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行政院國家科學委員會補助專題研究計畫 ■ 成果報告 □期中進度報告

樣澱粉β引發腦血管內皮細胞凋亡之分子機制探討(3/3)

Molecular mechanism of amyloid β-induced cerebral endothelial cell apoptosis (3/3)

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英文摘要。

關鍵字：Alzheimer's disease (AD); amyloid peptide (Aβ); cerebral amyloid angiopathy, (CAA); cerebral endothelial cells (CECs); apoptosis; Apoptosis Signal-Regulating Kinase1(ASK1)。

A pathological hallmark of Alzheimer's disease (AD) is accumulation of amyloid-β peptide (Aβ) in senile plaques. Aβ has been implicated in neuronal and vascular degeneration in AD because of its cytotoxic effects on neurons and endothelial cells (ECs). In the present study, we used a murine cerebral endothelial cell (CEC) line to explore the signaling cascade involved in Aβ-induced CEC death. Coimmunoprecipitation and western blotting indicated that $A\beta_{25-35}$, a cytotoxic fragment of Aβ, triggered dephosphorylation of Ser-967 on Apoptosis signal-regulating kinase 1 (ASK1), resulting in dissociation of the ASK1.14-3-3 complex. ASK1 has been shown to be associated with TRAF2 and ASK1 kinase activity was concomitant increased transiently by $A\beta_{25-35}$. MTT assay and flowcytometric analysis reveal that the apoptotic effect of $A\beta_{25-35}$ is attenuated in cells transfected with TRAF2, ASK1, MKK3, p38MAPKα dominant negative mutants (TRAF2, ASK1, p38MAPK inhibition) or in the presence of SB 203580 (a p38 MAPK inhibitor). In addition, ASK1-mediated p38MAPK activation was subsequent activated upon $A\beta_{25-35}$ treatment. $A\beta_{25-35}$ treatment also led to p53 phosphorylation at ser 15 and subsequent DNA binding. The expression of Bax, a proapoptotic Bcl2 family protein downstream of p53, was significantly elevated due to $A\beta_{25-35}$ treatment. Transfection of ASK1 dominant mutant suppressed $A\beta_{25-35}$-activated p38MAPK and p53 phosphorylation, Bax expression and resulted in attenuation of CEC death. Furthermore, p38MAPK inhibition or Bax knockdown using a Bax RNAi strategy reduced Bax expression and subsequent CEC death after $A\beta_{25-35}$ exposure. These results suggest that $A\beta_{25-35}$ activated an apoptotic cascade involving ASK1-p38MAPK-p53 signaling pathway, followed by p53 DNA binding and Bax induction, resulting in CEC death.

中文摘要。

關鍵字：阿茲海默式症(Alzheimer's disease, AD); 樣澱粉β 胜肽(amyloid β peptide, Aβ); 腦血管樣澱粉病變(cerebral amyloid angiopathy, CAA); 腦內皮細胞(cerebral endothelial cells, CECs); 細胞凋亡(apoptosis); Apoptosis Signal-Regulating Kinase1(ASK1)。

阿茲海默症(Alzheimer's disease, AD)的病理特徵為樣澱粉β 胜肽 (amyloid β peptide)在腦部血管老化斑塊(senile plaques)的大量堆積。由於樣澱粉β 胜肽會對神經以及內皮細胞產生細胞毒性，因此樣澱粉β 胜肽 (amyloid β peptide) 被認為與阿茲海默症的神經及血管退化性病徵有相當的關連性。最近的研究發現，樣澱粉β 會誘導腦血管內皮細胞產生細胞凋亡，然而其作用機制則尚未研究清楚。因此在本年度的計劃中我們將進一步探討樣澱粉β 胜肽引發腦內皮細胞凋亡的訊息傳遞路徑。希望此計劃的完成可進一步瞭解澱粉β 胜肽 調控腦內皮細胞凋亡的機轉，並且發展出治療樣澱粉β 誘發腦血管疾病的治療方針。

利用共同免疫沉澱以及西方點墨法測定發現澱粉β 可以使得 Apoptosis signal-regulating kinase 1 (ASK1)在胺基酸殘基 ser 967 的位置磷酸化程度降低，並導致 ASK1 和 14-3-3 兩蛋白所形成的複合物分離。同時在澱粉β 的刺激下，短時間內可見到 ASK1 和 TRAF2 形成複合物並伴隨著 ASK1 激酶活性的增加。利用 MTT 測定分析，我們發現樣澱粉β 會濃度依賴性的降低腦內皮細胞的細胞存活率。同時在細胞轉染(transfection) TRAF2DN、ASK1DN、MKK3DN、以及 p38MAPKαDN 時則會抑制澱粉β 所降低的細胞存活率，進一步利用流式細胞儀分析發現選擇性的 p38MAPK 和 JNK 抑制劑，SB203580 及 SP600125 可以抑制樣澱粉β所引起的腦部內皮細胞凋亡。這結果指出澱粉β可能是透過活化 TRAF2、ASK1、p38 MAPK 和 JNK 而使得腦部內皮細胞進行細胞凋亡。再者，澱粉β 引起 p38MAPK 激酶活性的增加會被轉染不活化態 ASK1(ASK1)所抑制。澱粉β 也會增加轉錄因子 p53 蛋白在胺基酸殘基 ser15 位置的磷酸化以及和 DNA 結合的能力。受 p53 蛋白轉錄調控的促進細胞凋亡蛋白 Bax 在澱粉β 刺激下也會顯著增加。ASK1DN 轉染會抑制澱粉β誘導腦部內皮細胞 p38MAPK 活性的增加、p53 在胺基酸殘基 ser 15 的磷酸化、Bax 表現的增加以及細胞凋亡。同時，抑制 38MAPK 訊息路徑或是利用 RNAi 技術都可降低 Bax 表現並抑制澱粉β引起的細胞凋亡。因此在本年度的研究中我們推測樣澱粉β可能是透過活化 ASK1/TRAF2/p38MAPK/p53 訊息傳遞路徑進而增加 p53 和 DNA 結合使得 Bax 表現增加而引起細胞凋亡。

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ASK1 in Amyloid β Peptide-Induced Cerebral Endothelial Cell Apoptosis

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Running head: Amyloid β peptide-induced CEC apoptosis

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Abstract

A pathological hallmark of Alzheimer's disease (AD) is accumulation of amyloid-β peptide (Aβ) in senile plaques. Aβ has also been implicated in vascular degeneration in cerebral amyloid angiopathy (CAA) because of its cytotoxic effects on non-neuronal cells including cerebral endothelial cells (CECs). We explore the role of apoptosis signal-regulating kinase 1 (ASK1) in Aβ-induced death in primary cultures of murine CECs. Aβ induced ASK1 dephosphorylation, which could be prevented by selective inhibition of protein phosphatase 2A (PP2A), but not PP2B. ASK1 dephosphorylation resulted in its dissociation from 14-3-3. ASK1, released from 14-3-3 inhibition, stimulated p38 mitogen-activated protein kinase (p38MAPK) activity, leading to p53 phosphorylation. P53, a pro-apoptotic transcription factor, in turn transactivated the expression of Bax, a pro-apoptotic protein. Transfection with various dominant negative mutants (DNs) including ASK1 DN, MAPK kinase 3 (MKK3) DN, MKK6 DN and p38MAPK DN suppressed Aβ-induced p38MAPK activation, p53 phosphorylation and Bax upregulation and partially prevented CEC death. Bax knockdown using a *bax* RNAi strategy also reduced Bax expression and subsequent CEC death. These results suggest that Aβ operates an ASK1-MKK3/6-p38MAPK-p53-Bax cascade to cause CEC death in a PP2A-dependent manner.

Introduction

Amyloid-β peptide (Aβ), a 39-43-amino acid cleavage product of amyloid precursor protein, is thought to be the primary mediator of neuronal degeneration in Alzheimer's disease (AD) (Yankner et al., 1989). Prevailing evidence has demonstrated that Aβ induces apoptosis in various cell types such as neurons (Yankner et al., 1989; Behl et al., 1994), cerebrovascular smooth muscle cells (Davis-Salinas and Van Nostrand, 1995), oligodendrocytes (Xu et al., 2001b; Lee et al., 2004), astrocytes (Yang et al., 2004) and cerebral endothelial cells (CECs) (Xu et al., 2001a; Yin et al., 2002; Yin et al., 2006). CECs and astrocytes constitute the blood brain barrier (BBB) to shield the brain from damage by harmful circulating toxins or deleterious cellular elements. As a result, CEC apoptosis appears to be a contributing factor in Aβ-induced cerebrovascular degeneration (Yang et al., 2004) which is characterized by cerebral amyloid angiopathy (CAA). However, the molecular mechanism of Aβ-induced CEC apoptosis has not been fully delineated.

