and the control peptide equally penetrated neuronal cells after 24-h treatment (Figure 5a). To determine whether BIP is able to suppress oligomeric A β -induced neuronal cell death in slice culture, either BIP or control peptide were co-applied with oligomeric A β . After 24-h treatment of oligomeric A β and the peptide, the treatment of BIP significantly prevented PI uptake, whereas the control peptide did not affect the level of PI uptake (Figure 5b), suggesting BIP specifically blocks neuronal cell death induced by oligomeric A β . Consistent with the PI uptake results, both Nissl staining and caspase-3 immunoblot analyses also showed that BIP significantly suppressed oligomeric A β -induced neuronal loss and caspase-3 activation, but control peptide failed to prevent neuronal cell death (Figures 5c and d). We also found that the treatment of BIP suppresses oligomeric A β -induced conformational change of Bax (Figure 6). These results further support the hypothesis

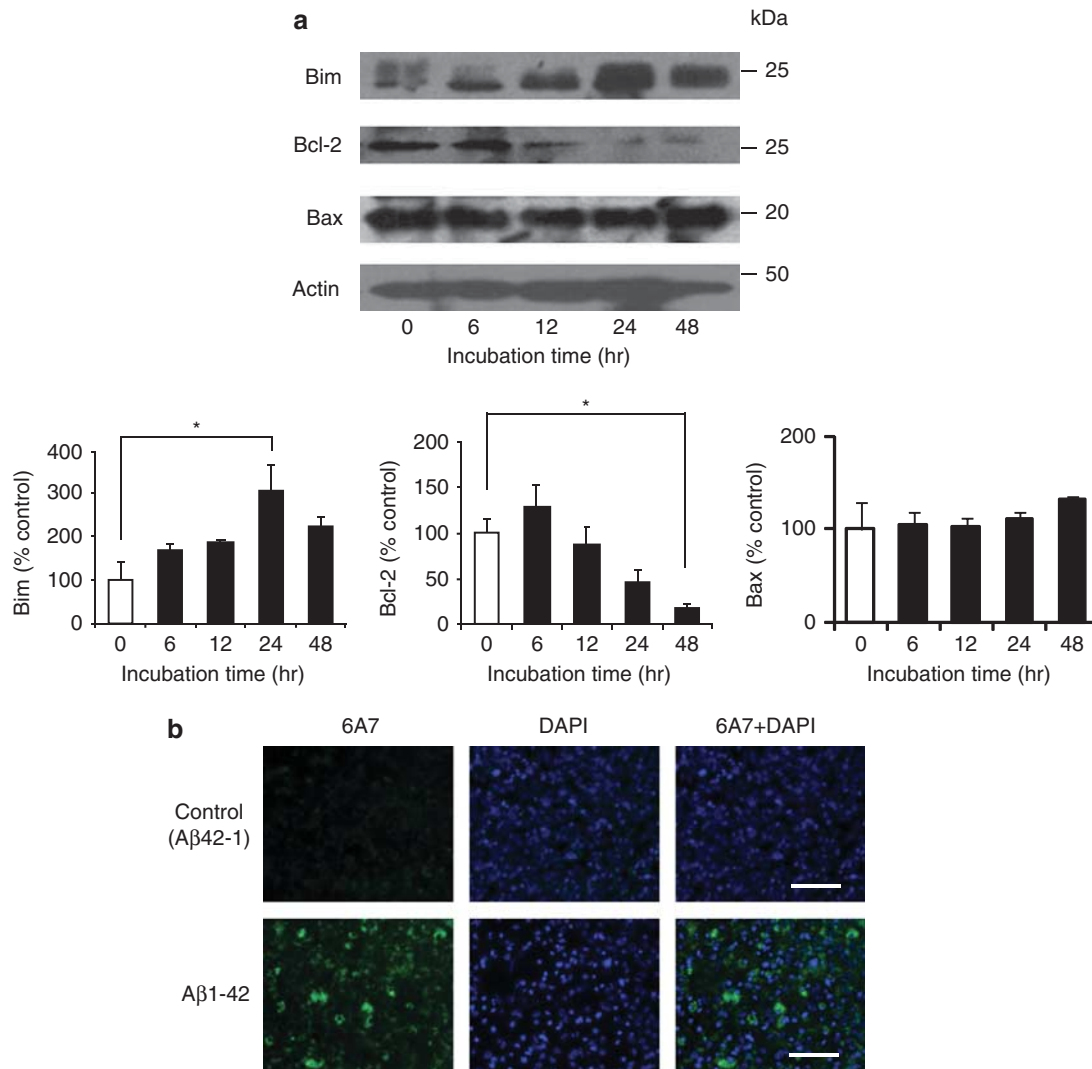


Figure 2 Oligomeric A β differentially regulates Bim, Bcl-2 and Bax in the hippocampal slice culture. **(a)** Representative western blots showed oligomeric A β induced upregulation of Bim and downregulation of Bcl-2 in a time-dependent manner. Bax levels were not changed by oligomeric A β treatment. Actin was used as internal loading control. The values of each band were normalized to that of actin and shown as a relative value of each group compared with the non-treatment slices. The indicated comparisons are significant at * $P < 0.05$, $n = 4$. **(b)** Immunocytochemistry analysis demonstrated the increased number of positive cells stained with 6A7 antibody, which specifically detect the active form of Bax, 24 h after oligomeric A β treatment in hippocampal slice cultures. Conversely, control peptide (A β_{42-1}) did not induce the active form of Bax (green: 6A7, blue: DAPI). Scale bar, 100 μ m

that the activation of Bax by oligomeric A β is an essential mechanism of oligomeric A β -induced neuronal cell death.

Discussion

In this study, we demonstrate that oligomeric A β altered the expression levels of Bcl-2, Bim and Bax, and that the genetic or pharmacological ablation of Bax activity suppresses oligomeric A β -mediated neurotoxicity in both *ex vivo* and *in vivo*. These results clearly indicate that Bax has an essential role in the induction of neuronal cell death caused by oligomeric A β .

In healthy cells, Bax is located in the cytosol or loosely associated to mitochondria and endoplasmic reticulum.¹⁸ Bax translocation to the mitochondria, which occurs in cells with apoptotic stresses, is thought to lead to mitochondrial

dysfunction and release of cytochrome c and subsequent apoptosis.¹⁹ Before its translocation to mitochondria, Bax changes its conformation that exposes the N-terminal residues.²⁰ This conformational change is believed to be necessary for membrane insertion of Bax at mitochondria and multimerization of Bax.²¹ The present study demonstrates that oligomeric A β induced the N-terminal exposure of Bax in neurons and that the inhibition of this event by BIP rescues neurons from oligomeric A β 's neurotoxicity, suggesting the activation of Bax by its conformational change is a key element of oligomeric A β -induced neurotoxicity.

Although the molecular mechanism(s) of Bax activation has not been clearly defined, multiple pro-apoptotic proteins (e.g., Bim) and anti-apoptotic proteins (e.g., Bcl-2) are known to regulate the activation of Bax thorough heterodimerization.²² In fact, consistent with the previous findings, our current data

