—Regular Article—

Beta-Asarone Attenuates Neuronal Apoptosis Induced by Beta Amyloid in Rat Hippocampus

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Neurodegenerative disorders, such as Alzheimer's disease (AD), is associated with the loss of neuronal cells, and it has been suggested that apoptosis is a crucial pathway in neuronal loss in AD patients. Recent evidence suggests that amyloid beta peptide (Aβ) induces neuronal apoptosis in the brain and in primary neuronal cultures. In this study, we investigated the impact of β-asarone against the apoptosis induced by Aβ in rat hippocampus. The results showed that intrahippocampal injections of Aβ (1–42) caused apoptosis in rat hippocampus. Oral administration of β-asarone (12.5, 25, or 50 mg/kg) for 28 d reverse the increase in the number of terminal deoxynucleotidyl transferase dUTP nick-end labeling positive cells in the hippocampus tissue. Mitochondrial dysfunction is a hallmark of β-amyloid (Aβ)-induced neuronal toxicity in AD. Therefore, we investigated nuclear translocation of apoptosis induction factors. Our results showed that β-asarone afforded a beneficial inhibition on both mRNA and protein expression of Bad, Bax, and cleavage of caspases 9 in rat hippocampus following intrahippocampal injections of Aβ (1–42). Our further investigation revealed that ASK1, p-MKK7, and p-c-Jun were significantly decreased after β-asarone treatment, implicating that the modulation of ASK1/c-JNK-mediated intracellular signaling cascades might be involved in therapeutic effect of β-asarone against Aβ toxicity. Taken together, these results suggest that β-asarone may be a potential candidate for development as a therapeutic agent for AD.

Key words——Alzheimer's disease; apoptosis; β-asarone; β-amyloid; c-Jun

INTRODUCTION

Alzheimer's disease (AD) is one of the most common neurodegenerative diseases and the most frequent cause of dementia in the elderly.[1] However, the cause of AD is still unknown and the treatment is therefore only palliative. AD is characterized by the presence of amyloid plaques in specific areas of the brain,[2] and finally by the atrophy of the affected brain regions, which results from extensive losses of synapses and neurons.[3] Recent studies have shown that in AD brains and in cultures of neurons exposed to Aβ, the dying cells display the characteristics of apoptosis.[4–5] The role of apoptosis in Aβ-induced toxicity suggests that its modulation may slow the neurodegenerative process.

Mitogen-activated protein kinases (MAPK) are essential components of eukaryotic signal transduction networks that enable cells to respond appropriately to extracellular signals and stresses.[6] MAPK is activated by sequential protein phosphorylation through a MAPK module. In the case of the c-Jun N-terminal kinase (JNK) cascade, apoptosis-signal-regulating kinase (ASK) 1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) that activates MAP kinase kinase (MKK) 7, which in turn activates JNK, a member of MAPK.[7] The MAPK signal transduction cascade plays pivotal roles in many cellular processes including cell growth, differentiation, and apoptosis.[8] It has also been demonstrated that the JNK/c-Jun pathway is hyperactive in the AD brain.[9] ASK1 activation is a key mechanism in Aβ-induced neurotoxicity, which plays a central role in Alzheimer's disease.[10]

The acetylcholinesterase inhibitors,[11] such as donepezil, antioxidants such as vitamin E,[12] and inhibitors to NMDA excitotoxicity[13] are clinically used in AD therapy, because they marginally delay disease progression. However, currently there is no curative therapy for AD.[14] *Acorus tatarinowii* Schott has been found to be effective in the management of amnesia.[15] β-asarone (for its structure, *see* Fig. 1), and the major ingredient of *Acorus tatarinowii* Schott, might be a very promising drug to treat neurodegeneration disorders, including AD.