Reversible protein phosphorylation catalysed by protein kinases and protein phosphatases regulates numerous cellular processes including apoptosis (Hunter, 2000). Recent studies have highlighted a major role of serine/threonine protein phosphatases including protein phosphatase 2A (PP2A), in apoptosis (Ray et al., 2005). PP2A, a member of the ceramide-activated protein phosphatases (CAPPs) has been implicated in Aβ-induced apoptosis through the ceramide-PP2A-Bim cascade (Yin et al., 2006). Bim is a member in the "BH3-only proteins", a subgroup of Bcl-2 apoptotic regulators which contain only one of the bcl-2 homology regions (BH3). In response to apoptotic stimuli, BH3-only proteins translocate to the mitochondrial membrane from other cellular

compartments to interfere with the function of anti-apoptotic Bcl-2 family members, leading to apoptotic cell death (Huang and Strasser, 2000). In addition to protein phosphatase, the findings that MAP kinase, a serine/threonine-specific protein kinase, is activated by Aβ independent of a well established BH3-only death paradigm, raise the possibility that a BH3-independent signaling processes via the MAP kinase pathway may also contribute to Aβ-induced CEC death.

Apoptosis signal-regulating kinase 1 (ASK1) has been implicated in the MAP kinase signaling events leading to cell death (Matsuzawa et al., 2002). Heightened oxidative tension is an important feature in apoptosis involving the ASK1-MAP kinase pathway. Aβ-induced cell death is also characterized by increased oxidative stress (Yankner et al., 1989; Behl et al., 1994). Reactive oxygen species (ROS) have been shown to activate ASK1 to induce apoptosis (Kanamoto et al., 2000; Nishitoh et al., 2002; Noguchi et al., 2005). ASK1 activity is tightly controlled by multiple mechanisms including phosphorylation (Morita et al., 2001), oligomerization (Gotoh and Cooper, 1998) and protein-protein interactions. The kinase and pro-apoptotic activities of ASK1 are inhibited by a number of associated proteins, including 14-3-3 proteins (Zhang et al., 1999), the reduced form of thioredoxin (Trx) (Saitoh et al., 1998), Cdc25A (Zou et al., 2001), Hsp 72(Park et al., 2002). ASK1 can also bind to TNF receptor-associated factor 2 (TRAF2) or ASK1-interacting protein 1(Zhang et al., 2003) and these associations are required for TNF-induced ASK1 activation (Nishitoh et al., 1998).

ROS generation and resultant mitochondrial dysfunction are causally related to Aβ-induced apoptotic neuronal (Behl et al., 1994; Hensley et al., 1994) as well as CEC death (Xu et al., 2001a; Yin et al., 2002). The present study was undertaken to explore

the contribution of a major apoptosis paradigm, namely the ASK1-MAP kinase pathway, in Aβ-induced CEC death. In this communication, we provide experimental evidence to support the contention that activation of the ASK1-MKK3/6-p38MAPK-p53-Bax pathway contributes to Aβ-induced CEC apoptosis. This finding raises the possibility that more than one apoptotic pathways may be in operation downstream of PP2A in Aβ-induced CEC death.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco-BRL (Grand Island, NY); the enhanced chemiluminescence (ECL) detection kit from Amersham Pharmacia Biotech (Piscataway, NJ); amyloid peptide ($A\beta_{1-40}$), and a cytotoxic fragment ($A\beta_{25-35}$), protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase antibodies, and rabbit polyclonal antibodies specific for ASK1, p53, p38MAPK, GAPDH, α-tubulin, Bax, 14-3-3 and hemagglutinin (HA) from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against p53 phosphorylated at Ser 15, ASK1 phosphorylated at Ser 967 or p38MAPK phosphorylated at Ser/Thr residues from New England Biolabs (Beverly, MA); all reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) from Bio-Rad Laboratories (Richmond, CA); okadaic acid (OA) from Upstate Biotechnology(Lake Placid, NY); SB203580 from Calbiochem (San Diego, CA); [γ-^{32}P] ATP (6,000 Ci/mmol) from Amersham Biosciences; Lipofectamine Plus^{TM} from Life Technologies (Gaithersburg, MD); and all other chemicals from Sigma Chemical Co. (St. Louis, MO).

The hemagglutinin (HA)-tagged expression constructs for catalytically inactive ASK1-K709E (ASK1 DN) and pcDNA were derived as described previously (Chen et al., 2003). MAPK kinase 3 (MKK3) DN, MKK6 DN, and p38MAPK DN were kindly provided by Dr. CM Teng (National Taiwan University, Taipei, Taiwan).

Mouse CEC primary culture

Mouse CECs were prepared as previously described (Xu et al., 1992; Yin et al., 2002). Briefly, mouse CECs migrating from isolated microvessel preparations were pooled to form a proliferating cell culture that was maintained in DMEM, with high glucose and L-glutamine supplemented with 10% FBS, 0.5 mg/ml heparin, and 75 μg/ml endothelial cell growth supplements. Mouse CECs (between passage 4-15) uniformly positive for factor VIII, vimentin, and characteristic bradykinin receptors (>95% endothelial cell purity), were grown to 85-95% confluence before use.

Cell viability assay

Cell viability was measured by the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as described previously (Hsu et al., 2002; Yin et al., 2002).

Flow cytometric analysis

CECs were cultured in 6-cm petri dishes. After reaching confluence, cells were treated with vehicle or Aβ with or without specific inhibitors for 30 min, then washed twice with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4), and re-suspended in ice-cold 70% ethanol at 0°C overnight. Cells were washed for 5 min with 0.4 ml phosphate-citric acid buffer (pH 7.8) containing 50 mM Na_2HPO_4, 25 mM citric acid and 0.1% Triton X-100 and subsequently stained with 1.5 ml propidium iodide (PI) staining buffer containing 0.1% Triton X-100, 10 mM PIPES, 100 mM NaCl, 2 mM $MgCl_2$, 100 μg/ml RNase A and 50 μg/ml PI for 30 min in the dark before flow cytometric analysis. Cells were filtered on a

nylon mesh filter. The samples were analyzed by the FACScan and Cellquest program (Becton Dickinson). Each experiment was repeated at least three times.

Plasmid DNA transfection

For transfection, 10^5 CECs were seeded onto 12-well plates and transfected with Lipofectamine Plus with 1 μg of pcDNA, ASK1 DN, MKK3 DN, MKK6 DN or p38MAPK DN. After 24 h, the medium was replaced by fresh DMEM and stimulated with Aβ (20 μM) for various time periods.

Suppression of bax expression

bax suppression was achieved by transfecting cells with pSiREN-*bax*, a plasmid that generates small interfering RNAs that target *bax* mRNA for degradation. To produce pSiREN-*bax*, the following complementary oligonucleotides were annealed and cloned into BamHI/EcoRI-digested pSiREN (BD Biosciences Clontech, CA): *bax* sense, 5'-gatccgactggtgctcaaggccctgttcaagagacagggccttgagcaccagctttttg-3'; *bax* antisense, 3'-gctgaccacgagttccgggacaagttctctgtcccggaactcgtggtcagaaaaaacttaag-5'. A control RNAi was also constructed by cloning custom-synthesized oligonucleotides (BD Biosciences Clontech, CA) into BamHI/EcoRI-digested pSiREN.

