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Beta-asarone attenuates beta-amyloid-induced apoptosis through the inhibition of the activation of apoptosis signal-regulating kinase 1 in SH-SY5Y cells

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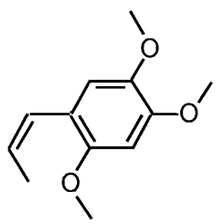
Beta-amyloid (Aβ) toxicity has been postulated to initiate synaptic loss and subsequent neuronal degeneration seen in Alzheimer's disease (AD). We previously demonstrated that β-asarone improves cognitive function by suppressing neuronal apoptosis *in vivo*. In this study, we assessed the neuroprotective effects of β-asarone against the toxicity of Aβ in relation to the mitochondria-mediated cell death process, and to elucidated the role of the ASK1/MKK7/JNK and mitochondrial pathways in β-asarone-induced neuroprotection in SH-SY5Y cells. Our results show that β-asarone afforded protection against Aβ-induced toxicity by inhibiting apoptosis in SH-SY5Y cells. This result was also confirmed by caspase-9 and caspase-3 activity assays. Expression of p-ASK1, p-MKK7, p-JNK, Bax, Bad, and cytochrome *c* release decreased after pretreatment with β-asarone in SH-SY5Y cells exposed to $A\beta_{1\text{-}42}$. Interestingly, these effects of β-asarone against $A\beta_{1\text{-}42}$ insult were enhanced by ASK1 siRNA. These findings suggest that β-asarone prevents $A\beta_{1\text{-}42}$-induced neurotoxicity through attenuating neuronal apoptosis, and might be a potential preventive or therapeutic agent for AD.

1. Introduction

Approximately 10% of people older than 65 years and about 50% of those older than 85 years have Alzheimer disease (AD). At present, this represents approximately 4 million persons in the United States and 15 million people worldwide (Gruber-Baldini et al. 2007). Extracellular accumulation of β-amyloid (Aβ), the defining proteinaceous component of senile plaques, is strongly implicated in AD pathogenesis (Hung et al. 2009). Previous studies have shown that Aβ-induced toxicity involves oxidative stress, inflammation, and perturbation of calcium homeostasis (Fernández et al. 2009). In addition, both necrotic and apoptotic processes are thought to occur in primary neurons and neuronal cell lines after exposure to Aβ as well as in AD brains (Li et al. 2009; Matsui et al. 2006). Aβ has been implicated as the primary neurotoxic factor in the pathogenesis of AD (Zhao et al. 2009). As a result, therapeutic strategies that address the toxicity of Aβ may foster novel developments for the treatment of AD.

Mitogen-activated protein kinases (MAPKs) are essential components of eukaryotic signal transduction networks that enable cells to respond appropriately to extracellular signals and stresses (Michnick et al. 2006). MAPK is activated by sequential protein phosphorylation through a MAPK module. Apoptosis signal–regulating kinase 1 (ASK1) is a ubiquitously expressed MAPK kinase kinase (MAPKKK) that activates MAP kinase kinase (MKK) 7, which in turn activates the c-Jun NH2-terminal kinase (JNK) (also known as stress-activated protein kinase) signaling cascades (Matsuura et al. 2002). It has also been demonstrated that the JNK pathway is hyperactive in the AD brain (Ma et al. 2009). ASK1 activation is a key mechanism in Aβ-induced neurotoxicity, which plays a central role in AD (Hashimoto et al. 2003).

A cetylcholinesterase inhibitors are clinically used in AD therapy, because they marginally ameliorate cognitive deficits (Santoro et al. 2010). However, to date, only palliative treatments of the symptoms are available for AD (Relkin 2007). *Acorus tatarinowii* Schott has been found to be effective in the management of amnesia (Liao et al. 2005). Several lines of evidence have suggest that β-asarone, the major ingredient of *Acorus tatarinowii* Schott, has neuroprotective effects *in vitro* and *in vivo* (Limón et al. 2009; Cho et al. 2002). We previously demonstrated that the β-asarone improves cognitive function in the beta-amyloid hippocampus injection rats (Geng et al. 2010). However, so far, whether β-asarone might be beneficial to AD by suppressing the apoptosis still remains to be elucidated. In this study, we investigate effect of β-asarone on the $A\beta_{1\text{-}42}$-induced neuronal apoptosis and identify signaling protein kinase cascades that may be responsible for the putative effect of β-asarone.



2. Investigations and results

2.1. Beta-asarone reduces Aβ_{1-42}-induced SH-SY5Y cells death

MTT assay revealed that Aβ_{1-42} significantly increased SH-SY5Y cells death in a dose-dependent manner up to 20 μM, with no further decrease being observed at 100 μM concentrations (data not shown). Aβ_{1-42}-induced (20 μM) cells death was gradually increased in a time-dependent manner up to 24 h, with no further decrease in 48 h. Pretreatment of 10 μg/mL β-asarone for 24 h significantly decreased cell death induced by Aβ_{1-42} (Fig. 1A). Thus, we selected 24 h time and 20 μM concentration of Aβ_{1-42} for the remainder of the experimental paradigms. Beta-asarone significantly prevented the SH-SY5Y cells death induced by Aβ_{1-42} in dose-dependent manner from 10 μg/mL up to 100 μg/mL (Fig. 1B). Since 10 μg/mL of β-asarone was the minimum concentration to result in a significant preventive effect, this concentration of β-asarone was employed for the remainder of the experimental paradigms.