Because apoptosis inhibitors can prevent cell death even in the continued presence of the apoptosis-inducing trigger, apoptosis pathways are attractive tar-

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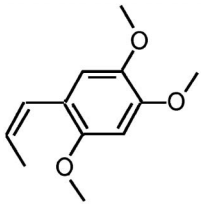

Fig. 1. Chemical Structure of β-asarone

gets for therapeutic intervention.[16] So far, whether β-asarone might be beneficial to AD by suppressing the apoptosis still remains to be elucidated. The aims of this study were, therefore, to investigate the effect of β-asarone on the Aβ-induced neuronal apoptosis and to identify signaling protein kinase cascades that may be responsible for the putative effect of β-asarone.

MATERIALS AND METHODS

Animals Male Sprague-Dawley (SD) rats (weight range: 220 to 240 g) were purchased from the Beijing Vital River Experimental Animals Technology Ltd. (Beijing, China), and were housed 6 per cage in a light-controlled room (lights on from 7:00 AM to 7:00 PM) at a temperature of 22±2℃ and humidity of 50±5% with food and water *ad libitum* feeding. All studies were previously approved by the Animal Care and Use Committee of Qiqihar Medical University. Rats were assigned randomly into 7 groups: the normal control group, sham control group, the Aβ-injection group, Aβ plus donepezil group, Aβ plus β-asarone (12.5 mg/kg) group, Aβ plus β-asarone (25 mg/kg) group, and Aβ plus β-asarone (50 mg/kg) group.

Aβ(1–42)-induced AD Rat Model The Aβ (1–42) (Sigma, St. Louis, MO. USA) were dissolved in 35% acetonitrile/0.1% trifluoroacetic acid at a concentration of 10 μg/μl and incubated at 37℃ for 7 d to allow for fibril formation. The rats were anesthetized with sodium pentobarbital (40 mg/kg, i.p.), and placed on a stereotaxic instrument (David Kopf Instruments, Tujunga, CA). The scalp of each rat was incised, and the skull was adjusted to place bregma and lambda on the same horizontal plane. Small burr holes were drilled and Aβ (1–42) (1 μl=10 μg) or the vehicle (1 μl) were injected into the bilateral hippocampuses (−3.0 mm anteroposterior, ±2.2 mm medial-lateral, −2.8 mm dorsal-ventral from the dura, according to bregma)[17] at a rate of 0.2 μl/min with a 5 μl Hamilton syringe with 27-gauge stainless steel needle. The hole was blocked with dental acrylic cement and scalp was then closed with suture. Sham group rats received the same surgical procedures with injection of identical volume vehicle (acetonitrile/0.1% trifluoroacetic acid). After wound sutured, the rats were allowed to recover from surgery for 3 days. Donepezil hydrochloride (0.33 mg/kg) (Sigma, St. Louis, MO. USA) (referred to simply as donepezil) and β-asarone (Sigma, St. Louis, MO. USA) (12.5, 25, or 50 mg/kg) were administered intragastricly once daily for 28 days from three days after Aβ (1–42) hippocampus injection.

Tissue Processing Rats were anesthetized and transcardially perfusion-fixed with 4% formaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). Brains were removed quickly and postfixed with the same fixation solution overnight at 4℃. Postfixeds brain were embedded in papaffin, and 5-micrometer sections were obtained using a rotary microtome and placed on pretreated slides.

Evaluation of Apoptosis with Terminal Deoxynucleotidyl Transferase-mediated dUTP Nick-end Labeling (TUNEL) Staining For detection of *in situ* DNA fragmentation, TUNEL staining was performed using an TACSR TdT *In Situ* Apoptosis Detection Kits (Trevigen Inc., Gaithersburg, Md., USA) according to the manufacturer's protocol. In brief, tissue sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity. After adding the equilibration buffer, sections were treated with terminal deoxynucleotidyltransferase (TdT) and digoxigenin-dNTPs for 60 min at 37° C. Specimens were then treated with anti-digoxigenin-peroxidase for 30 min at 37° C, colorized with 3,3′-diaminobenzidine (DAB) substrate. Finally, slides were rinsed, dehydrated, and mounted. A negative control was prepared by omitting the TdT enzyme to control for non-specific incorporation of nucleotides or binding of enzyme-conjugate. The specimens were examined using a bright-field microscope (Zeiss Axioskop; Carl Zeiss GmbH, Jena, Germany) and the data expressed as the number of TUNEL-positive cells/mm^2 in at least five random high-power fields.