Western blot analysis

To determine the expressions of ASK1, ASK1 phosphorylated at Ser967,14-3-3, p38MAPK, p38MAPK phosphorylated at Ser/Thr residues, p53, p53 phosphorylated at Ser15, and Bax in CECs using α-tubulin and GAPDH, as the internal controls, proteins

were extracted and analyzed by Western blotting as previously described (Yin et al., 2002; Chen et al., 2004). Briefly, CECs were cultured in 6-cm dishes. After reaching confluence, cells were treated with vehicle or specific inhibitors followed by Aβ. After incubation, cells were washed twice in ice-cold PBS and solubilized in extraction buffer containing 10 mM Tris (pH 7.0), 140 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, 0.5% Nonidet P-40, 0.05 mM pepstatin A and 0.2 mM leupeptin. Samples of equal amounts of protein (60 μg) were subjected to SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane that was later incubated in TBST buffer (150 mM NaCl, 20 mM Tris-HCl and 0.02% Tween 20 (pH 7.4)) containing 5% non-fat milk. Proteins were incubated with first specific primary antibodies and then horseradish peroxidase-conjugated secondary antibodies. Specific bands were detected based on enhanced chemiluminescence per manufacturer's instructions. Quantitative data were obtained using a computing densitometer with scientific imaging systems (Eastman Kodak Co.).

Immunoprecipitation and protein kinase assays

CECs were grown in 6-cm dishes. After reaching confluence, cells were treated with 20 μM Aβ for the indicated time intervals. After incubation, cells were washed twice with ice-cold PBS, lysed in 1 ml of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 125 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 25 mM β-glycerophosphate, 50 mM NaF and 100 μM sodium orthovanadate, and centrifuged. The supernatant was immunoprecipitated overnight with polyclonal antibodies against ASK1 or p38MAPK in the presence of

protein A/G-agarose beads. The beads were washed three times with lysis buffer and two times with kinase buffer containing 20 mM HEPES (pH 7.4), 20 mM $MgCl_2$ and 2 mM dithiothreitol. The kinase reactions were performed by incubating immunoprecipitated complex with 20 µl of kinase buffer supplemented with 20 µM ATP and 3 µCi of [γ-^{32}P] ATP at 30°C for 30 min. To assess ASK1 and p38MAPK activities, 50 µg/ml of myelin basic protein (MBP) was added to serve as the substrate. The reaction mixtures were analyzed by 15% SDS-PAGE followed by autoradiography.

Co-immunoprecipitation (Co-IP)

CECs were grown in 6-cm dishes. After reaching confluence, cells were treated with 20 µM Aβ for the indicated time intervals. The cells were harvested, lysed in 1 ml of PD buffer (40 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.1% Nonidet P-40, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 300 µM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml leupeptin and 1 mM dithiothreitol), and centrifuged. The supernatant was immunoprecipitated overnight with specific antibodies against ASK1 in the presence of protein A/G-agarose beads at 4 °C. The immunoprecipitated complex was washed three times with PD buffer. The samples were fractionated on 15% SDS-PAGE, transferred to a polyvinylidene difluoride membrane and subjected to immunoblotting with antibodies specific for 14-3-3.

Preparation of nuclear extracts and EMSA

CECs were grown in 6-cm dishes. After reaching confluence, cells were treated with 20 µM Aβ for the indicated time intervals. The nuclear protein fractions were then

prepared as described previously (Xu et al., 2001b; Chen et al., 2004). Briefly, cells were washed with ice-cold PBS, and then centrifuged. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES, 10 mM KCl, 0.5 mM DTT, 10 mM aprotinin, 10 mM leupeptin, and 20 mM PMSF) for 15 min on ice, and vortexed for 10 s. The nuclei were pelleted by centrifugation at 15,000 ×g for 1 min. A pellet containing nuclei was resuspended in hypertonic buffer (20 mM HEPES (pH 7.6), 25% glycerol, 1.5 mM $MgCl_2$, 4 mM EDTA, 0.05 mM DTT, 20 mM PMSF, 10 mM aprotinin, and 10 mM leupeptin) for 30 min on ice. The supernatants containing the nuclear proteins were collected by centrifugation at 15,000 ×g for 2 min and stored at −70 °C. A double-stranded oligonucleotide probe containing the p53 sequence (5′-GAACATGTCTAAGCATGCTG-3′; Santa Cruz) was end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. The nuclear extract (2.5-5 μg) was incubated with 1 ng of a ^{32}P -labeled p53 probe (50,000-75,000 cpm) in 10 μl of binding buffer containing 1 μg poly(dI-dc), 15 mM HEPES (pH 7.6), 80 mM NaCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol at 30 °C for 25 min. DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on 6% polyacrylamide gels. The gels were vacuum-dried and subjected to autoradiography with an intensifying screen at −80 °C.

Statistical analysis

Results are presented as mean ± SEM from at least three independent experiments. One-way analysis of variance, followed by Bonferroni's multiple range tests when appropriate, was used to determine the statistical significance of the difference between the means. A p value less than 0.05 was considered statistically significant.

Results

ASK1 activation in Aβ-induced CEC apoptosis

As reported previously, $A\beta_{1-40}$ and $A\beta_{25-35}$ have equal potency for inducing apoptosis in CECs (Xu et al., 2001a). In the present study, we also demonstrated that the potency of $A\beta_{1-40}$ in causing CEC death was similar to that of $A\beta_{25-35}$ as determined by the MTT assay (data not shown). Thus, this study was conducted using mainly $A\beta_{25-35}$ with key experiments confirmed with $A\beta_{1-40}$. ASK1 activation is a pivotal mechanism in a broad range of cell death paradigms (Nishitoh et al., 2002). To explore whether ASK1 activation contributes to Aβ-induced CEC death, cells were transiently transfected with hemagglutinin (HA) epitope-tagged ASK1 DN prior to Aβ treatment. In preliminary studies, we first confirmed that the protein encoded by ASK1 DN plasmid was expressed in transfected CECs. Based on immunoblotting using specific antibodies to HA or ASK1, HA-tagged ASK1 DN protein was highly expressed in ASK1 DN-transfected cells compared to the control group (transfected with pcDNA) (data not shown). Aβ induced death in CECs could be partially but significantly reduced with transfection of ASK1 DN (cell viability: ASK1 DN, 77.4 ± 3.6 %; pcDNA, 56.2 ± 5.0 %, $p<0.05$, Figure 1A). To further elucidate whether ASK1 activation is involved in the signaling cascade of Aβ-induced cell death, ASK1 kinase activity was measured after Aβ exposure. Treatment with Aβ rapidly increased ASK1 kinase activity in CECs as early as 5 min and returned to basal level within 1 h (Figure 1B). We next explored the mechanism by which Aβ induces ASK1 activation. Dissociation of ASK1 from inhibitory protein, 14-3-3, may lead to ASK1 activation. Phosphorylation of the ASK1 Ser967 residue was proposed to

be a major mode in the regulation of ASK1 binding to 14-3-3 (Zhang et al., 1999). We examined whether the extent of ASK1 Ser967 phosphorylation is altered after Aβ exposure. Aβ caused a marked decrease in ASK1 Ser967 phosphorylation as early as 2 min after Aβ exposure (Figure 1C). Co-immunoprecipitation was then employed to confirm the hypothesis that Aβ-induced ASK1 dephosphorylation was accompanied by the dissociation of the ASK1-14-3-3 complex. As shown in Figure 1D, Aβ rapidly caused ASK1 dissociation from 14-3-3. This response began as early as 2 min after Aβ exposure. These findings together suggest that ASK1 Ser-967 dephosphorylation and subsequent ASK1 dissociation from 14-3-3 were rapid and reversible signaling processes in response to Aβ-induced stress in CECs. However, the mechanism that regulates ASK1 dephosphorylation at the Ser-967 residue remains to be identified. It is conceivable that Aβ may activate a protein phosphatase that dephosphorylates Ser-967, leading to ASK1 activation. Protein phosphatase 2A (PP2A) was recently shown to participate in Aβ-induced CEC death (Yin et al., 2006). We, therefore, explore whether PP2A is involved in ASK1 dephosphorylation. Okadaic acid, a selective PP2A inhibitor, reduced ASK1 dephosphorylation in CECs treated with Aβ. Cyclosporin A, a specific PP2B/calcineurin inhibitor, was without effect (Figure 1E). These results suggest that PP2A may be specifically responsible for Aβ-induced dephosphorylation of ASK1 Ser-967 in CECs.

