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ARTICLE

β-Ecdysterone protects SH-SY5Y cells against β-amyloid-induced apoptosis via c-Jun N-terminal kinase- and Akt-associated complementary pathways

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Abstract

Recently, the significantly higher incidence of Alzheimer's disease (AD) in women than in men has been attributed to the loss of neuroprotective estrogen after menopause. Does phytoestrogen have the ability to protect against amyloid-β (Aβ) toxicity? The aim of this study was to evaluate hypothesis that β-ecdysterone (β-Ecd) protects SH-SY5Y cells from Aβ-induced apoptosis by separate signaling pathways involving protein kinase B (Akt) and c-Jun N-terminal kinase (JNK). Here, we demonstrate that phytoestrogen β-Ecd inhibits Aβ-triggered mitochondrial apoptotic pathway, as indicated by Bcl-2/Bax ratio elevation, cytochrome *c* (cyt *c*) release reduction, and caspase-9 inactivation. Interestingly, β-Ecd upregulates Bcl-2 expression in SH-SY5Y cells under both basal and Aβ-challenged conditions, but downregulates Bax expression only in Aβ-challenged conditions. Subsequently, Akt-dependent NF-κB activation is required for Bcl-2 upregulation, but not Bax downregulation, in response to β-Ecd, which was validated by the use of LY294002 and Bay11-7082. Notably, β-Ecd attenuates the Aβ-evoked reactive oxygen species (ROS) production, apoptosis signal-regulating kinase 1 (ASK1) phosphorylation and JNK activation without altering the basal ASK1 phosphorylation and JNK activation. ROS-scavenging by diphenyleneiodonium (DPI) abrogated the ability of β-Ecd to alter the activation of ASK1. Simultaneously, inhibition of JNK by SP600125 abolished β-Ecd-induced Bax downregulation in Aβ-challenged SH-SY5Y cells, whereas LY294002 failed to do so. Consequently, β-Ecd possesses neuroprotection by different and complementary pathways, which together promote a Bcl-2/Bax ratio. These data support our hypothesis and suggest that β-Ecd is a promising candidate for the treatment of AD.

Introduction

Alzheimer's disease (AD) is the most common form of dementia among the elderly [1]. Diverse lines of evidence suggest that β-amyloid (Aβ) peptide has a causal role in its pathogenesis [2]. Treating AD is the single biggest unmet medical need in neurology. Current drugs improve symptoms, but do not have profound disease-modifying effects [3]. The development of an effective therapeutic intervention for AD is crucial for public health.

Apoptosis is a physiological response that occurs during development of the nervous system. Despite the differences in clinical manifestations and neuronal vulnerability, aberrant apoptosis seems to play a key role in the progression of several neurodegenerative disorders [4]. The incidence of apoptosis is elevated in analysis of postmortem AD brains [5]. Aβ induces neuronal apoptosis in the brain [6], in transgenic mice [7], and in neuronal cell cultures [8]. Mitochondrial cascade hypothesis proposes that mitochondrial dysfunction is the primary event in AD pathology involving free radicals production and apoptosis triggering [9].

Though it remains unclear whether oxidative stress is a major cause or merely a consequence of mitochondrial dysfunctions associated with AD [10], supplementation

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with antioxidants is reported to be beneficial especially in the early stages of the disease [11]. A variety of natural antioxidants have evolved in plants, including medicinal herb, to respond to natural oxygenic atmosphere [12]. Indeed, it has been well documented that various plant-derived components, including phytoestrogen, possess their ability to reduce oxidative stress [13].

Reactive oxygen species (ROS) are not only toxic to neurons, but also to second messengers in intracellular signal transduction [14]. Accumulating evidence indicates that apoptosis signal-regulating kinase 1 (ASK1), a mediator of the c-Jun N-terminal kinase (JNK) pathway, which regulates mitochondria-dependent apoptosis, is highly sensitive to oxidative stress [15]. The protein kinase B (Akt) has been shown to play a pivotal role in neuroprotection by regulating the Bcl-2 family of proteins [16]. Pharmacological inhibition of oxidative stress confers protection from AD-related neurotoxin Aβ toxicity [17].

Because depletion of 17-β-estradiol is an established risk factor for AD in post-menopausal women [18], phytoestrogen have become interesting candidates for a possible prevention and/or treatment of AD [19]. Phytoestrogen β-Ecdysterone (β-Ecd) derived from the *Achyranthes bidentata* BI., which is used in folk medicine to improve memory function, has been reported to exhibit neuroprotective effects and antioxidant properties [20]. It is reasonable to postulate that β-Ecd may be beneficial for AD.

Because our previous work has shown that the neuroprotective effects of β-Ecd is based on its antioxidant properties [21], this study was initiated to address two questions: (i) does β-Ecd prevents Aβ-Induced apoptosis? (ii) if so, which signaling pathwaysare involved in β-Ecd-mediated neuroprotection in a Aβ toxicity context? To address these issues, we hypothesize that β-Ecd would prevent or attenuate Aβ-induced apoptosis via association of Akt and JNK-mediated cell signaling pathway. Our findings support the hypothesis and suggest that β-Ecd may be a promising candidate for the treatment of AD.

Material and methods

Cell culture and treatment

Human neuroblastoma cells (SH-SY5Y) obtained from the Shanghai Institute of Cell Biology (Shanghai, China) were grown in Dulbecco's modified Eagle's medium, supplemented with 1% penicillin–streptomycin and 10% fetal calf serum, and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Cultured SH-SY5Y cells were pre-treated β-Ecd for 1 h and then exposed to Aβ(25–35) (Sigma, St. Louis, MO) for 24 h. For inhibitor studies, SH-SY5Y cells pre-treated with JNK inhibitor SP600125, Akt inhibitor LY294002, NF-κB inhibitor Bay11-7082, or NADPH oxidase inhibitor diphenyleneiodonium (DPI) for 30 min before the indicated treatment. For ASK1 knockdown studies, cells transfected with ASK1 short hairpin RNA (shRNA) plasmid, then allowed to incubate for 24 h, and treated as described above. To avoid any reversible action, the inhibitors were maintained throughout the duration of the experiment.

Analyses of cell viability

The number of viable cells was quantified using a MTS/PMS reagent based CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega) according to the manufacturer's instructions.

Detection of apoptosis

Apoptosis was determined using the Annexin V-FITC/propidium iodide (PI) Apoptosis Detection Kit (KeyGEN BioTECH, Nanjing, China) according to the instructions from the manufacturer. The population of Annexin V-positive cells was evaluated by flow cytometry (BD Biosciences, San Jose, CA), as described previously [22].