RNA Isolation and Real-time PCR Total RNA was extracted from 100 mg of frozen hippocampus tissues using RNAiso Reagent kit (Takara Biotechnology, Dalian, China), and cDNA was synthesized with SYBR ExScript™ RT-PCR kit (Takara Biotechnology, Dalian, China) according to the

manufacturer's protocol. Reverse transcription was carried out as follows: 42℃ for 15 min, 95℃ for 2 min (one cycle). cDNA stored at −20℃ for PCR.

Real-time PCR was performed in a 50 μl of reaction solution. The following sequences were used as primers: ASK1 sense primer, 5′-CTG TGC TAA TGA CCT GCT TGT TG -3′, and ASK1 antisense primer, 5′-TAC CCG AGA GTT TGG GCT GT; MKK7 sense primer, 5′-GGC TCC CGT CAA CCT TGT TC-3′, and MKK7 antisense primer, 5′-GGT ACC CTG TCT GCT TCA TGA TCT C; c-Jun sense primer, 5′-GGG AAC AGG TGG CAC AGC TTA-3′, and c-Jun antisense primer, 5′-GCA ACT GCT GCG TTA GCA TGA; Bad sense primer, 5′-ACA CGC CCT AGG CTT GAG GA-3′, and Bad antisense primer, 5′-GGC TCA AAC TCT GGG ATC TGG A; Bax sense primer, 5′-AGA CAC CTG AGC TGA CCT TGG AG-3′, and Bax antisense primer, 5′-GTT GAA GTT GCC ATC AGC AAA CA; caspase-9 sense primer, 5′-CTG AGC CAG ATG CTG TCC CAT A-3′, and caspase-9 antisense primer, 5′-CCA AGG TCT CGA TGT ACC AGG AA; GAPDH sense primer, 5′-GAC AAC TTT GGC ATC GTG GA -3′, and glyceraldehyde phosphate dehydrogenase (GAPDH) antisense primer, 5′-ATG CAG GGA TGA TGT TCT GG. The thermal profile was as follows: 1 cycle of 95℃ for 10 s; 40 cycles of 5 s at 95℃ and 31 s at 60℃. Reactions were performed in a ABI7300 real-time PCR system (Applied Biosystems, CA), and threshold cycle (Ct) data were collected using the Sequence Detection Software version 1.2.3 (Applied Biosystems, CA).

Real-time PCR assay was performed in triplicate for each samples to ensure reproducibility. The relative quantification of gene expression was analysed by the $2^{-\Delta\Delta Ct}$ method.[18] The fold change in target gene cDNA relative to the GAPDH internal control was determined by:

Fold change$=2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct=(Ct_{\text{target gene}}-Ct_{GAPDH})-(Ct_{control}-Ct_{GAPDH})$

Western Blot Cytoplasm proteins were isolated from 120 mg of frozen hippocampus tissues using Cytoplasmic Protein Extraction kit (Beyotime Biotechnology, Haimen, China), and protein concentrations were determined using the BCA Protein Assay kit (Beyotime Biotechnology, Haimen, China) according to the protocol provided by the manufacturer, then they were aliquoted and stored.

100 μl of supernatant was added to an equal volume of 2×SDS sample buffer and boiled for 5 min at 100℃. The samples were then stored at −80℃ 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℃ with ASK1 antibody (2000:1), MKK7 antibody (2000:1), p-MKK7 antibody (1500:1), c-Jun antibody (1500:1), p-c-Jun antibody (2000:1), Bax antibody (1500:1), Bad antibody (1500:1), caspase-9 antibody (2000:1), or GAPDH antibody (3000:1) 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 and was used 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).