p38MAPK activation in Aβ-induced CEC apoptosis

ASK1 belongs to the MAPKKK family and activates the JNK and p38MAPK pathways via MKK4/7 and MKK3/6, respectively (Ichijo et al., 1997). JNK-mediated

signaling cascade has previously been shown to participate in Aβ-induced CEC death (Yin et al., 2002). In the present study, we focused on the role of p38MAPK signaling cascade in Aβ-induced CEC death. We examined whether p38MAPK signaling events are involved in Aβ-induced CEC apoptosis using flowcytometry. As shown in Figure 2A, the percentage of propidium iodide (PI)-stained cells in the apoptotic region (Apo, sub-G0/G1 peak, subdiploid peak) was significantly increased following 20 μM Aβ treatment (Figure 2A, c), as compared to the vehicle-treated group (Figure 2A, a) The compiled data are shown in the lower panel in Figure 2A (Aβ: 64.2 ± 4.6 %; vehicle, 7.2 ± 2.3 %, $p<0.05$). Aβ-induced CEC apoptosis was attenuated by SB203580, a p38MAPK inhibitor, (Figure 2A, d and lower panel: Aβ plus SB203580, 45.2 ± 2.6 %, $p<0.05$ as compared to Aβ alone). The time course of Aβ-induced p38MAPK phosphorylation is shown in Figure 2B. Aβ increased p38MAPK phosphorylation in a time-dependent manner. In parallel, using myelin basic protein (MBP) as a p38MAPK substrate, a time-dependent increase in p38MAPK activity was also observed in Aβ-treated CECs (Figure 2C). To further ascertain the linkage between ASK1 and p38MAPK signaling cascade downstream of Aβ, we determined the effect of ASK1 DN on Aβ-induced p38MAPK activation. As shown in Figure 3A, transfection with ASK1 DN significantly reduced Aβ-induced p38MAPK activation. ASK1 activation of p38MAPK is via the MKK3/6 pathway (Ichijo et al., 1997). MKK3 DN and MKK6 DN also attenuated Aβ-induced p38MAPK activation (Figure 3B).

We also used MKK3 DN, MKK6 DN and p38MAPKα DN to block MKK3/6-p38MAPK signaling pathway in order to confirm whether the MKK3/6-p38MAPK cascade participates in Aβ-induced CEC death. As shown in Figure

3C, transfection with MKK3 DN, MKK6 DN and p38MAPKα DN attenuated Aβ-induced CEC death with the extent of cell death reduced from 43.8 ± 5.0 % to 23.8 ± 4.4 %, 21.8 ± 5.1 % and 19.1 ± 4.0 %, respectively. Together these findings suggest that ASK1-MKK3/6-p38MAPK cascade is in operation in Aβ-induced CEC death.

p53 activation in Aβ-induced CEC apoptosis

p53 is a transcription factor which plays a key role in the regulation of cell viability downstream of MAP kinase. Activation of p53 entails phosphorylation of its serine residues (Dumaz and Meek, 1999; Meek, 1999). To explore the role of p53 in Aβ death signaling downstream of MAP kinase, we examined the effect of Aβ on p53 phosphorylation at the Ser15 residue. Figure 4A shows that Aβ caused an increase in p53 phosphorylation at Ser15 residue in a time-dependent manner. The phosphorylation began at 10 min, peaked at 2 h, and declined toward the basal level 6 h after Aβ treatment. Phosphorylation at Ser15 residue is responsible for p53 binding to its cognate DNA binding sequence (Dumaz and Meek, 1999). The nuclear extracts of CECs with and without Aβ treatment were subjected to EMSA using p53-specific oligonucletides as the probe. As shown in Figure 4B, p53-specific DNA-protein binding activity, low in vehicle treated CECs, was markedly increased 1-2 h after Aβ treatment (Figure 4B). Pretreatment of nuclear extracts with specific antibody against p53 reduced p53-specific DNA-protein binding activity, demonstrating the specificity of p53-DNA binding activity (Figure 4C).

ASK1 DN was then employed to block ASK1 signaling in order to determine whether ASK1 mediates Aβ-induced p53 activation. As shown in Figure 5A, ASK1 DN significantly suppressed Aβ-induced p53 phosphorylation at Ser15 residue. Similarly, both MKK3 DN and MKK6 DN were effective in attenuating Aβ-induced p53

phosphorylation at the Ser15 residue (Figure 5B). Furthermore, SB 203580, a p38MAPK inhibitor, also inhibited Aβ-induced p53 activation (Figure 5C). These results support a causal role of the ASK1-MKK3/6-p38MAPK signaling cascade in Aβ-induced p53 activation.

Bax upregulation in Aβ-induced CEC apoptosis

p53 has been shown to induce apoptosis by causing mitochondrial dysfunction via transactivation of Bax expression (Jeffers et al., 2003; Hastak et al., 2005). We, therefore, examined whether Aβ is capable of inducing Bax expression in CECs. As shown in Figure 6A, Aβ elevated cellular level of Bax in a time-dependent manner. To further confirm that Aβ-induced cell death was mediated by Bax, Bax expression was silenced using RNA interfering (RNAi) strategy. RNAi experiments revealed that the basal Bax level in CECs was only slightly affected by *bax* RNAi. In contrast, *bax* RNAi suppressed Aβ-induced Bax expression (Figure 6B). Furthermore, transfection of CECs with *bax* RNAi attenuated Aβ-induced apoptosis by 55.9 ± 4.2 % (Figure 6C).

We then tested the hypothesis that Aβ may induce Bax expression through the ASK1-MKK3/6-p38MAPK-p53 signaling cascade. As shown in Figure 7A, ASK1 DN noticeably inhibited Aβ upregulation of Bax expression in CECs. Moreover, MKK3 DN, MKK6 DN (Figure 7B) and p38MAPK inhibitor, SB203580, (Figure 7C) also attenuated Aβ-induced Bax expression respectively. These findings support the contention that Aβ activates the ASK1-MKK3/6-p38MAPK-p53 signaling cascade to induce Bax expression, resulting in CEC apoptosis.

Discussion

Aβ has been implicated as the primary neurotoxic factor in the pathogenesis of AD. Results from our present study, similar to those reported previously (Xu et al., 2001a; Yin et al., 2002; Yang et al., 2004; Yin et al., 2005), show that Aβ is also cytotoxic to CECs. Aβ-induced apoptosis is a multifactorial process involving excessive formation of reactive oxygen species (ROS) (Schapira, 1996), alteration of intracellular calcium homeostasis (Kriem et al., 2005), mitochondrial dysfunction and caspases activation (Hitomi et al., 2004). However, the precise molecular mechanism responsible for the apoptotic action of Aβ remains to be fully characterized. We demonstrated that the activation of the ASK1-MKK3/6-p38MAPK signaling cascade followed by p53 activation and Bax expression contributes to Aβ-induced CEC death.

Phosphorylation of the ASK1 Ser967 residue is required for the formation of the ASK1-14-3-3 complex to keep ASK1 inactive (Zhang et al., 1999). However, the signaling pathways that control ASK1 function through Ser967 remain unresolved. Activation of an unknown protein phosphatase is required for TNF-α-induced ASK1 activation by dephosphorylating ASK1 Ser967 (Zhang et al., 2003). Goldman et al further demonstrated that an okadaic acid-sensitive phosphatase is required for H2O2 dephosphorylation of ASK1 Ser967 in COS7 cells (Goldman et al., 2004). We have shown that Aβ-induced CEC death was causally related to PP2A activation (Yin et al., 2006). In the present study, we demonstrated that okadaic acid, a specific inhibitor of PP2A, inhibited Aβ dephosphorylation of ASK1 Ser967. Cyclosporin A, a specific inhibitor of PP2B, however, failed to block Aβ-induced ASK1 Ser967 dephosphorylation.

These finding suggest that PP2A may play a pivotal role in ASK1 dephosphorylation and the subsequent signaling events.

Activated ASK1 plays a critical role in apoptosis by stimulating the downstream signaling events including the activation of MKK3/6 and p38MAPK in sequence (Yamaguchi et al., 2004; Holasek et al., 2005). Whether the p38MAPK signaling pathway participates in Aβ-induced CEC death has not been previously demonstrated. We show in the present study that p38MAPK was activated and causally related to Aβ-induced CEC death. In addition, we noted that Aβ-induced p38MAPK activation and subsequent CEC death was mediated by ASK1 and MKK3/6. These findings are consistent with the observation that the selective inhibitor of p38MAPK and dominant negative mutants of ASK1, MKK3 and MKK6 diminished Aβ-induced p38MAPK activation and CEC death.