Caspase activity assay

Measurements of caspase activities in cells were performed using the commercially available Caspase-Glo 3/7 Assay (Promega, Madison, WI), Caspase-Glo 8 Assay (Promega), Caspase 9 Activity Colorimetric Assay Kit (Biobox Biotech, Nanjing, China), and Caspase-12 Fluorometric Assay Kit (BioVision) according to the manufacturer's instructions.

Measurement of ROS

Intracellular ROS were estimated using the fluorescent probe 2′,7′-dichlorfluorescein-diacetate (DCFH-DA) provided in the OxiSelect Intracellular ROS Assay Kit obtained from Cell Biolabs Inc. (San Diego, CA). The DCF emission was recorded by fluorimetry (excitation at 485 nm, emission at 538 nm).

Calpain activity assay

Calpain activity was measured by using a commercially available calpain activity assay kit from Biovision (Milpitas, CA) as suggested by the manufacture's protocol. The level of AFC released from fluorogenic substrate Ac-LLY-AFC was measured by fluorometry.

DNA fragmentation assay

DNA fragmentation was quantitatively evaluated by Cell Death Detection ELISA^{PLUS} Assay Kit (Roche Molecular Biochemicals, Indianapolis, IN) that uses mouse monoclonal antibodies against DNA and histones, according to the manufacturer's instructions.

Nuclear factor kappa B (NF-κB) activity assay

NF-κB DNA-binding activity was analyzed using the commercially available TransAM NF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA) on nuclear extracts following the manufacturer's instructions. This kit is based on an enzyme-linked immunosorbent assay (ELISA) format and oligonucleotide containing the NF-κB consensus-binding sequence (5′-GGGACTTTCC-3′) immobilized on a 96-well plate. The active forms of NF-κB in the nuclear extracts were bound to the oligonucleotides and detected colorimetrically.

Western blot

Cell lysates from SH-SY5Y cells were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. Blots were incubated with the desired primary antibodies and then incubated with peroxidase-conjugated secondary antibodies in Tris buffered saline (TBS)-Tween containing non-fat dry milk, as previously described. The antigen–antibody complexes' chemiluminescence was detected by using the enhanced chemiluminescence system detection kit (Pierce, Rockford, lL).

Real-time RT-PCR

Total RNA was extracted from SH-SY5Y cells using Trizol Reagent (Invitrogen Corp., Carlsbad, California) according to the manufacturer's recommendation. Reverse transcription was performed using primeScript RT reagent kit (Takara Biotechnology, Dalian, China). Real-time PCR was performed with an Agilent Stratagene M×3005P (Agilent Stratagene, CA) with the SYBR premix Ex Taq (Takara) and the following primers: Bax: (F) 5′-ACC AGG GTG GTT GGG TGA GAC T-3′; (R) 5′-CAC CAC TGT GAC CTG CTC CAG A-3′. Bcl-2: (F) 5′-CGC CAA CAA ATA TGC AGA AG-3′; (R) 5′-TGG TGC ATC AGC AAC AAT G-3′. β-actin: (F) 5′-CAT GTA CGT TGC TAT CCA GGC-3′; (R) 5′-CTC CTT AAT GTC ACG CAC GAT-3′. Real-time RT-PCR data were analyzed according to the $2^{-\Delta\Delta ct}$ method as described [23].

Kinase assays

Akt and JNK Kinase assays were respectively performed using Akt Kinase Assay Kit (Nonradioactive) and SAPK/JNK Kinase Assay Kit (Nonradioactive) obtained commercially from Cell Signaling Technology (Beverly, MA) according to the manufacturer's instructions. Briefly, cells were treated as indicated and lysed. Cell lysates were immunoprecipitated with immobilized p-Akt mAb or p-JNK mAb, and then the immune complexes were incubated with GSK-3 fusion protein or c-jun fusion protein as a substrate in the presence of ATP. Phosphorylation of GSK-3α/β at Ser21/9 or phosphorylation of c-Jun at Ser63 was measured by western blot to quantify phosphorylated substrates.

Reporter gene activity assays

SH-SY5Y cells were transiently co-transfected with firefly luciferase reporter plasmid (pGMNF-κB-lu or PGMER-lu, Genomeditech, Shanghai, China) and renilla luciferase reporter plasmid pRL-TK (Promega) by using FuGENE 6 Transfection Reagent (Promega). Estrogen receptor (ER)-luciferase activity or NF-κB-luciferase activity was measured using a Dual-Glo Luciferase Assay System (Promega), following the protocol provided by the manufacturer.

Statistics

Unless indicated differently, the results are presented as means ± SD of at least three independent experiments. Statistical analyses were performed with SPSS software. Differences among means were assessed by ANOVA followed by Student-Newman–Keuls post hoc test. Statistical significance was accepted for P values of <0.05.

Results

β-Ecd inhibits Aβ-triggered cyt c release, caspase-9 activation, and apoptosis in SH-SY5Y cells

As shown in Fig. 1a, β-Ecd attenuated the Aβ-induced increases in cytotoxic cell death in a dose-dependent manner at 1–10 μM, and the cytoprotective effect reached a plateau at 10 μM with a 35.4% increase in cell viability over Aβ-challenged SH-SY5Y cells. Therefore, 10 μM was chosen as the maximum concentration of β-Ecd for use throughout subsequent experiments. A β-Ecd kinetic–response curve was created (Fig. 1b), and Aβ-challenged SH-SY5Y cells were incubated with β-Ecd (10