We also determined LDH release as an indicator of cytotoxicity by a colorimetric assay, and results revealed that exposure to Aβ_{1-42} alone induced a significant increase in LDH release compared with control by 2.6 fold. Relative to Aβ_{1-42} alone, β-asarone (10 μg/mL) showed 20% decrease in Aβ_{1-42}-induced LDH release. Beta-asarone alone had no effect on LDH release from the SH-SY5Y cells (Fig. 1C).

2.2. Beta-asarone prevents Aβ_{1-42}-induced apoptosis through the ASK1-dependent mechanism

SH-SY5Y cells apoptosis was quantified by staining cell with annexin-V-FITC/PI (Fig. 2A). Quantitative analysis of annexin V-positive cells revealed that treatment of SH-SY5Y cells with 20 μM Aβ_{1-42} for 24 h evoked cell apoptosis as indicated by the percentage of annexin V-positive cells, with approximately 5.2 times that of control. Pretreatment of SH-SY5Y cells with 10 μg/mL β-asarone, prior to Aβ_{1-42} exposure, significantly decreased cell apoptosis by 35%.

Knock-down of ASK1 expression by RNAi markedly reduced the ASK1 phosphorylation, and importantly enhanced the protective effect of β-asarone against Aβ_{1-42}-induced apoptosis (Fig. 2B). It should be noted that ASK1 expression was not totally blocked in cells transfected with ASK1 RNAi. This may be attributed in part to a 60–70% transfection efficiency of the ASK1 RNAi in these cells (data not shown). We further determined the SH-SY5Y cells death by MTT, and results showed that cell treated with Aβ_{1-42} exhibited an increase in cells death, whose response was significantly depressed by the β-asarone pretreatment. Knock-down of ASK1 enhanced the protective effect of β-asarone against Aβ_{1-42}-induced death (Fig. 2C). These results suggest that the ASK1 is required for neuroprotective effect of β-asarone in SH-SY5Y cells.

2.3. ASK1/JNK signal pathway is involved in neuroprotective effect of β-asarone

It has been shown that ASK1 is an important factor for induction of apoptosis through the activation of JNK (Nadeau et al. 2009), and MKK7 is an essential component of the JNK signal transduction pathway activated by Aβ (Xu et al. 2009), so the changes of ASK1, MKK7, and JNK phosphorylation by Aβ_{1-42} treatment were studied in SH-SY5Y cells. As shown in Fig. 4D and 2E, treatment with 20 μM Aβ_{1-42} increased phosphorylation of ASK1, MKK7, and JNK with control by 1.4, 2.5, and 1.3 fold, respectively. Beta-asarone negatively regulates ASK1, MKK7, and JNK phosphorylation in SH-SY5Y cells. However, under the same treatment, we did not detect any significant difference in total ASK1, MKK7, and JNK protein expression (data not shown), and suggest that the alteration of ASK1, MKK7, and JNK phosphorylation were not due to enhanced expression of the ASK1, MKK7, and JNK proteins. Knock-down of ASK1 expression by RNAi markedly enhanced the inhibitory effect of β-asarone on p-ASK1, p-MKK7, and