A number of studies have indicated that p53 plays an important role in promoting cell apoptosis by influencing the transcription of pro-apoptotic genes (el-Deiry et al., 1995). Indeed, p53 is a tightly regulated protein. Aβ was recently shown to promote phosphorylation-mediated stabilization of p53 and subsequent cortical neuron death (Fogarty et al., 2003). p53 phosphorylation by p38MAPK contributed to NO-induced apoptosis (Kim et al., 2002). In agreement with this observation, we noted that the dominant negative mutants of ASK1, MKK3 and MKK6, or, SB203580, a selective p38MAPK inhibitor, prevented Aβ-induced p53 phosphorylation and CEC death. Thus, it is plausible that Aβ activates the ASK1-MKK3/6-p38MAPK cascade to cause p53 phosphorylation and subsequent cell death.

The Bcl2 family proteins regulate mitochondria-mediated apoptosis with the balance of the anti- and pro-apoptotic members arbitrating the life-or-death decisions. Bax, a pro-apoptotic member of the Bcl-2 family causes apoptosis by disrupting mitochondrial integrity. *bax* expression is induced by p53 in response to selected stress signals (Zhang et al., 2005). In the present study, *bax* RNAi attenuated Aβ-induced CEC apoptosis, suggesting that Bax expression is causally related to Aβ-induced CEC apoptosis. In addition to Bax, recent reports have indicated that BH3-only members of Bcl2 family, such as Bim (Yin et al., 2002) and Bad (Yin et al., 2005), also participate in Aβ-induced CEC death. The link between these pro-apoptotic Bcl-2 family proteins-mediated cell death pathways remains to be established. Moreover, transcription factors other than p53 may also contribute to Aβ-induced CEC apoptosis. These include AP-1 (Yin et al., 2002) and FKHRL1 (Yin et al., 2006). These observations explain, at least in part, why blocking the ASK1 signaling cascade could not completely abolish Aβ-induced CEC death.

As described above, PP2A appears to play an important role in activating ASK1. PP2A is a member in the ceramide-activated protein phosphatase (CAPP) family. We have previously established that Aβ-induced death of non-neuronal cells including CECs, astrocytes (Yang et al., 2004) and oligodendrocytes (Lee et al., 2004) involved activation of neutral sphingomyelinase to release ceramide from membrane sphingomyelin. Results from the present studies suggest that ceramide activation of PP2A may be involved in Aβ-induced death paradigms in CECs by dephosphorylating both Akt (Yin et al., 2006) as well as ASK1. Taken together, these results suggest that Aβ activation of PP2A may activate at least 2 separate pathways, one on the Akt-FKHRL1-Bim cascade (Yin et al.,

2006) and another on the ASK1-MAP kinase-p53-Bax cascade as reported here. The differential mechanisms of Aβ actions in driving these 2 separate death signaling pathways downstream of PP2A remain to be elucidated. It is likely that 2 different pathways culminating in Bim and Bax expression respectively are in cooperation. Both Bim and Bax are Bcl-2 family proteins. However, Bim is a pro-apoptotic protein belonging to the BH3-only subfamily while Bax is not. Whether Bim and Bax act in sequence or in synergy remains controversial (Willis and Adams, 2005). Additional works are needed to characterize the interrelationship between Bim and Bax in Aβ-induced CEC death

In conclusion, our present study demonstrated for the first time that Aβ initiates apoptosis may through the PP2A-ASK1-MKK3/6-p38MAPK signaling cascade to induce p53 activation and Bax expression in CECs (Figure 8).

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Figure legends

Figure 1. ASK1 in Aβ-induced CEC apoptosis. ***A.*** CECs were transiently transfected with pcDNA (mock transfection) or HA-ASK1-DN for 24 h. Following transfection, cells were treated with vehicle or 20 μM Aβ for another 48 h, and cell viability was determined by the MTT assay. Each column represents the mean ± S.E.M. of at least three independent experiments performed in duplicate. *, $P < 0.05$ as compared with the pcDNA group in the presence of Aβ ***B.*** CECs were treated with 20 μM Aβ for the indicated time intervals, and ASK1 kinase activity was assessed by *in vitro* kinase assay using MBP as a substrate. After SDS-PAGE, γ-^{32}P-labeled MBP was visualized by autoradiography. Immunoblotting confirming equal amount of immunoprecipitated ASK1 for each sample is shown in the lower panel. Data shown are representative in three separate experiments with similar results. **KA,** kinase assay; **IB,** immunoblotting. ***C.*** CECs were treated with 20 μM Aβ for various time intervals as indicated. Cell lysates were then prepared and subjected to immunoblotting with anti-pSer967-ASK1 antibody. Equal loading in each lane is reflected by similar intensities of ASK1 in the lower panel. *, $P < 0.05$ as compared with the control group. ***D.*** Cells were treated with 20 μM Aβ for 0-30 min, then immunoprecipitated with the anti-ASK1 antibody. The immunoprecipitated complex was then subjected to immunoblotting with an anti-14-3-3 antibody. Typical bands representative of three separate experiments with similar results are shown. Immunoblotting confirming equal amount of immunoprecipitated ASK1 for each sample is shown in the lower panel. **IP,** immunoprecipitation; **IB,** immunoblotting. ***E.*** CECs were pretreated with vehicle, 1-10 nM okadaic acid or 100 nM cyclosporine A for 30 min before treatment with 20 μM Aβ for another 10 min. Cell lysates were then

prepared and subjected to immunoblotting with anti-pSer967-ASK1 antibody. Equal loading in each lane is reflected by similar intensities of ASK1 in the lower panel. *, $P < 0.05$ as compared with the vehicle-treated group in the presence of Aβ

Figure 2. p38MAPK in Aβ-induced CEC apoptosis ***A.*** CECs were pretreated with vehicle or 10 μM SB203580, (specific p38MAPK inhibitor) for 30 min before treatment with 20 μM Aβ for another 48 h. The percentage of apoptotic cells was then analyzed by flowcytometric analysis of PI-stained cells as described in Material and Methods. Compiled results are shown in the lower panel. Each column represents the mean ± SEM of at least three independent experiments. *, $P < 0.05$ as compared with the group treated with Aβ alone. ***B.*** CECs were treated with 20 μM Aβ for 0-60 min and p38MAPK phosphorylation was determined by immunoblotting with anti-phospho-p38MAPK antibody. Equal loading in each lane is reflected by approximately similar intensities of p38MAPK in the lower panel. *, $P < 0.05$ as compared with the control group ***C.*** Cells were treated with 20 μM Aβ for 0-60 min, and p38MAPK activity was assessed by *in vitro* kinase assay using MBP as a substrate. After SDS-PAGE, γ-^{32}P-labeled MBP was visualized by autoradiography. Typical bands representative of three independent experiments with similar results are shown. **KA,** kinase assay; **IB,** immunoblotting.

Figure 3. ASK1, MKK3 and MKK6 in Aβ-induced p38MAPK activation and CEC death ***A.*** CECs were transiently transfected with pcDNA or ASK1 DN for 24 h. Following transfection, CECs were treated with vehicle or 20 μM Aβ for indicated time

intervals and harvested for assessing the level of p38MAPK phosphorylation by immunoblotting with an anti-phospho-p38MAPK antibody. Equal loading in each lane is reflected by approximately similar intensities of the GAPDH bands. Compiled results are shown in the lower panel. *, $P < 0.05$ as compared with the pcDNA (mock transfection) group in the presence of Aβ ***B.*** CECs were transiently transfected with pcDNA, MKK3 DN or MKK6 DN for 24 h. Following transfection, cells were treated with vehicle or 20 μM Aβ for 30 min and then harvested and the level of p38MAPK phosphorylation was determined as described above in ***A.*** Compiled results are shown in the lower panel. *, $P < 0.05$ as compared with the pcDNA (mock transfection) group in the presence of Aβ. ***C.*** CECs were transiently transfected with pcDNA, MKK3 DN, MKK6 DN, or p38MAPK DN for 24 h. Following transfection, cells were treated with vehicle or 20 μM Aβ for another 48 h, and cell viability was determined by the MTT assay. Each column represents the mean ± SEM of at least three independent experiments in duplicate. *, $P < 0.05$ as compared with the pcDNA (mock transfection) group in the presence of Aβ.