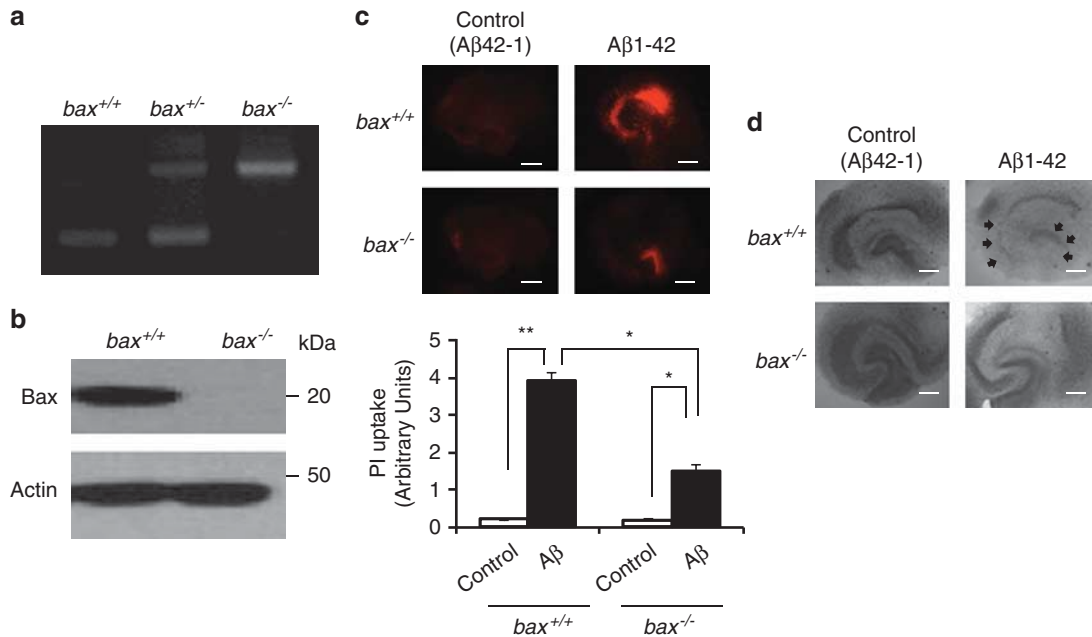


Figure 3 *bax*^{-/-} neurons are resistant to oligomeric A β neurotoxicity. (a) DNA was extracted from *bax*^{+/+} and *bax*^{-/-} mice, and each genotype was identified by PCR with the primer sets specifically detecting each genotype as described in the previous study. *bax*^{+/+} yields a 304-bp PCR product and *bax*^{-/-} yields a 507-bp PCR product. (b) Total protein (20 μ g) from whole brain was analyzed by immunoblot with anti-Bax antibody. Immunoblot analysis showed Bax in *bax*^{+/+} mouse samples at the expected molecular weight of 21 kDa, and no expression of Bax in *bax*^{-/-} samples. (c) Hippocampal slice culture were treated with oligomeric A β (500 nM) in the presence of PI (red) for 48 h. Representative data showed oligomeric A β -induced PI uptake was significantly reduced in the slice cultures from *bax*^{-/-} mice compared with *bax*^{+/+} mice. The PI uptake was quantitatively analyzed ($n=5$). Control A β ₄₂₋₁ peptide had no effect on PI uptake. Scale bar, 500 μ m. * $P<0.05$, ** $P<0.01$. (d) Neuronal cell loss by oligomeric A β (arrows) was significantly reduced in the hippocampal slice cultures from *bax*^{-/-} mice. Scale bar, 500 μ m