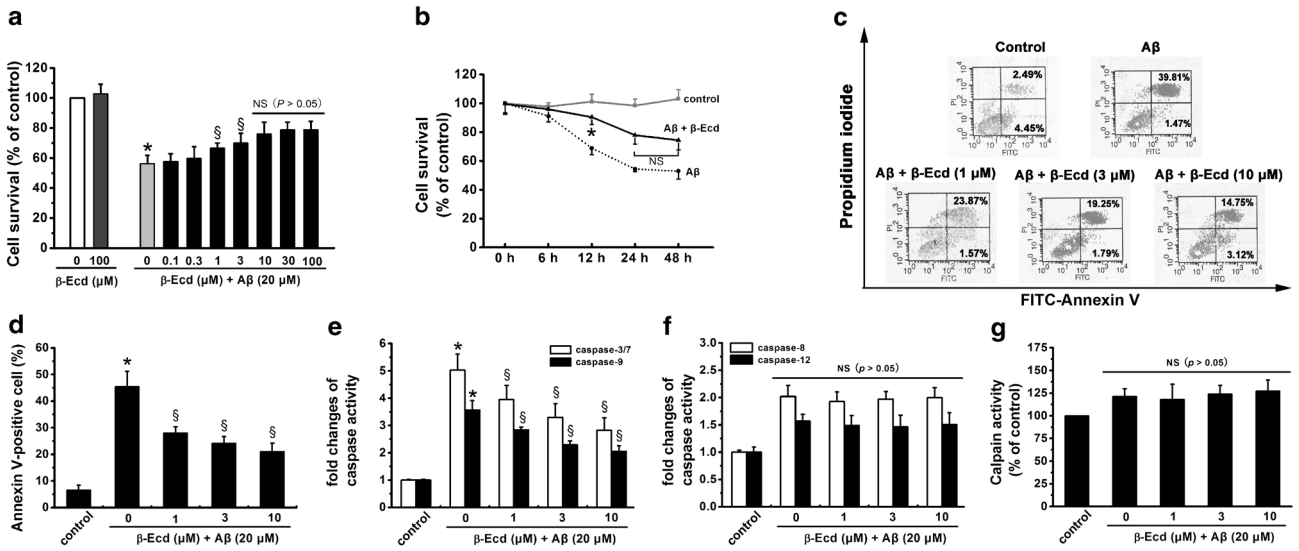

Fig. 1 β-Ecd attenuates Aβ-induced caspase-3 activation, cell viability reduction, and apoptosis in SH-SY5Y cells. SH-SY5Y cells were pre-treated with β-Ecd at increasing concentrations, as indicated, for 1 h before challenge with Aβ for an additional 24 h or indicated times. **a**, **b** Cell viability was determined by MTS assays. **c** Annexin V/FITC-PI staining and flow cytometric analysis of apoptosis. **d** Bar diagrams showing percentage of apoptosis. **e** Caspase-3/7 and caspase-9 activity were assessed by fluorometric and colorimetric assay, respectively. **f** Caspase-8 and caspase-12 activity were assessed by fluorometric assay. **g** Calpain activity was determined by fluorometric assay using Ac-LLY-AFC as the substrate. Data are expressed as means (SD) (three experiments for each condition). $^{*}p < 0.05$ versus control; $^{\S}p < 0.05$ versus Aβ alone treatment

μM) for 24 was found to be the optimum time, which was used in all of the experiments. We also confirmed these findings in primary cultured rat hippocampal neurons and PC12 cells, and obtained similar results (Supplementary Fig. S1).

Aβ-induced apoptosis is a key pathologic event in AD [24]. We next investigated whether β-Ecd inhibits Aβ-evoked apoptosis of SH-SY5Y cells. β-Ecd inhibited Aβ-induced apoptosis in SH-SY5Y cells in a dose-dependent manner, as shown by Annexin V-FITC/PI staining and flow cytometric analysis (Fig. 1c, d). Similar to the results obtained from Annexin V-FITC/PI staining, β-Ecd significantly blocked Aβ-induced increases in caspase-3/7 activity, another marker of apoptosis (Fig. 1e). The next question is which caspases are involved in β-Ecd-mediated neuroprotection. β-Ecd significantly decreased mitochondria-associated caspase-9 activity in SH-SY5Y cells exposed to Aβ (Fig. 1e), but did not significantly affect death receptors-associated caspase-8 and endoplasmic reticulum-associated caspase-12 activities (Fig. 1f). Again, β-Ecd treatment did not significantly alter calpain activity in Aβ-challenged SH-SY5Y cells (Fig. 1g). These results imply that the mitochondrial pathway, rather than the endoplasmic reticulum or death receptor pathway may contribute to the neuroprotective effect of β-Ecd.

β-Ecd elevates the ratio of Bcl-2/Bax in Aβ-challenged SH-SY5Y cells

Given that caspase-9 activation in the cytosol is the result of the loss of integrity of the outer mitochondrial membrane caused by proapoptotic members of the Bcl-2 family [25], we examined whether β-Ecd might alter expression of Bcl-2 and Bax proteins under basal conditions and in the context of Aβ challenge. Western blot analysis presented in Fig. 2a, b indicate that β-Ecd increased basal Bcl-2 expression, whereas the basal levels of Bax expression remained unchanged. Next, to explore whether β-Ecd affects the Aβ-induced changes in Bcl-2 and Bax expression, SH-SY5Y cells were pre-treated with 1, 3, and 10 μM β-Ecd for 1 h, followed by exposure to 20 μM Aβ(25–35) for 24 h. The results showed that β-Ecd pre-treatment attenuated both Aβ-induced Bcl-2 downregulation and Bax upregulation (Fig. 2c, d). Since Bcl-2 and Bax share the same events, including release of cyt *c* [26], we analyzed the expression of cytoplasm cyt *c* by western blot analysis. As depicted in Fig. 2e, β-Ecd attenuated release of cyt *c* induced by Aβ (25–35). As shown in Fig. 2f, g, β-Ecd dose-dependently upregulates Bcl-2 expression at transcription levels under both basal conditions and Aβ challenge context. However, β-Ecd downregulates Bax expression at transcription levels only in the context of Aβ challenge, suggesting that β-Ecd