Figure 4. p53 phosphorylation and p53 EMSA in Aβ-induced CEC apoptosis. ***A.*** CECs were treated with 20 μM Aβ for indicated time periods. Cells were then harvested and p53 phosphorylation at Ser15 residue was determined by immunoblotting with an anti-pSer15-p53 antibody. Equal loading in each lane is shown by the similar intensities of GAPDH. Compiled results are shown in the lower panel. *, $P < 0.05$ as compared with the control group ***B.*** CECs were incubated with 20 μM Aβ for 1 and 2 h. Following incubation, the nuclear protein fraction was prepared for EMSA as described in Materials and Methods. ***C.*** An anti-p53 antibody was included prior to EMSA to detect the specific

binding p53 binding activity. Data shown are representative of three independent experiments with similar results.

Figure 5. Involvement of ASK1, MKK3, MKK6 and p38MAPK in Aβ-induced p53 phosphorylation in CECs ***A.*** CECs were transiently transfected with pcDNA or ASK1 DN for 24 h. Following transfection, cells were treated with vehicle or 20 μM Aβ for 1 h and then harvested for immunoblotting to assess the level of p53 phosphorylation at Ser15 residue using an anti-pSer15-p53 antibody. Equal loading in each lane is reflected by approximately similar intensities of the α-tubulin bands. Compiled results are shown in the lower panel. *, $P < 0.05$ as compared with the pcDNA (mock transfection) group in the presence of Aβ ***B.*** CECs were transiently transfected with pcDNA, MKK3 DN or MKK6 DN for 24 h. Following transfection, cells were treated with vehicle or 20 μM Aβ for 1 h and then harvested and the level of p53 phosphorylation at Ser15 residue was determined as described in ***A.*** Equal loading in each lane is reflected by approximately similar intensities of the α-tubulin bands. Compiled results are shown in the lower panel. *, $P < 0.05$ as compared with the pcDNA (mock transfection) group in the presence of Aβ ***C.*** CECs were treated with 10 μM SB203580 (specific p38MAPK inhibitor) for 30 min, followed by vehicle or 20 μM Aβ for another 1 h and then harvested for assessing the level of p53 phosphorylation at Ser15 residue as described in **A.** Equal loading in each lane is reflected by approximately similar intensities of the α-tubulin bands. Compiled results are shown in the lower panel. *, $P < 0.05$ as compared with the pcDNA (mock transfection) group in the presence of Aβ

Figure 6. Bax expression in Aβ-induced CEC apoptosis. ***A.*** CECs were treated with 20 μM Aβ for 24-72 h and then harvested for assessing the extent of Bax expression by immunoblotting with a specific antibody against Bax. Compiled results are shown in the lower panel. *, $P < 0.05$ as compared with the control group ***B.*** CECs were transfected with control RNAi or *bax* RNAi for 24 h. Following transfection, cells were treated with vehicle or 20 μM Aβ for another 48 h. The expression of Bax was then determined by immunoblotting with an anti-Bax antibody. Data shown are representative of three independent experiments with similar results. ***C.*** CECs were transiently transfected with control RNAi or *bax* RNAi for 24 h. Following transfection, cells were treated with vehicle or 20 μM Aβ for another 48 h, and cell viability determined by the MTT assay. *, $P < 0.05$ as compared with the control RNAi group in the presence of Aβ

Figure 7. ASK1, MKK3, MKK6 and p38MAPK in Aβ-induced Bax expression in CECs ***A.*** Cells were transiently transfected with pcDNA or ASK1 DN for 24 h. Following transfection, cells were treated with vehicle or 20 μM Aβ for another 48 h. Cells were then harvested and the extent of Bax expression was determined by immunoblotting with an anti-Bax antibody. Equal loading in each lane is shown by approximately similar intensities of the α-tubulin bands. Compiled results are shown in the lower panel. *, $P < 0.05$ as compared with the pcDNA (mock transfection) group in the presence of Aβ ***B.*** CECs were transiently transfected with pcDNA, MKK3 DN or MKK6 DN for 24 h. Following transfection, cells were treated with vehicle or 20 μM Aβ for another 48 h and then harvested for assessing the extent of Bax expression as decribed in ***A.*** Equal loading in each lane is shown by apprximately similar intensities of the α-tubulin bands.

Compiled results are shown in the lower panel. *, $P < 0.05$ as compared with the pcDNA (mock transfection) group in the presence of Aβ ***C.*** CECs were pretreated with vehicle or 10 μM SB203580 (specific p38MAPK inhibitor) for 30 min, followed by vehicle or 20 μM Aβ for another 48 h and then harvested for assessing the extent of Bax expression as described in **A.** Equal loading in each lane is shown by apprximately similar intensities of the α-tubulin bands. Compiled results are shown in the lower panel. *, $P < 0.05$ as compared with the pcDNA (mock transfection) group in the presence of Aβ

Figure 8. Schematic summary of ASK1-mediated apoptotic pathway in Aβ-induced CEC death. Aβ activates the PP2A-ASK1-MKK3/6-p38MAPK signaling cascade, leading to p53 activation and Bax expression in Aβ-induced CEC death. Approaches applied in the present studies are shown to support the causal role of each step in the cascade.

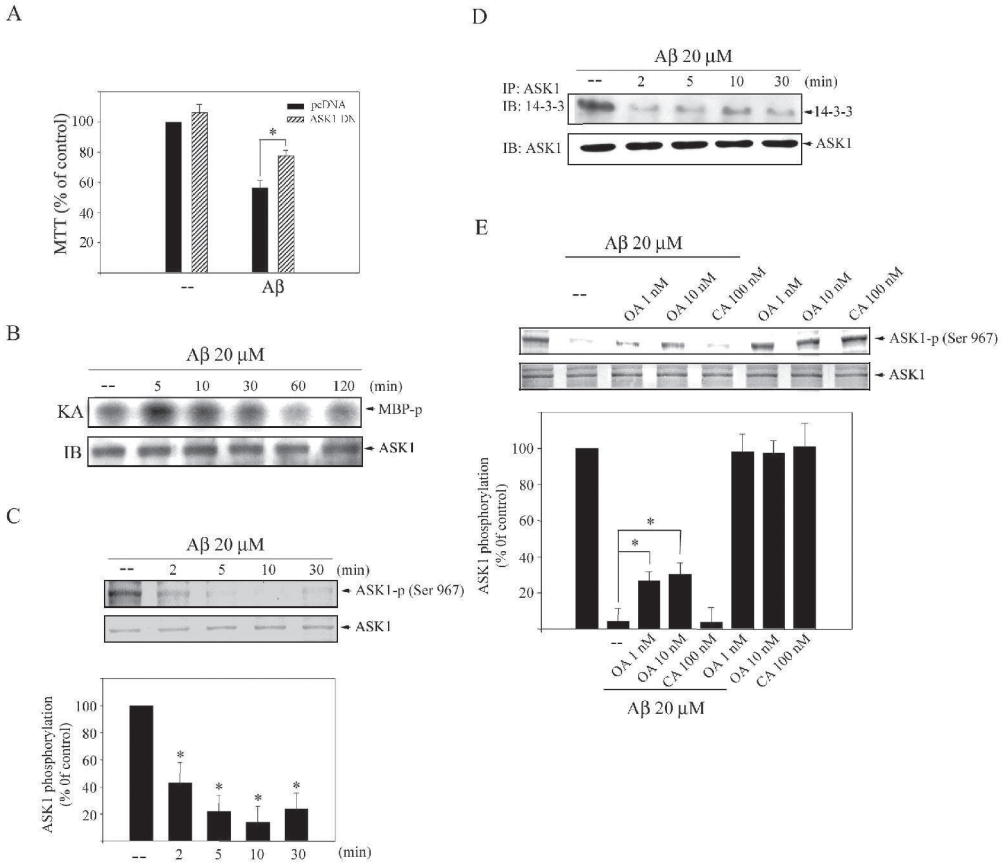
A
MTT (% of control)
pcDNA
ASK1 DN
Aβ
B
Aβ 20 μM
(min)
KA
MBP-p
IB
ASK1
C
Aβ 20 μM
(min)
ASK1-p (Ser 967)
ASK1
ASK1 phosphorylation (% 0f control)
D
Aβ 20 μM
(min)
IP: ASK1
IB: 14-3-3
14-3-3
IB: ASK1
ASK1
E
Aβ 20 μM
OA 1 nM
OA 10 nM
CA 100 nM
ASK1-p (Ser 967)
ASK1
ASK1 phosphorylation (% 0f control)