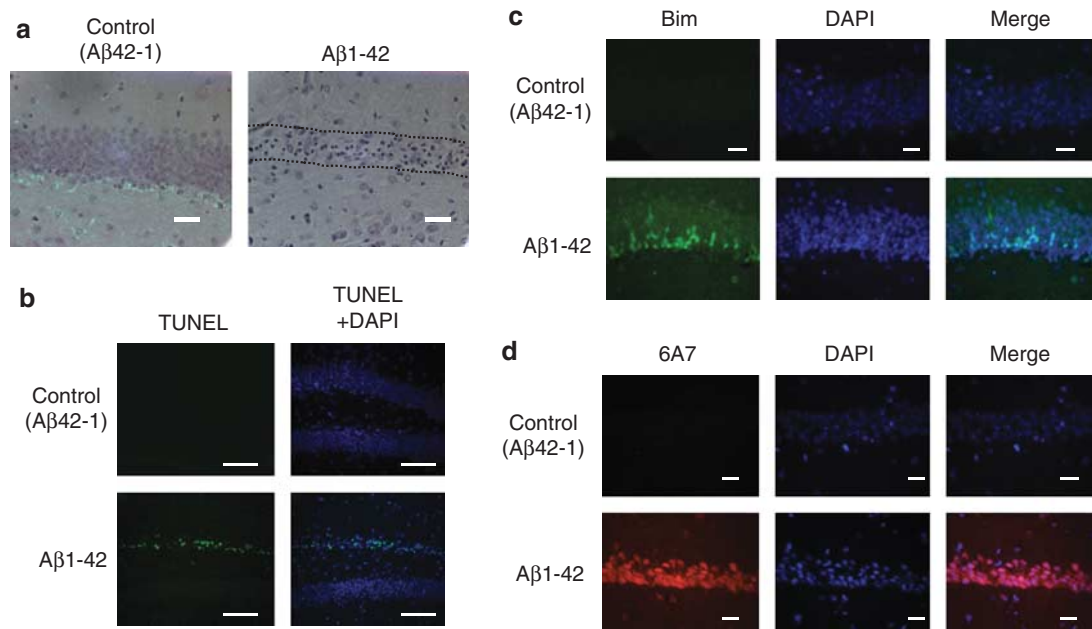


Figure 4 Intrahippocampal injection of oligomeric A β upregulates Bim and activates Bax. C57BL/6J mice were killed and the levels of Bim and active Bax were analyzed at 10 or 20 days after oligomeric A β injection. (a) At 20 days after oligomeric A β injection, neuronal cell loss in hippocampus was evident in A β injected mice in H&E staining, but not in control peptide-injected mice. The region of neuronal cell loss is indicated by dotted lines. Scale bar, 100 μ m. (b) The number of TUNEL-positive cells in hippocampus was dramatically increased after oligomeric A β injection whereas virtually no TUNEL-positive cell (green) was detected in control peptide-injected hippocampus tissues. Blue: DAPI. Scale bar, 200 μ m. (c) Immunoreactivity for Bim (green) was increased in hippocampus at 10 days after the oligomeric A β injection. Scale bar, 100 μ m. (d) The number of active Bax-positive cells detected by 6A7 antibody (red) was dramatically increased at 10 days after the oligomeric A β injection. Scale bar, 100 μ m compared with control peptide-injected mice