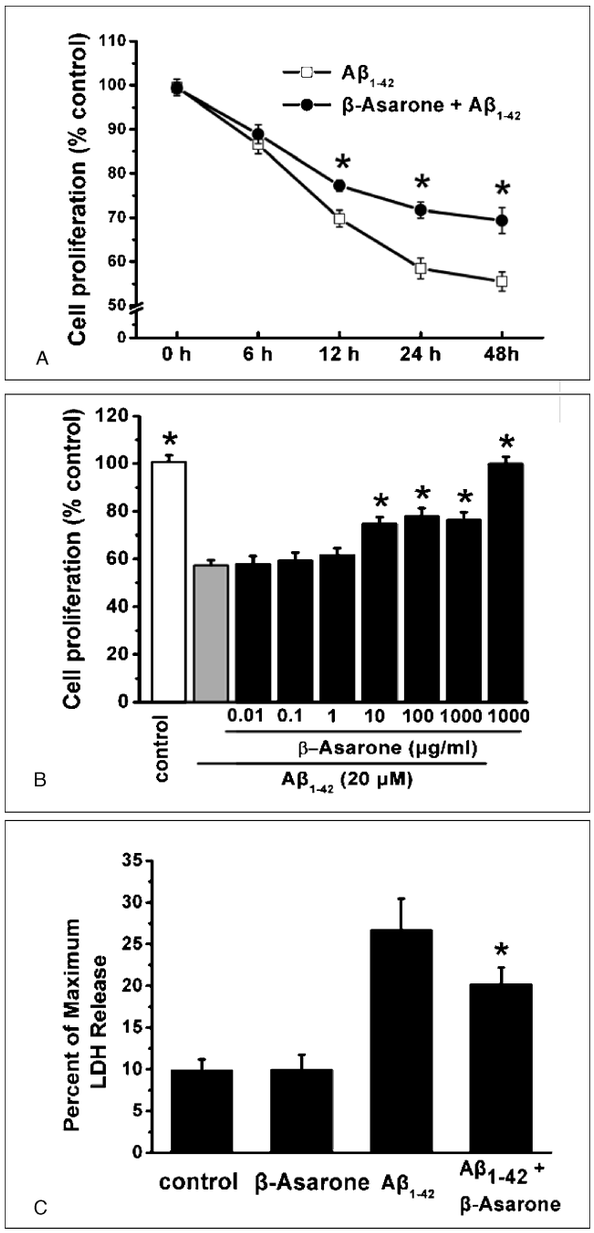


Fig. 1: Prevention of Aβ_{1-42}-induced cell death by β-asarone. A) β-asarone reduces Aβ_{1-42}-induced SH-SY5Y cells death in time-dependent manner. SH-SY5Y cells were pretreated with or without β-asarone (10 μg/mL) for 24 h and exposed to aggregated Aβ_{1-42} (20 μM) for the indicated times, and cell viability was determined by MTT. B) β-asarone reduces Aβ_{1-42}-induced SH-SY5Y cells death in dose-dependent manner. SH-SY5Y cells were pretreated with increasing concentrations (0.01-1000 μg/mL) of β-asarone for 24 h, followed by exposure to aggregated Aβ_{1-42} for 24 h, and cell viability was determined by MTT. C) Effects of preconditioning with β-asarone on LDH release in SH-SY5Y cells subjected Aβ insult. LDH release has been normalized to the maximal releasable amount obtained by incubating cells with 1% Triton X-100 for 30 minutes. Data obtained from three separate experiments and are expressed as mean ± S.D.; *, $P < 0.05$ compared to Aβ_{1-42} alone or that at the matched time point