Fig.1

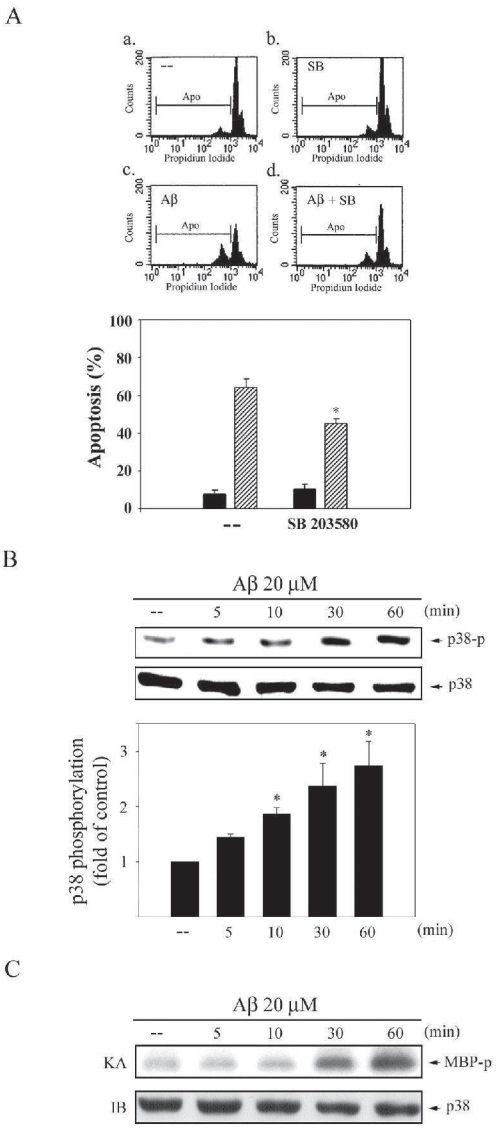
A
a.
--
Apo
b.
SB
Apo
c.
Aβ
Apo
d.
Aβ + SB
Apo
Counts
Propidium Iodide
Apoptosis (%)
100
80
60
40
20
0
--
SB 203580
B
Aβ 20 μM
-- 5 10 30 60 (min)
p38-p
p38
p38 phosphorylation
(fold of control)
3
2
1
-- 5 10 30 60 (min)
C
Aβ 20 μM
-- 5 10 30 60 (min)
KA
MBP-p
IB
p38

Fig. 2

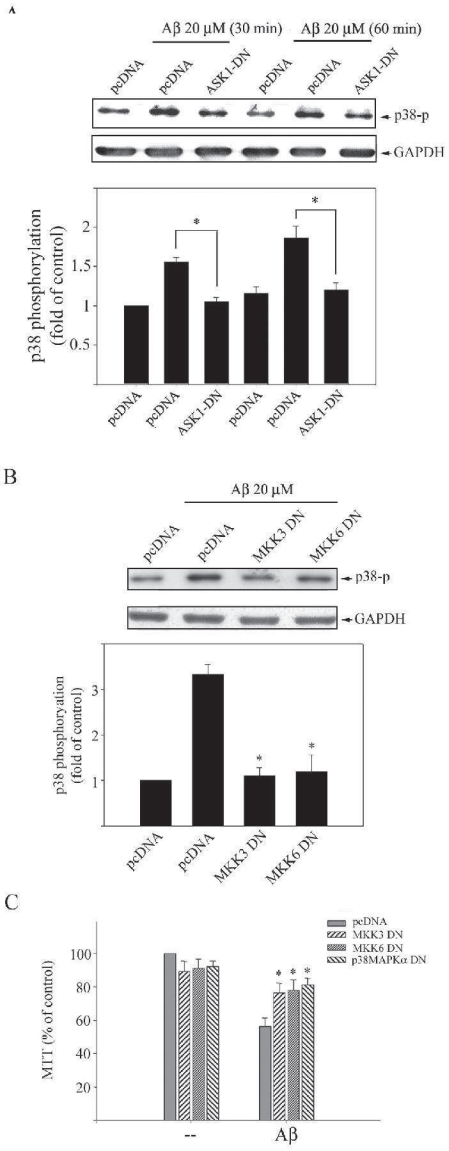
A
Aβ 20 μM (30 min)
Aβ 20 μM (60 min)
peDNA
peDNA
ASK1-DN
peDNA
peDNA
ASK1-DN
p38-p
GAPDH
p38 phosphorylation (fold of control)
2
1.5
1
0.5
*
*
peDNA
peDNA
ASE1-DN
peDNA
peDNA
ASK1-DN
B
Aβ 20 μM
peDNA
peDNA
MKK3 DN
MKK6 DN
p38-p
GAPDH
p38 phosphoryation (fold of control)
3
2
1
*
*
peDNA
peDNA
MKK3 DN
MKK6 DN
C
peDNA
MKK3 DN
MKK6 DN
p38MAPKα DN
MTT (% of control)
100
80
60
40
20
*
*
*
--
Aβ

Fig. 3

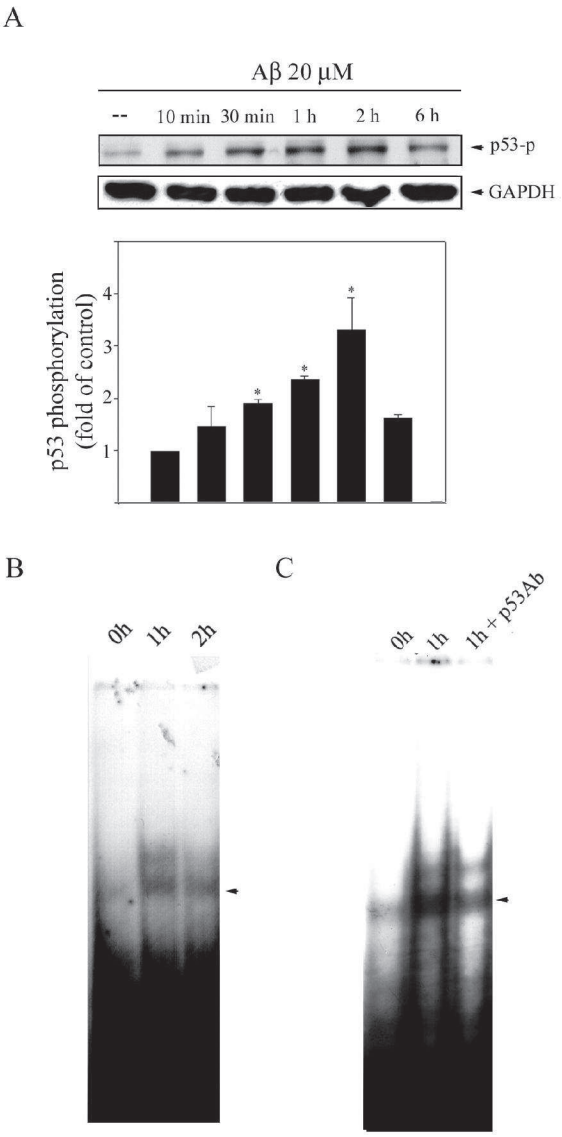
A
Aβ 20 μM
--
10 min
30 min
1 h
2 h
6 h
p53-p
GAPDH
p53 phosphorylation
(fold of control)
1
2
3
4
B
C
0h
1h
2h
0h
1h
1h + p53Ab