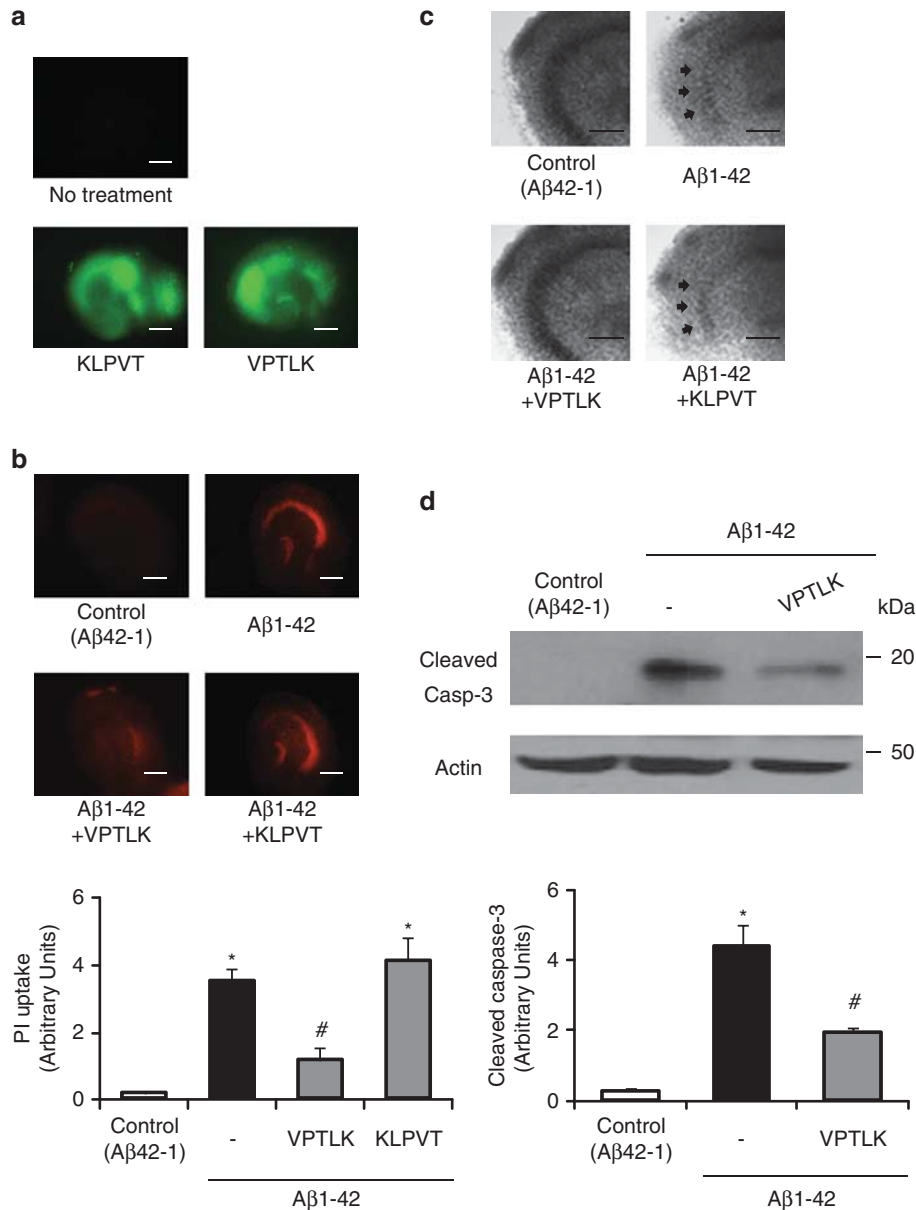


Figure 5 Bax-inhibiting peptide (BIP) suppresses neuronal cell death induced by oligomeric A β . Either BIP (VPTLK) or control peptide (KLPVT) was added to hippocampal slice cultures at the same time with oligomeric A β to examine its neuroprotective effect. (a) Cell permeability of both peptides was confirmed by green fluorescence in hippocampal slice cultures. Strong green fluorescence of both BIP- and negative-control peptide was found at 24 h after the peptide treatment, indicating both peptides are penetrated to neurons. (b) The intensity of PI in slices treated with oligomeric A β for 48 h was quantified as a marker of cell death. BIP significantly prevented oligomeric A β -induced PI uptake ($n=5$). Scale bar, 500 μ m. * $P<0.01$ versus control # $P<0.05$ versus A β only (-). A β_{42-1} peptide was used as a control. (c) Nissl staining demonstrated that the treatment of BIP significantly prevented neuronal cell loss induced by oligomeric A β . Arrows indicate the region of neuronal cell loss. (d) BIP suppressed the activation of caspase-3 (cleaved caspase-3) induced by oligomeric A β ($n=4$). * $P<0.01$ versus control, # $P<0.05$ versus A β only (-)

demonstrate that the level of Bim is significantly upregulated after oligomeric A β treatment, whereas anti-apoptotic protein Bcl-2 is downregulated, suggesting that oligomeric A β induces neuronal cell death through the alteration of the balance of Bcl-2 family proteins and consequent activation of Bax. Unlike most 'BH3-only' proteins, Bim can interact with all pro-survival Bcl-2 proteins with high affinity, and is one of the few BH3-only proteins that can directly activate Bax.