Statistical Analysis All values in the figures of present study indicate means±standard deviation (SD) and n represents the number of rats used in each experiment. 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). The data were analyzed by SPSS 13.0 software (SPSS Inc., Chicago, IL, USA), and $p<0.05$ was assessed as statistically significant.

RESULTS

***β*-Asarone Attenuates Neuronal Apoptosis in an A*β* (1–42)-injected AD Rat Model** To explore the effects of *β*-asarone on apoptosis induced by A*β* (1–42), TUNEL staining was used to identify apoptotic cells in the hippocampus. Control sections incubated without TdT enzyme showed no staining (results not shown). Brain sections from the sham and normal group showed very few TUNEL-positive nuclei, whereas there was an increase in TUNEL-positive

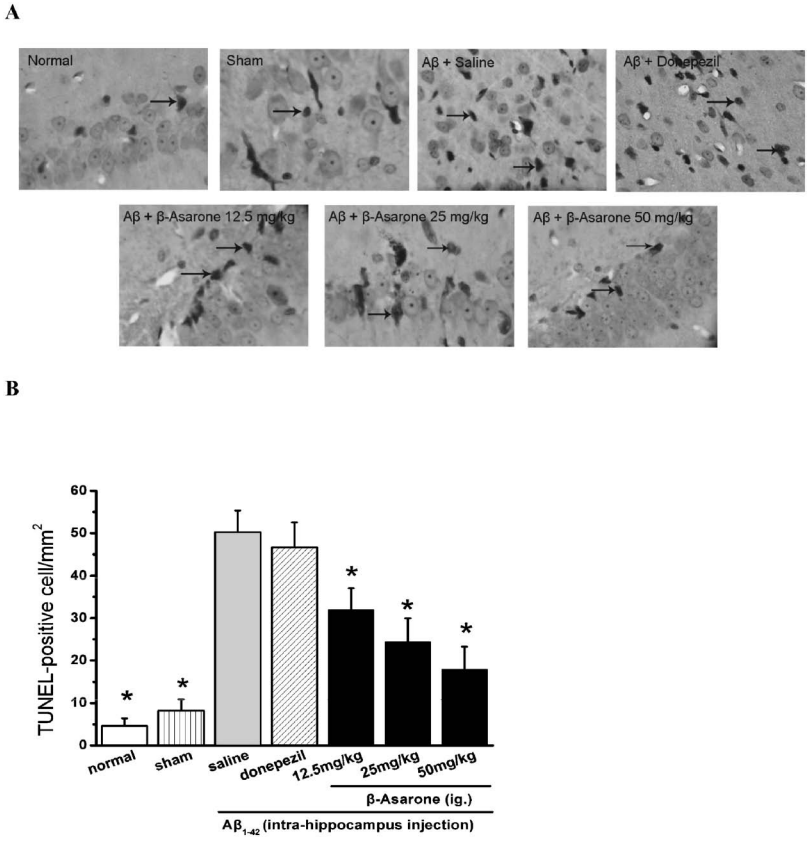

Fig. 2. β-Asarone Treatment Attenuation Neuronal Apoptosis Induced by Aβ (1–42) Injection

Treatment with β-asarone (12.5, 25, or 50 mg/kg) or donepezil (0.33 mg/kg) given *p.o.* was initiated 3 days after the Aβ (1–42) intrahippocampal injections into SD rat for 28 days. Detection of hippocampus apoptosis cells was carried out using TUNEL method. A) Representative sections of TUNEL-labeled cell in the hippocampus. TUNEL-positive pyramidal neurons undergoing apoptosis were observed in the hippocampus (arrowheads) (original magnifications, $\times$200). B) The bar chart describes the number of TUNEL-positive cells obtained from 5 rats per group, and data are expressed as means$\pm$S.D.; $^{*}p<0.05$ *vs.* Aβ (1–42)-injected rats.

cells in the Aβ (1–42) group. The increase in TUNEL-positive cells was inhibited in the Aβ (1–42) $+\beta$-asarone (12.5, 25, or 50 mg/kg) group (Fig. 2 (A)). Quantitative analysis of TUNEL-positive cells revealed that TUNEL-positive nuclei in CA1 of the hippocampus were reduced from $50.20\pm5.16/mm^2$ in Aβ (1–42) group to 32.00 ± 5.09, 24.40 ± 5.59, and $18.00\pm5.24/mm^2$ in Aβ (1–42) $+\beta$-asarone (12.5, 25, or 50 mg/kg) group (Fig. 2(B)) ($p<0.01$).