Fig. 4

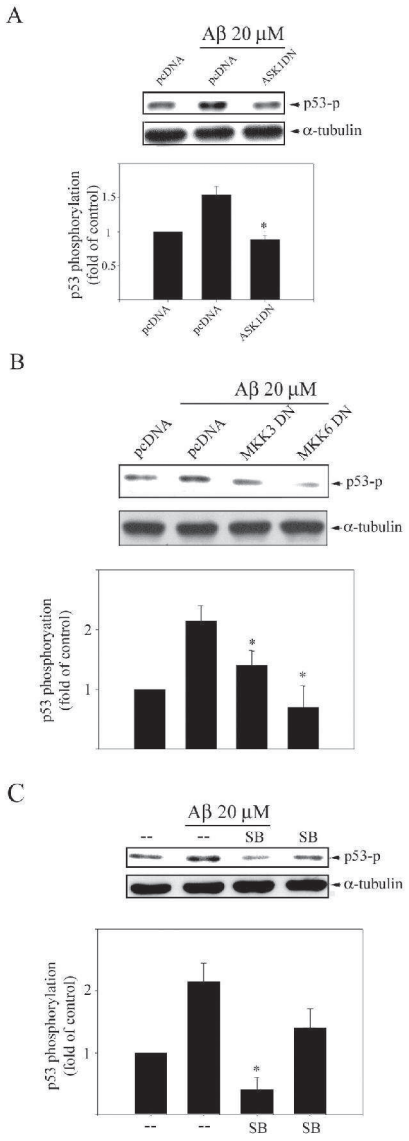
A
Aβ 20 μM
pcDNA
pcDNA
ASK1DN
p53-p
α-tubulin
p53 phosphorylation (fold of control)
1.5
1
0.5
*
pcDNA
pcDNA
ASK1DN
B
Aβ 20 μM
pcDNA
pcDNA
MKK3 DN
MKK6 DN
p53-p
α-tubulin
p53 phosphorylation (fold of control)
2
1
*
*
C
Aβ 20 μM
--
--
SB
SB
p53-p
α-tubulin
p53 phosphorylation (fold of control)
2
1
*
--
--
SB
SB

Fig. 5

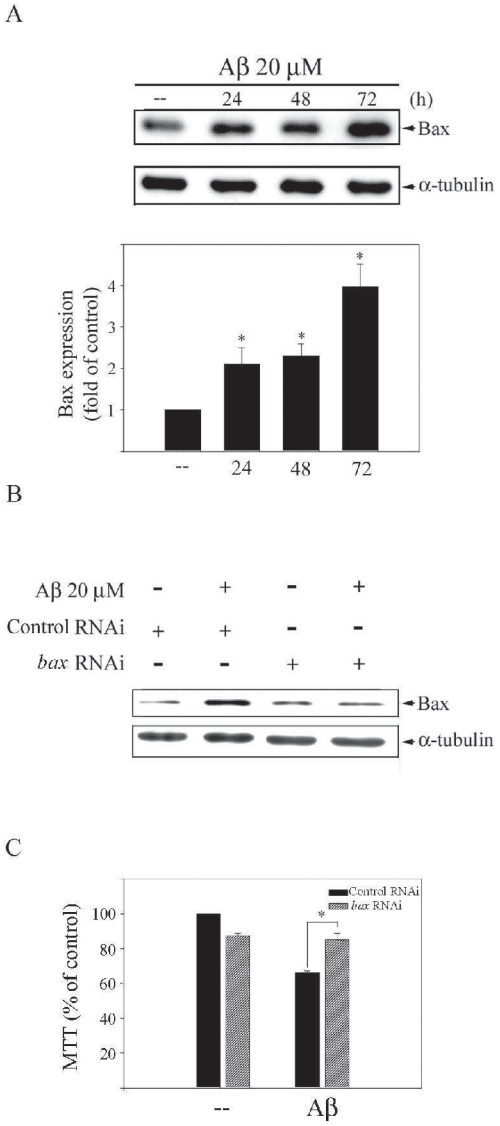
A
Aβ 20 μM
-- 24 48 72 (h)
Bax
α-tubulin
Bax expression (fold of control)
1 2 3 4
-- 24 48 72
B
Aβ 20 μM - + - +
Control RNAi + + - -
bax RNAi - - + +
Bax
α-tubulin
C
MTT (% of control)
20 40 60 80 100
Control RNAi
bax RNAi
--
Aβ

Fig. 6

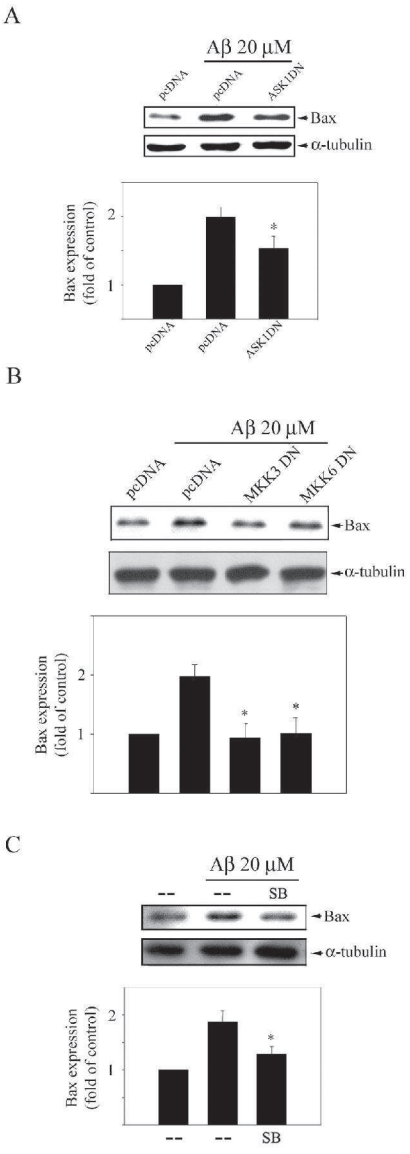
A
Aβ 20 μM
pcDNA
pcDNA
ASK1DN
Bax
α-tubulin
Bax expression
(fold of control)
2
1
*
pcDNA
pcDNA
ASK1DN
B
Aβ 20 μM
pcDNA
pcDNA
MKK3 DN
MKK6 DN
Bax
α-tubulin
Bax expression
(fold of control)
2
1
*
*
C
Aβ 20 μM
--
--
SB
Bax
α-tubulin
Bax expression
(fold of control)
2
1
*
--
--
SB

Fig. 7

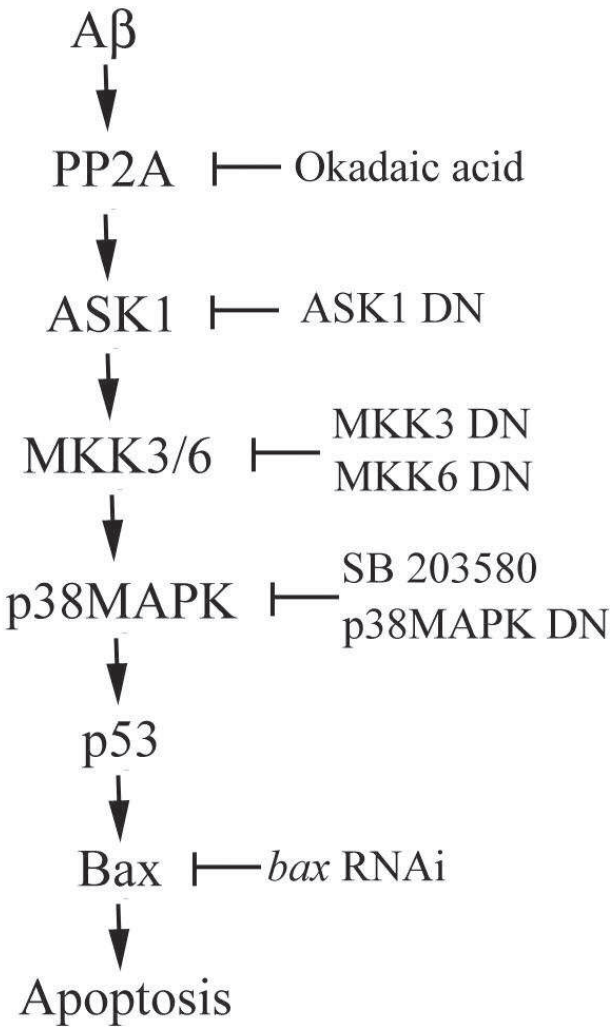
Aβ
PP2A
Okadaic acid
ASK1
ASK1 DN
MKK3/6
MKK3 DN
MKK6 DN
p38MAPK
SB 203580
p38MAPK DN
p53
Bax
bax RNAi
Apoptosis

Fig. 8