²³ For instance, several *in vitro* data suggested that the direct

physical interaction between Bim and Bax can drive Bax activation.^{24,25} Importantly, the ablation of Bim suppresses A β -induced cell death in primary neurons,¹³ and overexpression of anti-apoptotic proteins (Bcl-w) significantly protected neurons against A β -induced apoptosis.¹⁴ Previous reports suggested that overexpression of Bim in cultured neurons induces a rapid apoptotic death²⁶ and Bim is highly expressed in AD neurons.¹³ Bim is also known to induce Bax activation directly or indirectly.²⁷ Collectively, these data strongly

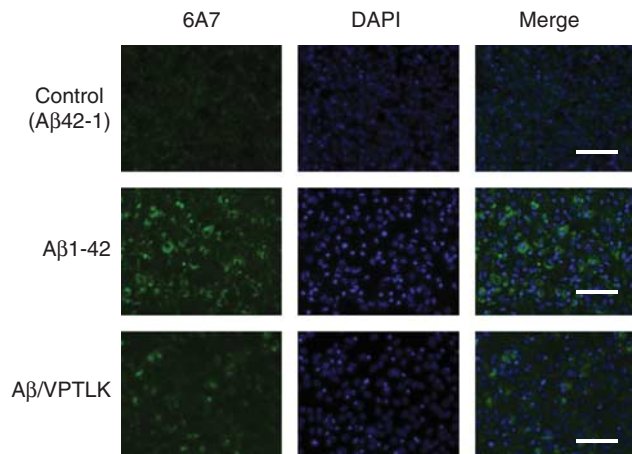


Figure 6 BIP prevents the conformational change of Bax induced by oligomeric A β . BIP (VPTLK) was treated to hippocampal slice cultures with oligomeric A β for 48 h. A β_{42-1} was used as a control. The slices were stained with 6A7 antibody for detecting active conformational change of Bax. In the BIP-treated slices, the number and intensity of 6A7-positive signal (green) was significantly decreased compared with the slices treated with A β only. Scale bar, 100 μ m

suggest that Bim might be an upstream regulator of Bax activation in oligomeric A β -induced neurotoxicity and the pathological role of Bim and Bax in neuronal cell loss in AD.