Involvement of Proapoptotic Bax and Bad Proteins in the Suppressive Effect of β-Asarone against Aβ (1–42)-induced Neuronal Apoptosis The proapoptotic Bax and Bad proteins play a key role in the apoptotic process. To investigate the effects of β-asarone on expression of proapoptotic Bax and Bad, we determined these proteins by western blotting. Bax and Bad protein levels in Aβ-injected rats were significantly increased as compared with that in sham-operated rats ($165.92\pm23.20\%$ *vs.* $100.84\pm15.40\%$; $152.14\pm10.37\%$ *vs.* $99.24\pm6.13\%$, $n=5$, $p<0.05$, respectively). Treatment with β-asarone (25 or 50 mg/kg) markedly decreased the expression of Bax ($114.04\pm11.46\%$ and $108.68\pm12.45\%$) and Bad ($103.12\pm8.81\%$, and $110.34\pm16.04\%$) compared with that in Aβ (1–42)-injected rats ($n=5$, $p<0.05$, respectively) (Fig. 3(A) and (B)). Consistent with the results of protein levels, real-time PCR revealed that β-asarone treatment (12.5, 25, or 50 mg/kg) significantly decreased Bax mRNA levels to 2.56 ± 0.67, 2.48 ± 0.44, and 2.04 ± 0.45 from 4.37 ± 1.19 of Aβ-injected rats, and decreased Bad mRNA levels to 3.03 ± 0.39, 2.85 ± 0.51, and 3.02 ± 0.58 from 5.56 ± 1.08 (Fig. 3(C)). In addition, up-regulation of Bax and Bad was not attenuated by donepezil.

Involvement of Caspase-9 Activition in the Suppressive Effect of β-Asarone against Aβ (1–42)-induced Neuronal Apoptosis Caspase-9, a key molecule in apoptotic signaling, is thought to be initiator caspases. To determine whether β-asarone suppresses neuronal apoptosis in rat hippocampus in-