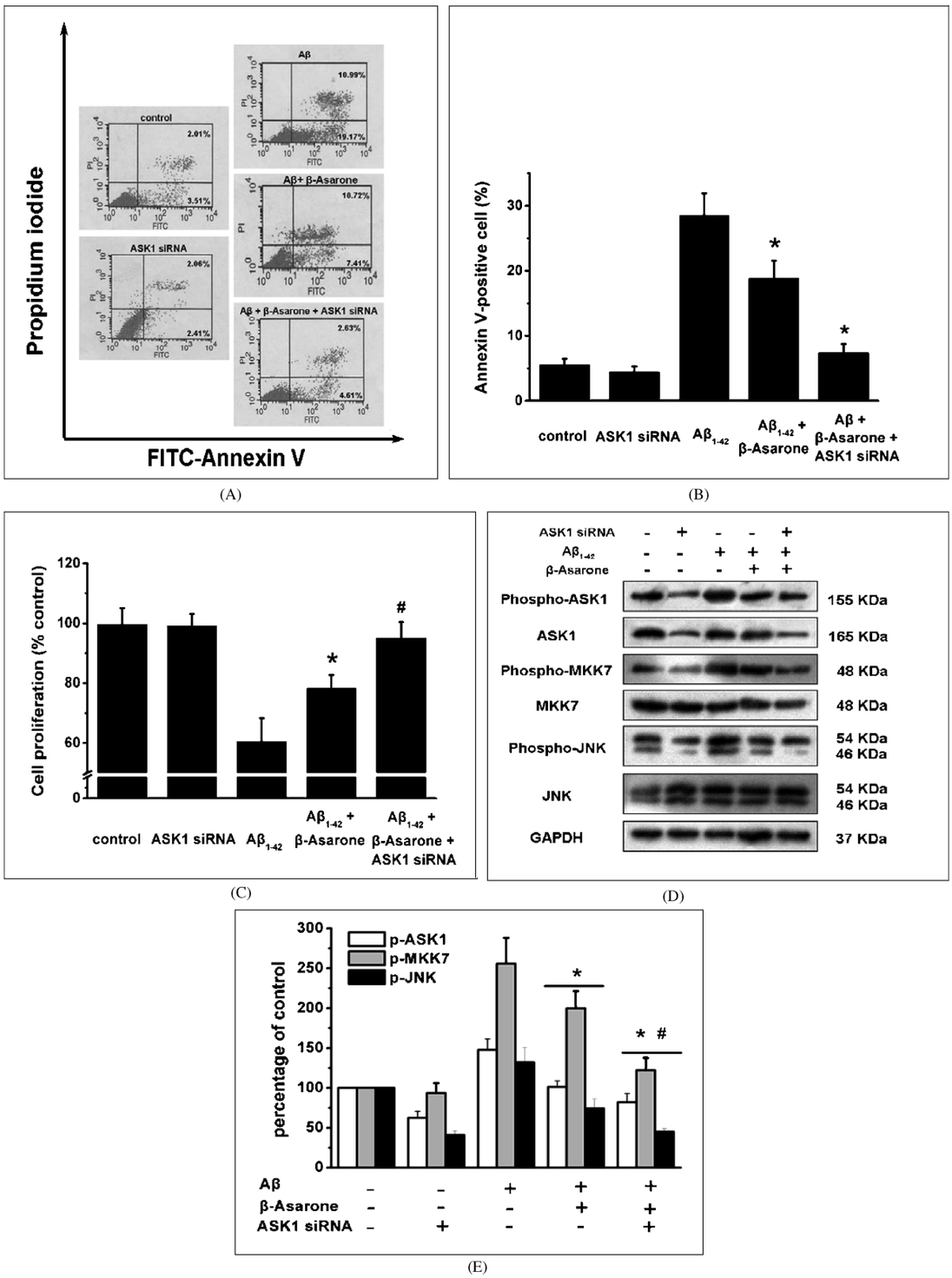


Fig. 2: Beta-asarone pretreatment attenuation $A\beta_{1\text{-}42}$-induced cell apoptosis. After transient transfection, SH-SY5Y cells were pre-incubated with 10 μg/mL β-asarone, which was followed 24 h later by exposure to 20 μM aggregated $A\beta_{1\text{-}42}$ for 24 h. Cells apoptosis was measured by labeling cells with annexin-V-FITC and counterstaining with propidium iodide (PI). A) Annexin-V-FITC/PI double staining of SH-SY5Y cells. The numbers indicate the percentage of cells in each quadrant (lower left: $FITC^-/PI^-$, intact cells; lower right: $FITC^+/PI^-$, apoptotic cells; upper left: $FITC^-/PI^+$, necrotic cells; upper right: $FITC^+/PI^+$, late apoptotic cells). B) The bar chart describes the percentual distribution of apoptotic cells. Percentage of annexin V-positive cells analysis of FACS obtained from three separate experiments. C) Cell viability was determined by MTT. Results expressed as the percentage of cell survival obtained from three separate experiments, and untreated cells were assumed to be vital (100% viability). D) ASK1, p-ASK1, MKK7, p-MKK7, JNK, and p-JNK levels were determined by immunoblot analysis with antibody to ASK1, p-ASK1, MKK7, p-MKK7, JNK, and p-JNK. The loading of the lanes was normalized to levels of GAPDH. E) Quantitated results of p-ASK1, p-MKK7, and p-JNK are presented relative to control. All densitometric analysis of western blot obtained from three separate experiments. Data are expressed as mean ± S.D., *, $P < 0.05$ compared to $A\beta_{1\text{-}42}$ alone. #, $P < 0.05$ compared to $A\beta_{1\text{-}42}$ plus β-asarone treatment cells