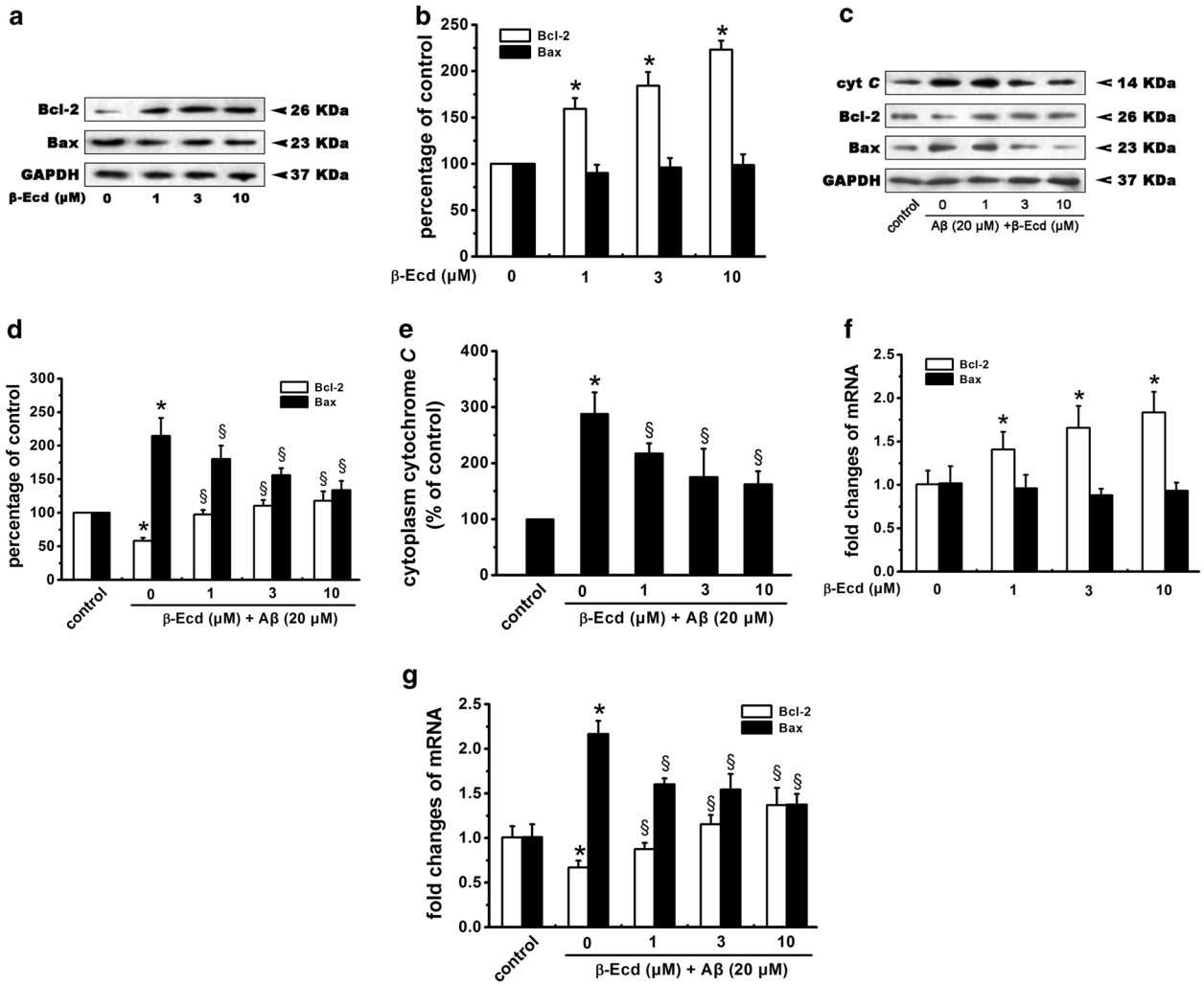

Fig. 2 β-Ecd increases Bcl-2/Bax ratio and suppresses mitochondrial pro-apoptotic pathway in Aβ-challenged SH-SY5Y cells. **a** SH-SY5Y cells were treated with β-Ecd at 1, 3, and 10 μM for 24 h. **a** Levels of Bcl-2 and Bax were analyzed by western blot. **b** The band intensity of Bcl-2 and Bax were quantified by densitometry and normalized to GAPDH. **c** SH-SY5Y cells were pre-treated with β-Ecd at 1, 3, and 10 μM for 1 h before challenge with Aβ for an additional 24 h. Levels of Bcl-2, Bax, and cyt *c* were analyzed by western blot. **d** The band intensity of Bcl-2 and Bax were quantified by densitometry and normalized to GAPDH. **e** The band intensity of cytoplasm cyt *c* was quantified by densitometry and normalized to GAPDH. **f** SH-SY5Y cells were treated with β-Ecd at 1, 3, and 10 μM for 24 h. RNA was extracted by the Trizol method, and the levels of Bcl-2 and Bax mRNA were determined by real-time PCR. **g** SH-SY5Y cells were pre-treated with β-Ecd at 1, 3, and 10 μM for 1 h before challenge with Aβ for an additional 24 h. RNA was extracted by the Trizol method, and the levels of Bcl-2 and Bax mRNA were determined by real-time PCR. Data are expressed as means (SD) (three experiments for each condition). $^{*}p < 0.05$ versus control; $^{§}p < 0.05$ versus Aβ alone treatment

acts in an Aβ challenge-dependent manner to regulate Bax expression.

β-Ecd activates the Akt–NF-κB pathway

An overwhelming body of evidence indicates that the mammalian Akt pathway activates the NF-κB signaling that elevated pro-survival Bcl-2 proteins [27]. Thus, the next question is whether Akt kinase activity is activated following β-Ecd treatment. As illustrated in Fig. 3a, b, β-Ecd constitutively activated Akt activity measured by an in vitro kinase assay under basal conditions and in the context of Aβ challenge, while Aβ had no significant effect on Akt activity. Since NF-κB activation can promote neuron survival by inducing the production of the anti-apoptotic proteins in the Bcl-2 family [28], we sought to determine whether β-Ecd inhibited NF-κB promoter activity in SH-SY5Y cells. This hypothesis was confirmed by an ELISA-based NF-κB DNA-binding activity assay, which showed that β-Ecd strongly increases the NF-κB DNA-binding activity under basal conditions through an Akt-dependent mechanism (Fig. 3c). Inhibition of Akt by LY294002 or blockade of NF-κB by Bay11-7082 abolished β-Ecd-induced upregulation of Bcl-2 in SH-SY5Y cells, whereas