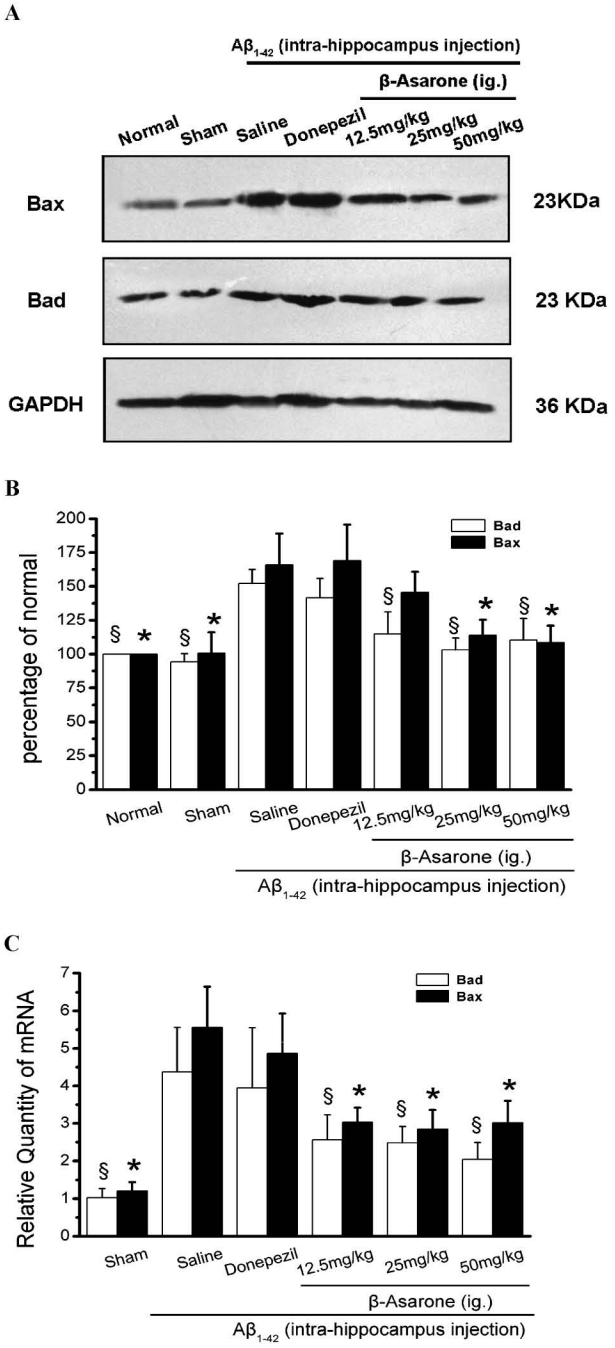

Fig. 3. β-Asarone Attenuation Aβ (1–42)-induced Upregulation of Bax and Bad

Treatment with β-asarone (12.5, 25, or 50 mg/kg) or donepezil (0.33 mg/kg) given *p.o.* was initiated 3 days after the Aβ (1–42) intrahippocampal injections into SD rat for 28 days. A) Bax and Bad levels were determined by western blot analysis wth antibodies 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 sham. Densitometric analysis of western blot obtained from 5 rats per group. C) Total RNA was isolated from hippocampus 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 5 rats per group, and data are expressed as means$\pm$S.D.; $^{*}p<0.05$, $^{\S}p<0.05$, *vs.* Aβ (1–42)-injected rats.

duced by Aβ (1–42) *via* caspase-9-mediated apoptosis pathways, we used western blot and real-time PCR to analyze mRNA and protein expression of caspase-9. The western blot result of caspase-9 in hippocampus showed Aβ (1–42)-injection result in activation of caspase-9 (171.03$\pm$41.08% in the Aβ (1–42) injected group *versus* 102.16$\pm$25.95% in sham group). Treatment with β-asarone (25 or 50 mg/kg) attenuated Aβ (1–42)-induced activation of caspase-9 in hippocampus (128.94$\pm$15.10% and 114.20$\pm$15.64%, approximately the inhibition being 24% and 33 7%, respectively) (Fig. 4(A) and (B)). Consistent with the results of protein levels, real-time PCR result revealed that β-asarone treatment (50 mg/kg) significantly reduced caspase-9 mRNA levels to 1.38$\pm$0.27 from 1.83$\pm$0.26 of Aβ-injected rats (Fig. 4(C)).

Effect of β-Asarone Attenuation Aβ (1–42)-induced Neuronal Apoptosis through a ASK1/c-Jun Signal Pathway To determine whether the ASK1/c-Jun pathway functions involvement in that β-asarone attenuate Aβ (1–42)-induced neuronal apoptosis, we assessed mRNA and protein of ASK1, p-MKK7, and p-c-Jun using western blot and real-time PCR analysis, respectively. Western blotting result revealed that ASK1, p-MKK7, and p-c-Jun protein was clearly up-regulated in Aβ-induced hippocampus in comparison with that sham-operated hippocampus. However, the expression of ASK1, p-MKK7, and p-c-Jun in Aβ-induced hippocampus was significantly down-regulated when the rats were treated with β-asarone (12.5, 25 or 50 mg/kg) (Figs. 5(A) and (B), 6 (A) and (B), 7(A) and (B)). Donepezil (0.33 mg/kg) had no effect on those proteins expression. Real-time PCR result revealed that ASK1 and MKK7 mRNA expression in hippocampus tissues was significantly deceased when rats were treated with β-asarone compared with control (Figs. 5(C) and 6(C)), but mRNA expression of c-Jun was not altered significantly (Fig. 7(C)).