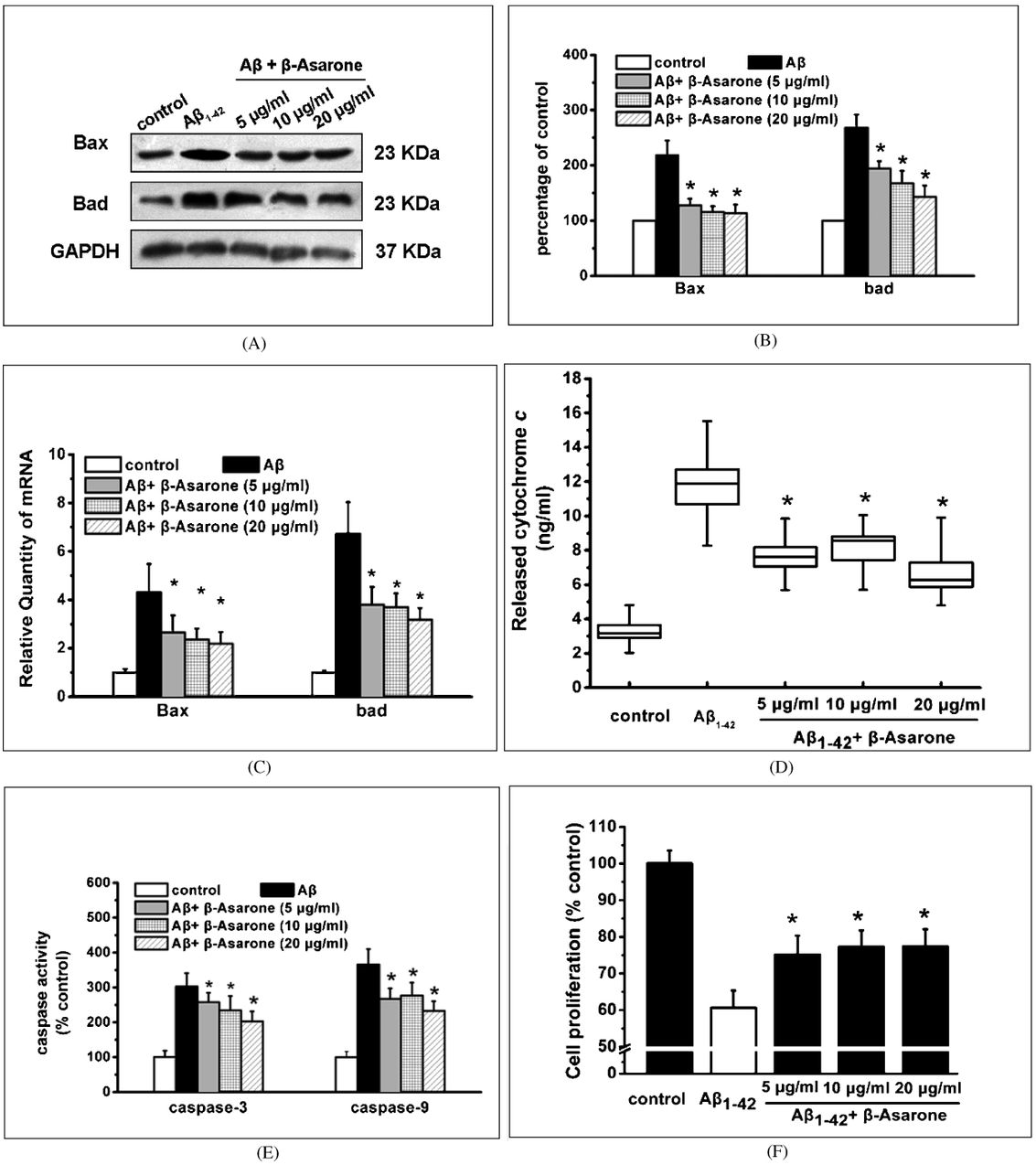


Fig. 3: Beta-asarone inhibition $A\beta_{1-42}$-induced activation of mitochondrial apoptotic pathways. SH-SY5Y cells were pretreated with 10 μg/mL β-asarone. Following 24 h incubation, aggregated $A\beta_{1-42}$ (20 μM) was added. A) Bax and Bad levels were determined by immunoblot analysis with antibody to Bax and Bad. The loading of the lanes was normalized to levels of GAPDH. B) Quantitated results of Bax and Bad are presented relative to control. Densitometric analysis of western blot obtained from three separate experiments. C) Total RNA was isolated from SH-SY5Y cells using RNAiso reagent and used for cDNA synthesis. The mRNA levels of Bax and Bad were detected by real-time PCR. $2^{-\Delta\Delta Ct}$ analysis of PCR obtained from three separate experiments. D) The level of cytosolic cytochrome *c* was measured by ELISA. Values obtained from three separate experiments. E) Activities of caspase-9 and caspase-3 were measured by a quantitative colorimetric assay. Values obtained from three separate experiments. F) Cell viability was determined by MTT. Results expressed as the percentage of cell survival obtained from three separate experiments, and untreated cells were assumed to be vital (100% viability). Data are expressed as mean ± S.D.; *, $P < 0.05$ compared to $A\beta_{1-42}$ alone

p-JNK. These results suggest that ASK1 acts as an up-stream regulator of neuroprotective effect of β-asarone.

2.4. Mitochondrial pathways are involved in the neuroprotective effect of β-asarone

Given that Bcl-2 family proteins, the best-characterized protein family involved in the regulation of apoptotic cell death, are important modulators of cells apoptosis induced by Aβ (Yao et al. 2007), we determined the effect of β-asarone on Bax and Bad protein levels. Western blot results (Fig. 3A) shows that β-asarone pretreatment decreases Bax and Bad protein levels in SH-SY5Y cells which increased significantly with $A\beta_{1-42}$ treatment (Fig. 3B). Consistent with the results of protein levels, real-time PCR results revealed that β-asarone pretreatment significantly decreases Bax and Bad mRNA levels (Fig. 3C). We further measured cytochrome *c* concentration in cytosolic fractions using a commercial ELISA kit. As shown in Fig. 3D, SH-SY5Y cells treated with $A\beta_{1-42}$ for 24 h showed an

increase in the cytosolic cytochrome *c* levels. Pretreatment with β-asarone attenuated the $A\beta_{1-42}$-induced increase in cytochrome *c* levels. We also took advantage of specific substrates for caspase-9 and caspase-3 to measure the activation of caspases in SH-SY5Y cells. Cellular caspase activity assay showed that treatment of SH-SY5Y cells with 20 μM $A\beta_{1-42}$ for 24 h strongly activated caspase-9 and -3 in SH-SY5Y cells. Pretreatment of SH-SY5Y cells with 10 μg/mL β-asarone, prior to $A\beta_{1-42}$ exposure, significantly suppress caspase-9 and caspase-3 activity (Fig. 3E). MTT analysis showed that $A\beta_{1-42}$ application alone resulted in a significant decrease in SH-SY5Y survival compared with control by 40%. Relative to $A\beta_{1-42}$ alone, β-asarone (5, 10, or 20 μg/mL) increased SH-SY5Y survival by 25%-28% during $A\beta_{1-42}$ exposure (Fig. 3F).

3. Discussion

Although presently approved anticholinesterases and memantine are primarily useful for symptomatic relief of the cognitive disturbances associated with AD, they seem to have a limited impact on disease progression and brain atrophy (Santoro et al. 2010). AD is a neurodegenerative condition characterized by progressive neuronal loss, which may be a consequence of the neurotoxic properties of the Aβ (Knowles et al. 1998). In the present study, we demonstrated that β-asarone exerted a neuroprotective effect against $A\beta_{1-42}$-induced neurotoxicity in SH-SY5Y cells. Beta-asarone specifically inhibits the $A\beta_{1-42}$-induced activation of the ASK1-MKK7-JNK signaling pathway, resulting in protection from $A\beta_{1-42}$-induced apoptosis in SH-SY5Y cells. To the best of our knowledge, there are currently no reports on ASK1-MKK7-JNK signaling cascade being involved in neuroprotective effect of β-asarone.

Acorus tatarinowii Schott has long been employed in the clinical treatment of AD in chinese herbal books. It has been reported to be responsible for various pharmacological actions on the central nervous system (CNS) (Liao et al. 2005). The more recent results of Hu et al. (2009) confirmed that β-asarone, a component isolated from *Acorus tatarinowii* Schott, passes easily through the blood brain barrier, and is well distributed in the brain. Beta-asarone, however, has not yet been evaluated for actions on AD and for its mechanism of action. LDH is a stable cytoplasmic enzyme present in all cells and is rapidly released into the cell culture supernatant when the plasma membrane is damaged. Thus, it can be used as a reliable biochemical index for neuronal plasma membrane damage (Sepp et al. 1996). MTT is an index of mitochondrial viability because it is reduced by metabolically active mitochondria (Abe and Saito 1998). Our results indicated that β-asarone exerted a significant induction of mitochondrial viability in SH-SY5Y cells and was effective in inhibiting LDH release to the extracellular medium from SH-SY5Y cells.