However, it remains unknown how oligomeric A β activates Bim and consequent neuronal cell death. Although future study will be required to determine the mechanism, one potential pathway might be c-Jun N-terminal kinase (JNK)-dependent phosphorylation, as JNK-mediated phosphorylation promotes Bax-dependent apoptosis.²⁸ As the activation of JNK by A β has been shown²⁹ and the active form of JNK has been reported to be increased in vulnerable neurons in AD,³⁰ it is plausible that the activation of JNK and subsequent activation of Bim/Bax-mediated apoptosis pathway might be the mechanism causing neuronal cell death in oligomeric A β -treated neurons and AD. Alternately, cyclin-dependent kinase 4 (Cdk4) has been suggested as an activator of Bim. Induction of Bim in nerve growth factor-deprived neuronal cells requires active Cdk4 and consequent reactivation of a series of genes suppressed by E2F.³¹ Among the reactivated genes, members of the myb transcription factor family, especially c-myc, seem to have a role in the activation of Bim. In previous studies, the inhibition of Cdk by various experimental approaches (i.e., chemical inhibitors, dominant-negative constructs, small interfering RNA) provide further evidence for the role of Cdk4 in the induction of Bim and neuronal cell death evoked by A β .^{13,15,32} Given that the elevated expression of cell cycle-related proteins including Cdk4 is evident in the vulnerable neurons in AD^{33–35} and A β -treated neuronal cells,^{32,36} an abortive reentry into the cell cycle might induce neuronal cell death by upregulating Bim/Bax apoptotic pathway. Supporting the causal role of cell cycle reentry in neuronal cell death, we previously found that dysregulation of cell cycle reentry results in neurodegeneration *in vivo*.³⁷

Although we did not examine mRNA level of Bim and its posttranslational change, a previous report demonstrated that fibrillar A β increases the level of Bim mRNA and protein.¹³ Thus, the increase of Bim expression by A β is likely mediated

by transcriptional regulation. However, the increased translational modification such as phosphorylation of Bim might also be the reason for the increase of band intensity in our western blot data (Figure 2a). In fact, the western blot bands for upregulated Bim have a slightly higher molecular weight and this change may come from the phosphorylation of Bim, as Bim is known to be phosphorylated by several kinases. Cdk1/Cyclin B1 is one of the kinases to phosphorylate Bim.³⁸ Interestingly, in our recent study (manuscript in preparation), we found that A β induced abnormal cell cycle upregulation as well as Cdk's activation and these events may have a role in posttranslational modification of Bim as seen in this study. Furthermore, the phosphorylation of Bim has been suggested to regulate Bax-dependent cell death.²⁸ Therefore, the modification may regulate the apoptotic activity in our model. In future study, we will pursue the link between Cdk and Bim in A β neurotoxicity.

The present study showing Bax activation by oligomeric A β and the resistance of *bax*^{-/-} neurons to oligomeric A β cell death suggest that Bax is a key mediator of oligomeric A β toxicity, and therefore BIP's protective effects against oligomeric A β can be translated as the result of Bax inhibition. BIP was developed as a Bax-inhibiting peptide, however, as usual in any type of drug, we cannot entirely exclude the possibility that BIP has unexpected target(s) in the cell, which can regulate cellular response to oligomeric A β . The future study developing therapeutics based on BIP will need further careful examination of the possibilities of the existence of the target(s) of BIP in addition to Bax.

In conclusion, in this study, we found that *bax*^{-/-} mice are resistant to oligomeric A β -induced neuronal cell death, suggesting the essential role of Bax in neurotoxicity of oligomeric A β . Furthermore, we show for the first time that BIP application prevents oligomeric A β -induced neuronal cell death, suggesting that BIP and its mimetics may be utilized to mitigate the progress of AD by rescuing neurons from Bax-induced cell death. Our study also suggests that oligomeric A β regulates the activity of Bim, Bcl-2 and Bax in neurons. Taken all together, it is highly likely that the activation of Bax by the regulation of Bim and Bcl-2 family is crucial for oligomeric A β -induced neuronal cell death.