DISCUSSION

We have clearly demonstrated for the first time in the current study that β-asarone treatment attenuated neuronal apoptosis induced by intrahippocampal injection of Aβ (1–42). β-asarone afforded a beneficial inhibition on expression of Bad, Bax, and cleavage of caspases 9 in rat hippocampus following intrahippocampal injections of Aβ (1–42). In addition, the modulation of ASK1/c-Jun-mediated intracellular

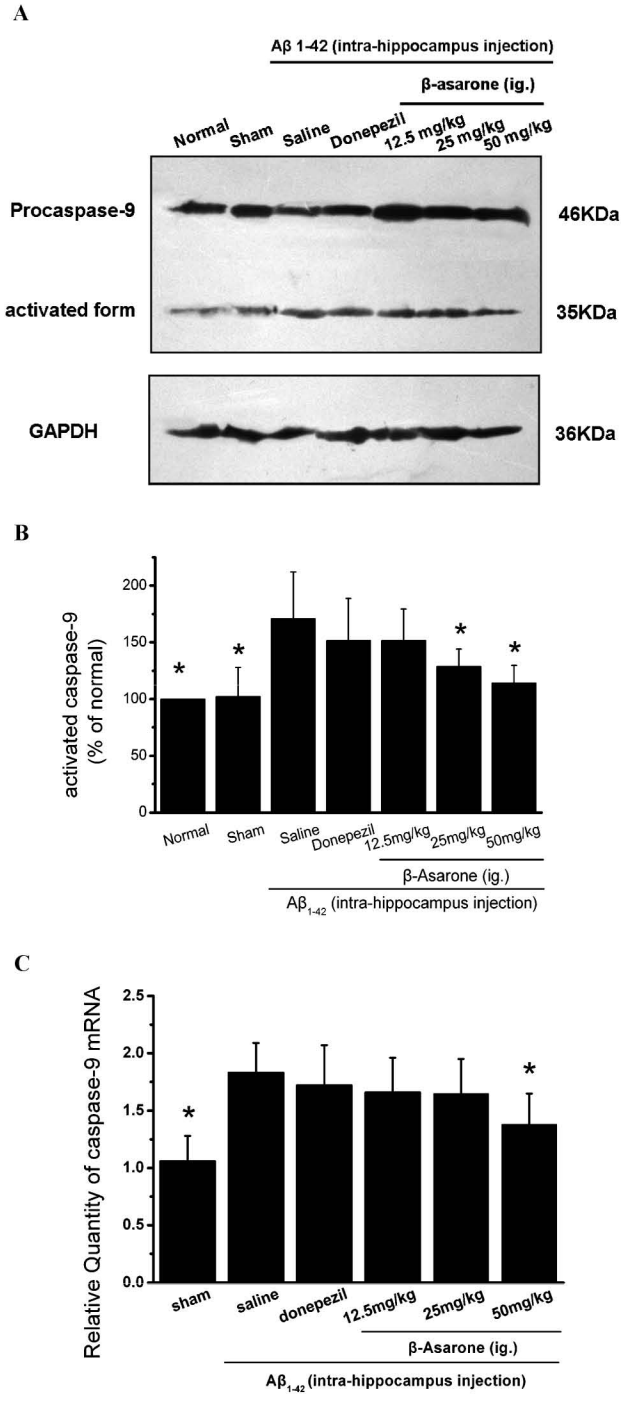

Fig. 4. β-Asarone Inhibition Aβ (1–42)-induced Caspase-9 Activition

Treatment with β-asarone (12.5, 25, or 50 mg/kg) or donepezil (0.33 mg/kg) given *p.o.* was initiated 3 days after the Aβ (1–42) intrahippocampal injections into SD rat for 28 days. A) Procaspase-3 and cleaved caspase-9 levels were determined by western blot analysis wth antibody to caspase-9. The loading of the lanes was normalized to levels of GAPDH. B) Quantitated results of activated caspase-9 are presented relative to sham. Densitometric analysis of western blot obtained from 5 rats per group. C) Total RNA was isolated from hippocampus using RNAiso reagent and used for cDNA synthesis. The mRNA levels of caspase-9 were detected by real-time PCR. $2^{-\Delta\Delta Ct}$ analysis of PCR obtained from 5 rats per group, and data are expressed as means±S.D.; $^{*}p<0.05$, $^{\S}p<0.05$, *vs.* Aβ (1–42)-injected rats.