Apoptosis, also known as programmed cell death, is dependent on intracellular pathways resulting in cellular commitment to a defined series of steps resulting in cell suicide (Carmen and Sinai 2007). There is accumulating evidence from *in vitro*, *in vivo*, and human studies suggesting that apoptosis is likely to have an essential role in pathogenesis of AD (Zhang et al. 2006; Ohyagi et al. 2005). Thus, identification of the apoptotic mechanisms operative in AD may provide insights into potential future therapeutic strategies for AD. Increased Aβ, one of the etiological factors in AD, appears to have a central role in neuronal apoptosis, because it can activate mitochondrial pathway (Ferreiro et al. 2007). Caspase-9 and caspase-3 belongs to a large family of cellular cystine proteases, known collectively as caspases for their preferential ability to cleave cellular substrates after aspartic acid residues. The cleavage of caspase-specific substrates results in the biochemical destruction of the cell and phenotypic changes associated with apoptosis (Yao et al. 2007). Caspase-9 is thought to be an initiator caspase. Activated caspase-9 cleaves and activates caspase-3, which is involved in the final execution phase of apoptosis (Villard et al. 2009). In the present study, we found that the number of apoptotic cells in the SH-SY5Y cells significantly increases after $A\beta_{1-42}$ treatment, which suggests the role of apoptosis in the development of AD. Beta-asarone preconditioning significant reduced the number of SH-SY5Y cells apoptosis. This result confirmed by levels of activated caspase-3 and caspase-9 which are known to measure the later stages of cell death.

Concerted actions of molecular signaling networks determine cell fates (He and Shen 2009). Within the many stress-responsive signaling pathways, the JNK signaling cascade is crucial for the maintenance of cell homeostasis and controls many cellular processes, including cell growth, transformation, differentiation and apoptosis (Junttila et al. 2008). There is very strong evidence linking the activation of JNK to neuronal loss in response to Aβ neurotoxicity (Chen et al. 2006). JNK are directly activated by the phosphorylation of tyrosine and threonine residues in a reaction catalyzed by the dual specificity MKK4 and MKK7. Studies *in vitro* have shown that MKK4 can also phosphorylate p38 MAPK, whereas MKK7 is specific toward kinases of the JNK subgroup (Zou et al. 2007). ASK1 activates the JNK and p38 pathways by directly phosphorylating and thereby activating their respective MKK7 and MKK4. Overexpression of wild-type or constitutively active ASK1 induces apoptosis in various cells through mitochondria-dependent caspase activation (Usuki et al. 2008). ASK1 is present in neuronal cells and has been implicated in a various types of neuronal cell death (Kim et al. 2005). Our results indicate that $A\beta_{1-42}$ treatment caused a increase expression of p-JNK in SH-SY5Y cells. Beta-asarone preconditioning inhibited activation of JNK. SH-SY5Y cells transfected ASK1 siRNA enhanced the inhibitory effects of β-asarone on $A\beta_{1-42}$-induced apoptosis, meanwhile, significantly enhanced inhibitive effect of β-asarone on $A\beta_{1-42}$-induced phosphorylation of JNK and MKK7, suggesting that the effect of β-asarone is mediated through ASK1-MKK7-JNK signaling pathway. Together, these findings provide realistic evidence that ASK1 was involved in neuroprotective effect of β-asarone.

The two best-studied pathways of caspase activation are the cell-surface–death- receptor pathway, i.e., Fas-mediated apoptosis, and the mitochondrion-initiated pathway. Many components of the mitochondrial apoptotic cascade appear to be involved in the neuronal toxicity of Aβ peptides (Antequera et al. 2009). Aβ can upregulate pro-apoptotic Bax expression or require Bax to mediate neurotoxicity (Wei et al. 2003). In addition, Bax protein levels have been reported to increase in AD brain (Bader et al. 2008). Another proapoptotic protein Bad is located in the cytosol but translocates to the mitochondria and forms a proapoptotic complex with Bcl-2 (Jiang et al. 2006). Bad and Bax have been reported to directly inhibit members of the caspase family, including caspases-9 which are potent effectors of neuronal death (Su et al. 2010). In the present study, we observed that $A\beta_{1-42}$ treatment caused an increased expression of the pro-apoptotic proteins Bax and Bad in SH-SY5Y cells. Beta-asarone preconditioning inhibits the increase in the expression of Bax and Bad. These results suggested that the mitochondrial pathway of cell apoptosis might be involved in preventive effect of β-asarone against $A\beta_{1-42}$ toxicity. In the mean time, the mRNA level of Bax and Bad were also up-regulated by $A\beta_{1-42}$ and were followed by the decreased expression in β-asarone treatment cells prior to Aβ abuse.

Mitochondrial cytochrome *c* is a water-soluble protein loosely attached in the mitochondrial intermembrane space. In response to a variety of apoptosis-inducing agents, cytochrome *c* is released from mitochondria to the cytosol (Xu et al. 2009).