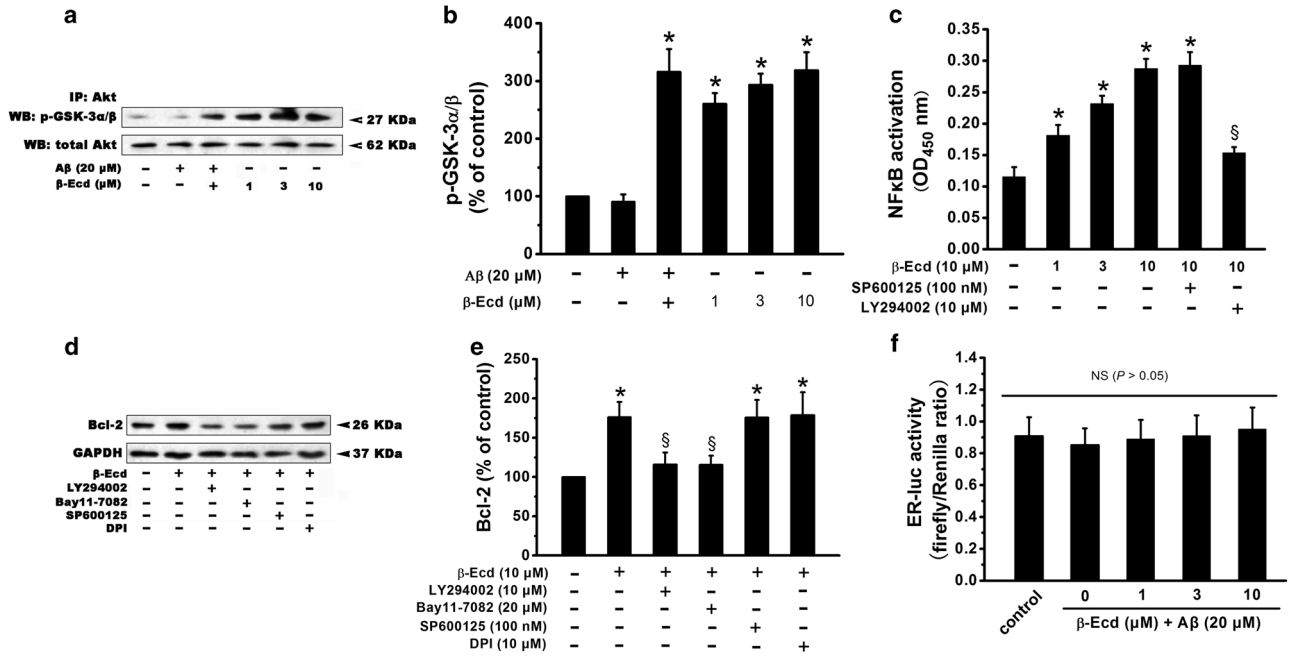

Fig. 3 β-Ecd induces constitutive Akt activation and NF-κB activity in SH-SY5Y cells. **a** SH-SY5Y cells were treated with β-Ecd for 24 h in the presence or absence of Aβ. Kinase activity of Akt immunoprecipitated with immobilized p-Akt mAb was determined using GSK-3 as a substrate. **b** The intensity of p-GSK-3α/β immunobands were quantified by densitometry and normalized to total Akt. **c** SH-SY5Y cells were treated with β-Ecd for 24 h in the presence or absence of LY294002 or SP600125. NF κB activity was colorimetrically evaluated using ELISA. **d** SH-SY5Y cells were pre-treated with or without LY294002, Bay11-7082, SP600125 or DPI for 30 min and then treated with β-Ecd for 24 h. Level of Bcl-2 was analyzed by western blot. **e** The band intensity of Bcl-2 was quantified by densitometry and normalized to GAPDH. **f** SH-SY5Y cells were co-transfected with plasmid PGMER-Lu and pRL-TK, and then treated as indicated and analyzed for luciferase activity. Data are expressed as means (SD) (three experiments for each condition). $^{*}p < 0.05$ versus control; $^{\S}p < 0.05$ versus Aβ alone treatment

SP60025 or DPI failed to do so (Fig. 3d, e), strongly suggesting that activated Akt–NF-κB signaling pathway is involved in the β-Ecd-induced expression of Bcl-2 gene under basal conditions. ER-dependent luciferase activity in SH-SY5Y cells showed a trend but not a statistically significant elevation in β-Ecd-treated versus untreated cells (Fig. 3f). ER specific antagonists ICI-182780 did not affect neuroprotection mediated by β-Ecd in Aβ-challenged SH-SY5Y cells, suggesting that β-Ecd exerts neuroprotective action through a ER-independent mechanism (Supplementary Fig. S2).

β-Ecd inhibits both ASK1 and JNK activation induced by Aβ in a ROS-dependent manner

The pro-apoptotic protein Bax plays a central role in the mitochondria-dependent apoptotic pathway, and Bax expression is regulated by JNK at a transcriptional level [29]. Next we tested if Aβ-induced JNK activation is reduced in SH-SY5Y cells in response to β-Ecd. As shown in the western blot analysis in Fig. 4a, b, β-Ecd inhibits JNK phosphorylation induced by Aβ. Given that ASK1 acts as an upstream regulator for the activation of JNK in brain of patients with AD [30], we next investigated whether β-Ecd inhibits ASK1 activation in Aβ-challenged SH-SY5Y cells. As expected, Aβ-induced ASK1 phosphorylation (that is, activation) in SH-SY5Y cells was attenuated by β-Ecd (Fig. 4c, d). ASK1 is required for ROS-induced JNK activation and apoptosis. We therefore examined whether β-Ecd might modulate ROS production in Aβ-challenged SH-SY5Y cells. As shown in Fig. 4e, β-Ecd significantly blocks Aβ-induced increases in ROS generation in a dose-dependent manner. However, ROS-scavenging actions of β-Ecd is independent on Akt/NF-κB pathway.

β-Ecd enhances the Bcl-2/Bax ratio through two independent and complementary pathways

To establish irrefutable cause-effect relationships among molecular events triggered by β-Ecd that result in mitochondria-mediated apoptosis in SH-SY5Y cells, we examined the effects of various kinase inhibitors. As illustrated in Fig. 5a, the data from the reporter assay indicated that β-Ecd treatment increased the activity of