Materials and Methods

Materials. Anti- β -actin antibody was obtained from Millipore (Billerica, MA, USA) and anti-cleaved-caspase-3 antibody was obtained from Cell Signaling Technology (Danvers, MA, USA). A β peptide (A β_{1-42}) and reverse control peptide (A β_{42-1}) were purchased from AnaSpec (Fremont, CA, USA). PI and other chemicals were obtained from Sigma (St. Louis, MO, USA).

Preparation of oligomeric A β . Soluble oligomeric A β was prepared from synthetic peptide according to a previous paper.³⁹ Briefly, 1 mg of A β_{1-42} peptide was dissolved in 120 μ l of hexafluoroisopropanol for 60 min at room temperature, and placed back on ice for 5–10 min. After evaporation of hexafluoroisopropanol overnight in the hood at room temperature, the peptide was dissolved in 40 μ l of fresh anhydrous DMSO, and further diluted to 5 mM stock solution. The stock peptide solution was then incubated for 24 h at 4 $^{\circ}$ C, and centrifuged at 14 000 \times g for 10 min at 4 $^{\circ}$ C. Supernatant was used as oligomeric A β . Before we treated slice culture with oligomeric A β , the oligomers were incubated at room temperature for 20 h.

Mouse strains. Bax KO mice (strain name: B6.129 \times 1-Bax^{t^{m1}Sjk/J}) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). This Bax KO mouse has C57BL/6 genetic background. Bax KO mice were crossed with wild-

type C57BL/6 mice, and *bax*^{+/-} mouse colony was generated. Bax KO as well as WT mice used for this study were generated by crossing *bax*^{+/-} mice in our mouse colony. Each experiment used a set of Bax KO and WT mice obtained from the same parent to minimize variations caused by genetical differences among mice. *bax*^{-/-} mice were genotyped by PCR, as described previously. The primers for the wild-type and KO Bax alleles were 5'-GAGCTGATCAGAACCATCATG-3' (sense) and 5'-CCGCTTCCATTGCTCAGCGG-3' (antisense). Conditions were set as follows: 94°C, 3 min (1 cycle); 94°C, 30 s, 63°C, 1 min, 72°C, 1 min (35 cycles); 72°C, 2 min (1 cycle). All protocols involving the use of mice were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University.

Electron microscopy. A β ₁₋₄₂ was adsorbed onto carbon films supported on Formvar (EMS, Hatfield, PA, USA) membrane-coated nickel grids. The excess buffered-protein solution was removed, and negatively stained with 2% uranyl acetate. Grids were then washed by touching the buffer and the excess buffer was immediately blotted using Whatman (Picataway, NJ, USA) filter paper. Grids were then air-dried and kept at room temperature. Negatively stained specimens were observed by a JEOL 1200EX electron microscope (JEOL, Tokyo, Japan) with 80 kV of electron acceleration voltage.

Preparation of organotypic hippocampal slice cultures. Organotypic hippocampal slice cultures were prepared as described previously.⁴⁰ Briefly, hippocampal slice cultures were prepared using 7–10-day-old mouse pups. Slices were cut at 400 μ m on a Mclwain tissue chopper, transferred to Millicell membrane inserts (0.4 μ m; Millipore), and placed in 6-well culture plates. The culture medium consisted of basal Eagle's medium with Earle's balanced salt solution, 20% heat-inactivated horse serum, enriched with 5.6 mM glucose. The medium was changed every other day. The effect of oligomeric A β (500 nM) was tested in the slices that had been maintained for 11–14 days *in vitro*. A β oligomer or reversed sequence of A β ₁₋₄₂ control peptide (A β ₄₂₋₁) was added to cultures in serum-free medium and, after the treatment, the hippocampal slices were rinsed twice in ice-cold phosphate-buffered saline (PBS), and then harvested by removing the Millicell membrane insert.

Assessment of neuronal cell death in organotypic hippocampal slice cultures. To determine neuronal cell death in the hippocampal slices, PI (5 μ g/ml) was added to the slice culture medium. Images were acquired through an AxioCam camera on an Axiovert 200M microscope (Zeiss, Oberkochen, Germany). Fluorescent intensity was measured using Image J (NIH, Bethesda, MD, USA). Hematoxylin and eosin (H&E) and Nissl staining was also performed for routine histochemical and morphological analyses.