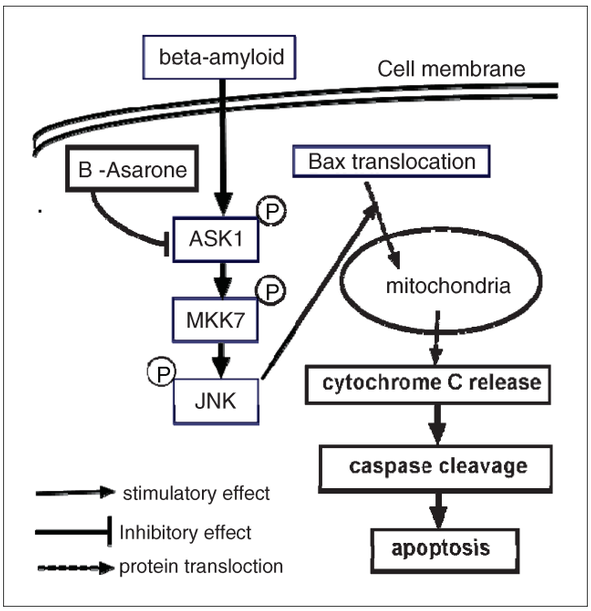


Fig. 4: Schematic representation of molecular mechanisms of β-asarone inhibits the apoptosis in SH-SY5Y cells induced by Aβ. Aβ induces ASK1 phosphorylation. ASK1 phosphorylation, in turn, increases MKK7 phosphorylation, which serve to increase JNK phosphorylation. JNK activation induces the mitochondrial pathway of apoptosis involving increased expression of Bax and Bad, followed by mitochondrial release of cytochrome *c*. Release of cytochrome *c* then further induces caspase-9 activation, leading caspase-3 activation and apoptosis. β-asarone reverse above-mentioned incident

Cytochrome *c* participates in the formation of a cytosolic complex. Within this complex, caspase-9 is activated, leading to the downstream activation of caspase-3. Activated caspase-3 ultimately leads to cell apoptosis (Zhang et al. 2010)**.** In the present study, we found that cytochrome *c* levels in cytosolic of SH-SY5Y cells treatment with $A\beta_{1\text{-}42}$ were significantly higher than in the control cells. Interestingly, the cytochrome *c* levels in cytosolic significantly decrease after β-asarone treatment as a manner similar to cells apoptosis. These findings imply that β-asarone might inhibit apoptosis involved in mitochondrion-initiated pathway in SH-SY5Y cells.

In conclusion, the current investigation suggests that β-asarone modulates Aβ-induced apoptosis by inhibiting the ASK1-MKK7-JNK signaling pathway in association with ASK1 phosphorylation. ASK1 siRNA inhibited ASK1 phosphorylation and enhanced the protective effect of β-asarone against $A\beta_{1\text{-}42}$-induced apoptosis. ASK1 phosphorylation in turn activates MKK7, which serve to activate JNK, thus active JNK induced apoptosis through mitochondria-dependent caspase activation (Fig. 4). Our findings suggest that β-asarone might be a potential drug to suppress neuronal cell apoptosis in AD.

4. Experimental

4.1. Cell culture

Human neuroblastoma SH-SY5Y cells obtained from the cell bank of Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China) were maintained in Dulbecco's modified Eagle medium (DMEM) (Hyclone, Logan, Utah, USA) supplemented with 10% fetal bovine serum, 50 units/mL penicillin (Invitrogen, Carlsbad, CA, USA), and 100 μg/mL streptomycin (Invitrogen). The cells were seeded in ø 60 mm dishes (Nalge Nunc Int., Rochester, NY, USA) at 1×10^4 cells/cm^2 and maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Before treatments, cells were washed with serum-free DMEM. All treatments were used under serum-free conditions.

4.2. Transfection

Transfection of SH-SY5Y cells was performed by Lipofectamine2000^{TM} (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Cells were cultured at 90% confluence in 6-well plates and were transfected with 10 nM ASK 1 siRNA (Santa Cruz Biotechnology, Santa Cruz, CA).

4.3. Determination of cell viability

Cell viability was measured by quantitative colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as described by Hertel et al. (1996). Briefly, the cells were cultured at a density of 5×10^4 cells per well in growth medium for 24 h in 96-well plates, and then pre-incubated with or without 0.01-1000 μg/mL or 10 μg/mL β-asarone, which was followed 24 h later by exposure to 20 μM aggregated $A\beta_{1\text{-}42}$ (Sigma, St. Louis, MO. USA) prepared as described previously (Szaingurten-Solodkin et al. 2009) for another 24 h or 0-48 h. Twenty five /well of MTT solution (final concentration, 500 μg/mL) was added and cells were incubated at 37 °C for 4 h. Supernatants were then aspirated and formazan crystals were dissolved with DMSO. The optical density of each well was determined at 570 nm using a microplate reader (Safire2, Tecan Group Ltd, Maennedorf, Switzerland).

4.4. Determination of cytotoxicity

SH-SY5Y cells were cultured as described above and then pre-incubated with or without β-asarone at concentrations of 10 μg/mL, which was followed 24 h later by exposure to 20 μM aggregated $A\beta_{1\text{-}42}$ for 24 h. Lactate dehydrogenase (LDH) release was measured by a cytotoxicity detection kit (Genmed, Westbury, NY) following the instructions in the protocol, and OD values were measured at 490 nm with a microplate reader (Safire2, Tecan Group Ltd, Maennedorf, Switzerland). Results were expressed as percentage of Triton X-100-induced LDH release.

4.5. Fluorescence activated cell sorting (FACS) analysis

SH-SY5Y cells were cultured at a density 1.5×10^5 cells per well in growth medium for 24 h in 96-well plates. After transient transfection, the cells pre-incubated with 10 μg/mL β-asarone, which was followed 24 h later by exposure to 20 μM aggregated $A\beta_{1\text{-}42}$ for 24 h. Cells were washed with ice-cold phosphate-buffered saline (PBS) and harvested by centrifugation at 500 × g for 5 min. Apoptosis was assessed using the Annexin V-FITC Apoptosis Detection Kit I (Becton Dickinson, San Jose, CA). Cells were washed twice with cold PBS and resuspended in binding buffer before addition of annexin V-FITC and propidium iodide (PI). Cells were vortexed and incubated for 15 min in the dark at room temperature before analysis using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, San Carlos, CA).