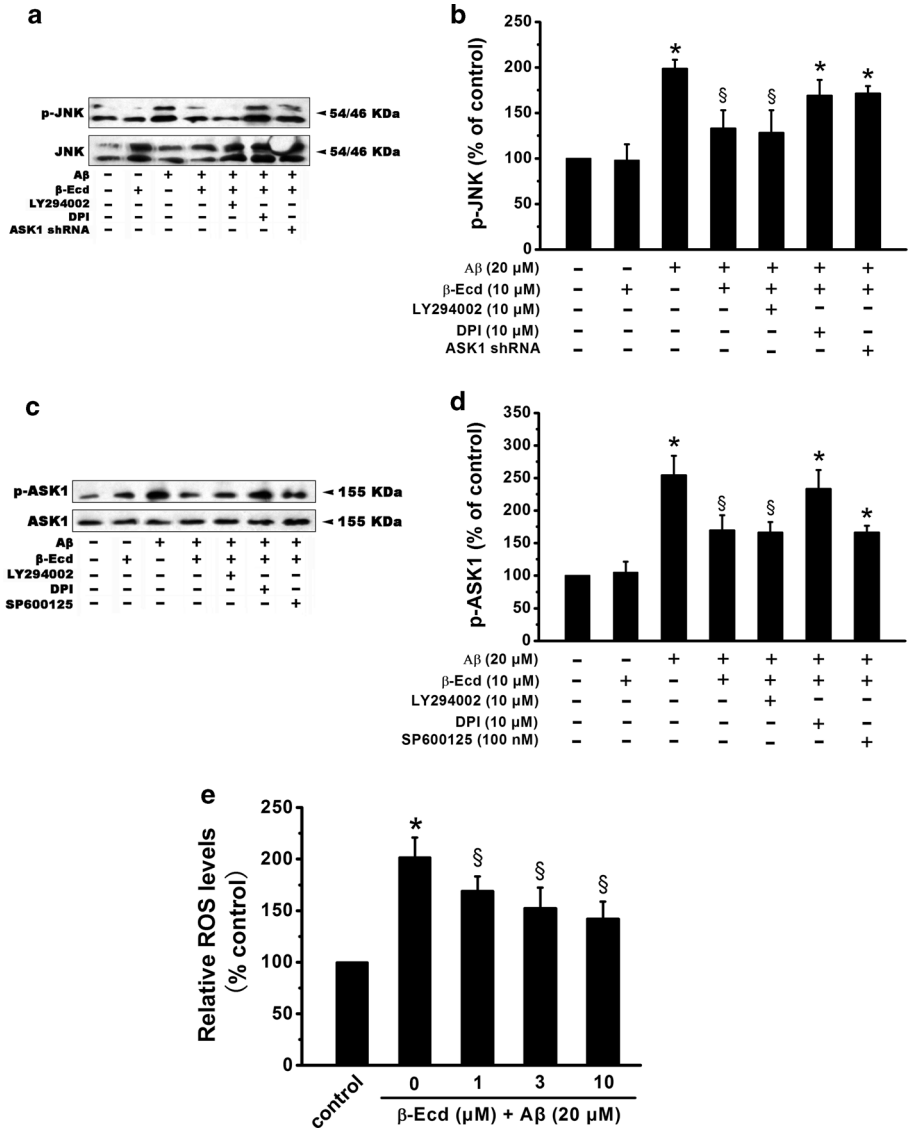

Fig. 4 β-Ecd inhibits JNK and ASK1 activation induced by Aβ in a ROS-dependent manner. After transient transfection with or without ASK1 shRNA plasmid, SH-SY5Y cells were pre-treated with or without LY294002, SP600125, or DPI for 30 min and then treated with β-Ecd in the presence or absence of Aβ for 24 h. **a** Level of p-JNK was analyzed by western blot. **b** The band intensity of p-JNK was quantified by densitometry and normalized to total JNK. **c** Level of p-ASK1 was analyzed by western blot. **d** The band intensity of p-ASK1 was quantified by densitometry and normalized to total ASK1. **e** SH-SY5Y cells were pre-treated with β-Ecd for 1 h before challenge with Aβ for an additional 24 h. Intracellular ROS were estimated using the fluorescent probe DCFH-DA by fluorometric assay. Data are expressed as means (SD) (three experiments for each condition). $^{*}p < 0.05$ versus control; $^{\S}p < 0.05$ versus Aβ alone treatment

the NF-κB promoter in the absence, but not in the presence, of Akt inhibitor LY294002 in SH-SY5Y cells. However, DPI or SP600125 failed to influence the increase of NF-κB activity induced by β-Ecd. These results suggest that Akt, but not ROS or JNK, is a major upstream mediator of DNA-binding activity of NF-κB in Aβ-challenged SH-SY5Y cells. Kinase assays indicates that ROS scavenger DPI or shRNA-induced ASK1 knockdown canceled the β-Ecd-mediated inactivation of JNK in Aβ-challenged SH-SY5Y cells, whereas LY294002 failed to do so (Fig. 5b, c).

Western blot analysis presented in Fig. 5d, e indicate that JNK or ROS is a prerequisite for the downregulation of Bax by β-Ecd, because β-Ecd-mediated inhibition of Bax is exclusively inhibited by the JNK inhibitor SP600125 well as a NADPH oxidase inhibitor DPI in SH-SY5Y cells exposed to Aβ(25–35). The Akt inhibitor LY294002 did not diminish the Bax downregulation in SH-SY5Y cells exposed to Aβ(25–35). These results suggest that the activation of the Akt and inhibition of JNK signaling pathways by β-Ecd are mediated by independent mechanisms. Blocking Akt–NF-κB pathway or ASK1–JNK pathway by pharmacological inhibitors significantly reduced the apoptosis-inhibiting effect of β-Ecd on Aβ-challenged SH-SY5Y cells but completely abolish that when dual inhibition of Akt and JNK (Fig. 5f), suggesting two separate pathways for neuroprotection mediated by β-Ecd.

Discussion

Despite much effort to discover a therapeutic strategy to prevent progression or to cure AD, available therapy is limited to the symptomatic treatment and its efficacy