Protein extraction and western blot analysis. After oligomeric A β treatment, the slices were rinsed twice with ice-cold PBS and then lysed in ice-cold cell lysis buffer (Cell Signaling Technology) with protease and phosphatase inhibitor cocktail (Roche, Indianapolis, IN, USA). The protein concentration was determined by BCA assay (Pierce, Rockford, IL, USA). The extracted proteins were separated on 10 or 12% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. The blots were blocked with 10% non-fat milk in TBS-T for 1 h at room temperature, and then treated with primary antibodies diluted with 1% non-fat milk and incubated overnight at 4°C. The following antibodies were used for western blot analysis: anti-caspase-3 (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Bim (1 : 1000; Stressgen, Farmingdale, NY, USA), anti-Bcl-2 (1 : 1000; Stressgen) and 6A7 (conformational specific Bax antibody) (1 : 1000; BD Pharmingen, San Diego, CA, USA).

Intrahippocampal injection of oligomeric A β . C57BL/6J mice (The Jackson Laboratories; 2–3-month-old) were anesthetized with pentobarbital and placed in a stereotaxic frame. Injection was made using a 10- μ l microsyringe (Hamilton, Reno, NV, USA). A volume of 1 μ l of oligomeric A β dissolved at 50 μ M in PBS was injected into the left hippocampus. Control animals were prepared identically and injected with the same volume and concentration of A β ₄₂₋₁ in PBS. Injections were made at stereotaxic coordinates from bregma; anteroposterior (AP) = 2.3 mm, mediolateral (ML) = 2.5 mm and dorsoventral (DV) = -2.5 mm according to a previous report.⁴¹ This corresponds to a site in the dorsal hippocampus in the apical dendritic zones of the CA1 region near the hippocampal fissure. Mice were killed 10 or 20 days after injection, brains dissected out, fixed in 10% buffered-formalin and paraffin-embedded. For brain tissue sections,

6- μ m-thick serial sections were cut, mounted onto slides and rehydrated according to standard protocols.

Immunocytochemistry. Cultured hippocampal slices were rinsed with ice-cold PBS once and fixed for 2 h with 4% paraformaldehyde in 0.1 M PBS.⁴² After washing with PBS, sections were permeabilized overnight with PBS containing 0.2% Triton X-100. At the end of the permeabilization blocking solution, 10% normal goat serum in PBS was applied for 4 h at room temperature. After washing with PBS, primary antibody was added and incubated for 24–48 h at 4°C. After thorough washing of the sections in PBS, a secondary antibody labeled with either Alexa Fluor (Life Technologies, Grand Island, NY, USA) 488 or 568 (1 : 300) was added and incubated for 4 h at room temperature. All of the experiments contained at least one sample incubated without a primary antibody to exclude non-specific signal. Nuclei were visualized with DAPI. Images were acquired through an AxioCam camera on an Axiovert 200M microscope (Zeiss). Images were then analyzed with the Axiovision software (Zeiss).

TUNEL analysis. Detection of 3'-OH termini of DNA strand breaks was performed using *in situ* cell death detection kit (Roche). Briefly, the tissue sections were treated with proteinase K (20 μ g/ml in 10 mmol/l Tris-HCl, pH 7.4) for 30 min at 37°C after rehydration. After rinsing with PBS, TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescence-labeled nucleotide was applied for 1 h 37°C. The samples were then washed and mounted using Aquamount (Southern Biotech, Birmingham, AL, USA).

Statistical analysis. Data were expressed as the means \pm S.E.; the number of independent experiments is indicated in the corresponding figure legend. Differences between groups were examined for statistical significance using one-way analysis of variance with an unpaired Student's *t*-test. A *P* value of < 0.05 indicated a statistically significant difference.

Conflict of Interest

Dr. Xiongwei Zhu was a consultant for and received grant support from Medivation. Dr. Mark A Smith was a consultant for Anavex Life Sciences Corporation, Eisai, Medivation, Neurotez and Takeda Pharmaceuticals; owned stock options in Aria Neurosciences, Neurotez, Panacea and Voyager, and received lecture fees from GSK, Medivation and Pfizer.

Acknowledgements. We thank Dr. Hisashi Fujioka for electron microscopy analysis. This work was supported by the National Institutes of Health (AG028679 to HL; AG031903 and P30CA043702 to SM); the Alzheimer's Association (IIRG-11-173471 to HL).

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