4.6. Cellular caspase activity assay

SH-SY5Y cells were cultured as described above, and then pre-incubated with or without β-asarone at concentrations of 5, 10, or 20 μg/mL, which was followed 24 h later by exposure to 20 μM aggregated $A\beta_{1\text{-}42}$ for 24 h. The cells were harvested in cell lysis buffer (25 mM HEPES, pH 7.4, 5 mM CHAPS, and 5 mM DTT). Ac-DEVD-AFC and Ac-LEHD-pNA were the substrates for caspase-3 and caspase-9, respectively. The experiments were performed according to the manufacturer's protocol (Millipore Corporation, Bedford, MA, USA).

4.7. RNA isolation and real-time PCR

SH-SY5Y cells were treated as described above for the cellular caspase activity assay and harvested by scraping into ice cold PBS 24 h later. Total RNA was isolated from cells using RNAiso Reagent kit (Takara Biotechnology, Dalian, China), and cDNA was synthesized with ExScript^{TM} RT kit (Takara Biotechnology, Dalian, China) according to the manufacturer' protocol. PCR were performed by using SYBR® Premix Ex Taq^{TM} in an ABI7300 real-time PCR system (Applied Biosystems, CA). The following sequences were used as primers for real-time PCR amplification: Bax sense primer, 5'- GCC ATC TGG ATG TGT ACG TG-3', and Bax antisense primer, 5'- TCC ACC TTG TTC CCT TTC AG; Bad sense primer, 5'- AGT GAC CTT CGC TCC ACA TC -3', and Bad antisense primer, 5'-CAC GGA TCC TCT TTT TGC AT-3'; and GAPDH sense primer, 5'-AGG CAA CTA GGA TGG TGT GG-3' and GAPDH antisense primer, 5'- TTG ATT TTG GAG GGA TCT CG-3'. The thermal profile was as follows: 1 cycle of 95 °C for 10 s; 40 cycles of 5 s at 95 °C and 31 s at 60 °C. Threshold cycle (Ct) data were collected using the Sequence Detection Software version 1.2.3 (Applied Biosystems, CA). The Ct represents the cycle number at which a fluorescent signal rises statistically above background. Real-time

PCR assay was performed in triplicate to ensure reproducibility. The relative quantification of gene expression was analysed by the $2^{-\Delta\Delta Ct}$ method (Arocho et al. 2006). The fold change in target gene cDNA relative to the GAPDH internal control was determined by:
Fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{target\ gene} - Ct_{GAPDH}) - (Ct_{control} - Ct_{GAPDH})$

4.8. Western blot

SH-SY5Y cells were treated as described above for the FACS analysis or cellular caspase activity assay. Cytoplasm proteins were isolated from SH-SY5Y cells using Cytoplasmic Protein Extraction kit (Beyotime Biotechnology, Haimen, China), and protein concentrations were determined using the BCA Protein Assay kit according to the protocol provided by the manufacturer (Beyotime Biotechnology, Haimen, China), then they were aliquoted and stored. One hundred microliter of supernatant was added to an equal volume of 2 × SDS sample buffer and boiled for 5 min at 100 °C. The samples were then stored at -80 °C until analyzed. Equal amounts of protein (100 μg/lane) were separated by 15% SDS-polyacrylamide gel electrophoresis and then electrotransferred onto a nitrocellulose filter membrane. After blocking for 4 h in a solution of 8 % nonfat dry milk in Tris-buffered saline containing 0.1% Tween (pH 7.6) at room temperature, membrane was then incubated overnight at 4 °C with primary antibody (ASK1, p-ASK1, MKK7, and p-MKK7 antibody, Cell Signaling Technology Inc., Beverly, MA; other antibody, Santa Cruz Biotechnology, Santa Cruz, CA) in concentrations of 1:1000 (p-ASK1), 1:1000 (ASK1), 1:1500 (MKK7), 1:1500 (p-MKK7), 1:1500 (p-JNK), 1:1500 (JNK), 1:1500 (Bax), 1:1000 (Bad), and 1:2500 (GAPDH) in Tris-buffered saline with 0.1% Tween 20 containing 8 % nonfat dry milk. After washing four times, the membrane were incubated with Horseradish Peroxidase Labeled Anti-Mouse IgG (10000:1; Medical Biological Laboratory Co., Nagoya, Japan) at room temperature for 2 h and again washed four times. The blots were developed using an ECL western blotting kit (Amersham Biosciences, Piscataway, NJ, USA) as recommended by the manufacturer. GAPDH was probed as an internal control to confirm that an equal amount of protein was loaded in each lane. Band intensities were quantified by an AlphaImager™ 2200 using the SpotDenso function of AlphaEaseFC™ Software version 3.1.2 (Witec, Littau, Switzerland).

4.9. Statistical analysis

All values in the figures of present study indicate means ± standard deviation (S.D.), and all determinations were repeated three times. The one way analysis of variance (ANOVA) was used to evaluate the difference among multiple groups followed by a post hoc test (Student-Newman-Keuls) when variable distributions were normal. Otherwise, the nonparametric Mann-Whitney U test was used. The data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL). Differences were considered significant at $P < 0.05$.

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