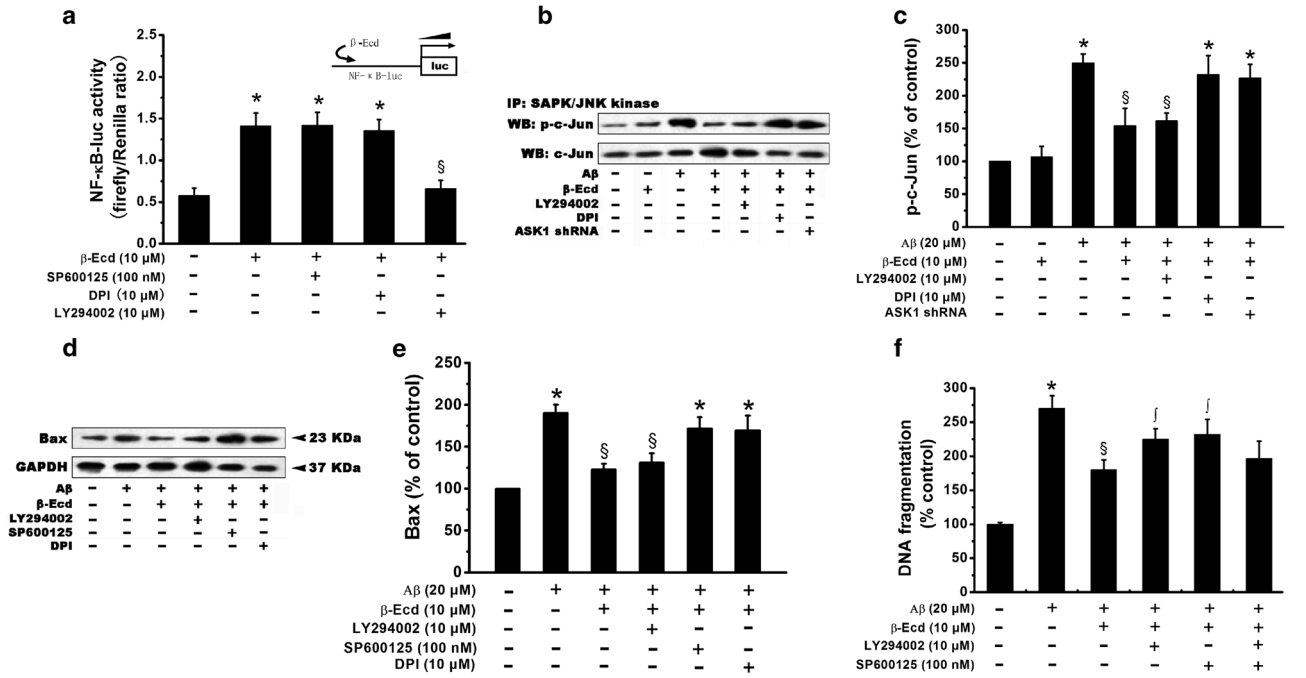

Fig. 5 Activation of the Akt and in activation of JNK signaling pathways by β-Ecd is mediated by independent mechanisms. After transient transfection with or without ASK1 shRNA plasmid, SH-SY5Y cells were pre-treated with or without LY294002, SP600125, or DPI for 30 min and then treated with β-Ecd in the presence or absence of Aβ for 24 h. **a** SH-SY5Y cells were co-transfected with plasmid pGMNF-κB-lu and pRL-TK, then treated as indicated and analyzed for luciferase activity. **b** Kinase activity of JNK immunoprecipitated with immobilized p-JNK mAb was determined using c-Jun as a substrate. **c** The intensity of p–c-Jun immunobands were quantified by densitometry and normalized to total c-Jun. **d** Level of Bax was analyzed by western blot. **e** The band intensity of Bax was quantified by densitometry and normalized to GAPDH. **f** DNA fragmentation was quantitatively evaluated by ELISA assay using mouse monoclonal antibodies against DNA and histones. Data are expressed as means (SD) (three experiments for each condition). $^{*}p < 0.05$ versus control; $^{\S}p < 0.05$ versus Aβ alone treatment. $^{\int}p < 0.05$ versus combined treatment with β-Ecd and Aβ

remains unsatisfactory at present [3]. The results of the present study demonstrate, for the first time, that β-Ecd prevents Aβ-induced mitochondrial-dependent apoptosis in SH-SY5Y cells via increasing cellular Bcl-2/Bax ratio. The upstream mechanism of the Bcl-2/Bax ratio enhancement by β-Ecd, a phytoestrogen, may be associated with the regulation of two separate pathways: (i) increased expression of anti-apoptotic Bcl-2 under basal conditions in an Akt-dependent manner and (ii) attenuation of ROS-dependent proapoptotic Bax upregulation that occurs under apoptotic challenge. We also demonstrated β-Ecd-mediated inhibition of Bax in Aβ-challenged SH-SY5Y cells by inhibiting JNK phosphorylation (activity) via ASK1 dephosphorylation (inactivity). Consistent with neuroprotection against Aβ-induced apoptotoic cell death in SH-SY5Y cells by β-Ecd, MTS assay showed identical results in primary cultured rat hippocampal neurons. If studies using an in vivo model of AD prove this beneficial effect, β-Ecd might be considered as an adjunct therapeutic strategy to combat neural demise in AD.

Plants, especially Chinese Material Medica, have been an important source for the discovery of novel therapeutically active compounds for AD [31]. Epidemiological evidence demonstrates that the prevalence of AD is higher in women than in men due to the loss of neuroprotective estrogen after menopause [32]. Therefore, we postulated that ER could mediate neuroprotective effect of phytoestrogen in the context of Aβ-challenged damage. However, the data presented in this report showed that β-Ecd did not modify ER-dependent luciferase activity, thus ruling out ER contribution.

According to the amyloid hypothesis, the neurotoxicity exerted by Aβ involves a number of cellular and molecular mechanisms, such as the generation of ROS, mitochondrial dysfunction and induction of apoptosis [33]. Inhibition of neuronal apoptosis may be an effective therapeutic strategies to attenuate Aβ-induced neurotoxicity and thus to improve neurological outcome in AD [34]. $A\beta_{1-42}$ is the major peptide constituent of amyloid plaques. $A\beta_{25-35}$, though not present in biological systems, is found to be at least as toxic as the full-length fragment. Therefore, $A\beta_{25-35}$ is widely used together with, or instead of, the endogenous fragment $A\beta_{1-42}$ in AD-relevant insults in vitro [35]. Our previous work shown that β-Ecd attenuated apoptosis in the

SH-SY5Y cells challenged with Aβ, an effect attributed to its antioxidant properties. The Bcl-2 family proteins are critical regulators of apoptosis regulating mitochondria-mediated apoptosis [36]. A novel finding of the current study is that the Bcl-2/Bax ratio elevation by β-Ecd might be the major working mechanism for suppressing Aβ-induced mitochondria-dependent apoptosis, which extended our previous observations [37].

In addition to mitochondrial dysfunction, endoplasmic reticulum stress and death receptor pathways have been suggested to be involved, at least partly, in the Aβ-mediated neurotoxicity [38]. However, we failed to observe a significant effect of β-Ecd on the calpain activity, caspase-8 activity (a death receptor apoptotic signaling related caspase), or caspase-12 (an endoplasmic reticulum-specific stress-activated caspase) activity in our model demonstrating some specificity for its action on the mitochondrial pathway. Our results suggest that the mitochondrial pathway, but not the endoplasmic reticulum and death receptor pathways, plays a major role in the neuroprotective properties of β-Ecd against Aβ-induced toxicity in SH-SY5Y cells.

What is the signal mechanism by which β-Ecd induces Bcl-2? Akt is ubiquitously expressed in mammals, but is initially present at low levels in the adult brain, and plays a critical role in the modulation of survival [39]. Bcl-2 is a downstream gene of Akt. In this study, we demonstrated that Aβ(25–35) did not significantly affect Akt activity in SH-SY5Y cells in our experimental conditions, which did not support our initial assumption that Aβ exposure will result in Akt kinase inactivation. Consistent with the protective function of Akt survival pathway, β-Ecd enhanced Akt kinase activation under basal conditions, suggesting that β-Ecd acts in a stimulus-independent manner to activate Akt signaling. β-Ecd-mediated inhibition of NF-κB promoter activity in Aβ-challenged cells were lost in response to Akt inhibitors LY294002. Blockade of NF-kB activation by the Bay11-7082 failed to abolish β-Ecd-mediated induction of Akt kinase activation, suggesting that β-Ecd induced NF-kB activation located downstream of Akt.

ROS play a physiological role in cell signaling and also a pathological role in various diseases [40]. An increasing number of studies have proposed a strong correlation between ROS-induced oxidative stress and the pathogenesis of AD [41]. ROS are major contributors to loss of neuronal apoptosis. Many antioxidant compounds have been demonstrated to protect the brain from Aβ neurotoxicity [42]. Given that serious adverse effects are related to synthetic antioxidants, recent research has been focused on natural products with antioxidant properties. Several signaling pathways, including ASK1 pathways, were identified as upstream pathways that regulate Bax [43]. However, it remains to be elucidated which signaling pathway involved in the beneficial effect of β-Ecd under conditions of oxidative stress. Results in this report indicate that ROS-scavenging is an upstream event that resulted in the downregulation of Bax in SH-SY5Y cells challenged with Aβ in an ASK1-dependent mechanism.

ASK1, an upstream activator of JNK signaling cascades, can be activated in response to oxidative stress and triggers various biological responses such as apoptosis in various cell types [44]. Accumulating evidence indicates that ASK1 plays a key role in the AD [45]. How does β-Ecd inhibition of ROS prevent the apoptotic effects of Aβ? The in vitro activation of ASK1/JNK pathway by Aβ observed in the SH-SY5Y cells is consistent with other published reports [46]. β-Ecd-mediated suppression of the activation ASK1 and JNK elicited by Aβ through a ROS-dependent mechanism, revealed by using pharmacological inhibitors DPI. In our cultures, no decrease in the basal level of phosphorylated (active) ASK1 or JNK could be detected as a result of exposure to β-Ecd alone. Importantly, shRNA-induced knockdown of ASK1 abolished β-Ecd-mediated JNK dephosphorylation in SH-SY5Y cells challenged with Aβ, whereas, SP600125 had no effect on β-Ecd-mediated ASK1 dephosphorylation, indicating that ASK1 is located upstream of JNK in this regulating pathway.

Our findings, however, must be interpreted in the context of certain limitations. First, SH-SY5Y cells are widely available and can provide a homogenous population of cells in large numbers, but in vitro models may not be clinically relevant. Second, although our findings clearly show that cellular antioxidant properties of β-Ecd is responsible for its neuroprotective effect in Aβ-challenged SH-SY5Y cell, whether it also contributes to memory improvement in vivo during AD is not clear. Third, neuroprotection mediated by β-Ecd require micromolar concentrations which would likely be impossible to achieve in the brain of AD patients due, in part, to its rapid metabolism and blood-brain barrier protection. It is noteworthy that as the in vivo system is multi-factorial, directly extrapolating in vitro conditions and results to the in vivo system might be misleading.

Crosstalk between Akt and JNK has been observed in vitro [47]. In our cultures, β-Ecd activated Akt signaling pathway and suppressed JNK signaling pathway. Does crosstalk between the Akt pathway and JNK pathway contributes to β-Ecd-mediated the mitochondria-associated apoptotic mechanisms? Results in this report indicate that β-Ecd-induced inhibition of JNK activity did not change by the addition of an Akt inhibitor LY294002. Similarly, inhibition of JNK with SP600125 failed to affect β-Ecd-induced activation of Akt. β-Ecd inhibition of apoptosis can be partly blocked not only by LY294002 but also by SP600125, and completely block by the combination of LY294002 with SP600125. All of these findings support the notion that the activating Akt signaling pathway and

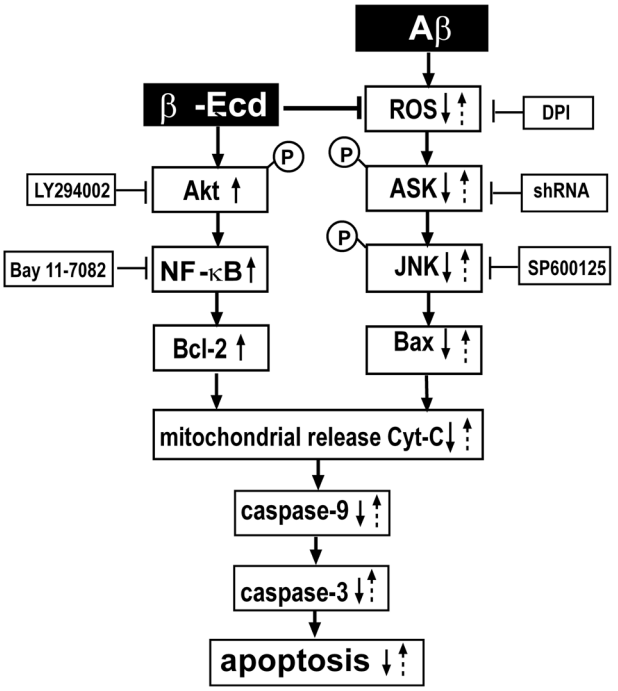

Fig. 6 Putative mechanisms whereby β-Ecd protects from Aβ-induced apoptosis. β-Ecd activates the NF-κB through Akt activation, resulting in upregulates of Bcl-2. β-Ecd inhibition of ROS production also inhibits Aβ-induced ASK1 phosphorylation, and this contributes JNK-dependent Bax downregulation. As a result, β-Ecd attenuates mitochondrial cyt *c* release, caspase-9 activation, and caspase-3 activation induced by Aβ. Upregulation of Bcl-2 and downregulation of Bax by β-Ecd is mediated by independent mechanisms. The dashed arrow indicates the actions of Aβ, while solid arrow represents the effects of β-Ecd

inhibiting JNK pathways by β-Ecd are mediated by independent mechanisms.

To explore the regulatory mechanisms of β-Ecd enhancement of Bcl-2/Bax ratio in Aβ-challenged SH-SY5Y cells, a model of molecular circuits is proposed in Fig. 6. β-Ecd induces basal Bcl-2 upregulation via activating Akt. In addition, β-Ecd-mediated ASK1 inactivation via ROS-dependent mechanism results in JNK dephosphorylation, and downregulation of Bax in SH-SY5Y cells challenged with Aβ. As a result of increasing Bcl-2/Bax ratio, attenuation of cyt *c* release suppresses caspase-dependent apoptosis. The present study provides in vitro evidence for our hypothesis. It bears emphasis that the underlying mechanisms are certainly more complex than what we describe here. Given the fact that the Bcl-2 family, made up of at least 25 proteins, includes both pro-life and pro-death proteins, our results do not exclude any other Bcl-2-dependent mechanism involved in the neuroprotection mediated by β-Ecd. Therefore, our results in this report suggest that β-Ecd may be a potential candidate for the treatment of AD, and warrant further preclinical studies in vivo.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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