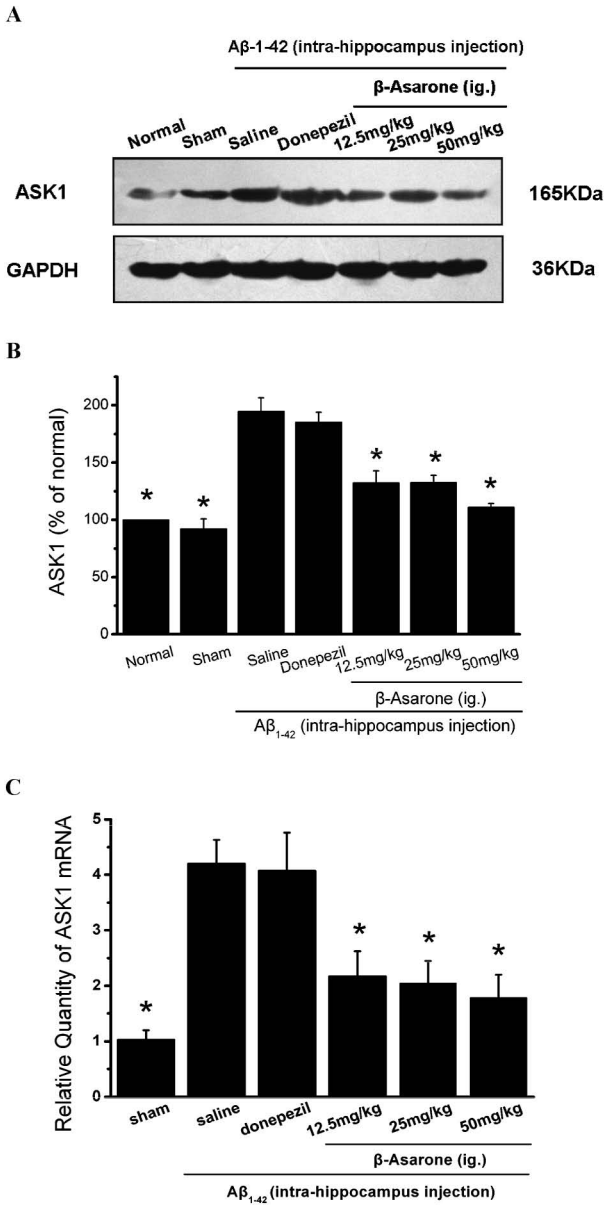

Fig. 5. β-Asarone Inhibition Aβ (1–42)-induced Upregulation of ASK1

Treatment with β-asarone (12.5, 25, or 50 mg/kg) or donepezil (0.33 mg/kg) given *p.o.* was initiated 3 days after the Aβ (1–42) intrahippocampal injections into SD rat for 28 days. A) ASK1 levels were determined by western blot analysis wth antibody to ASK1. The loading of the lanes was normalized to levels of GAPDH. B) Quantitated results of ASK1 are presented relative to sham. Densitometric analysis of western blot obtained from 5 rats per group. C) Total RNA was isolated from hippocampus using RNAiso reagent and used for cDNA synthesis. The mRNA levels of ASK1 were detected by real-time PCR. $2^{-\Delta\Delta Ct}$ analysis of PCR obtained from 5 rats per group, and data are expressed as means±S.D.; $^{*}p<0.05$, $^{\S}p<0.05$, *vs.* Aβ (1–42)-injected rats.

signaling cascades might be involved in therapeutic effect of β-asarone against Aβ toxicity.

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 vari-

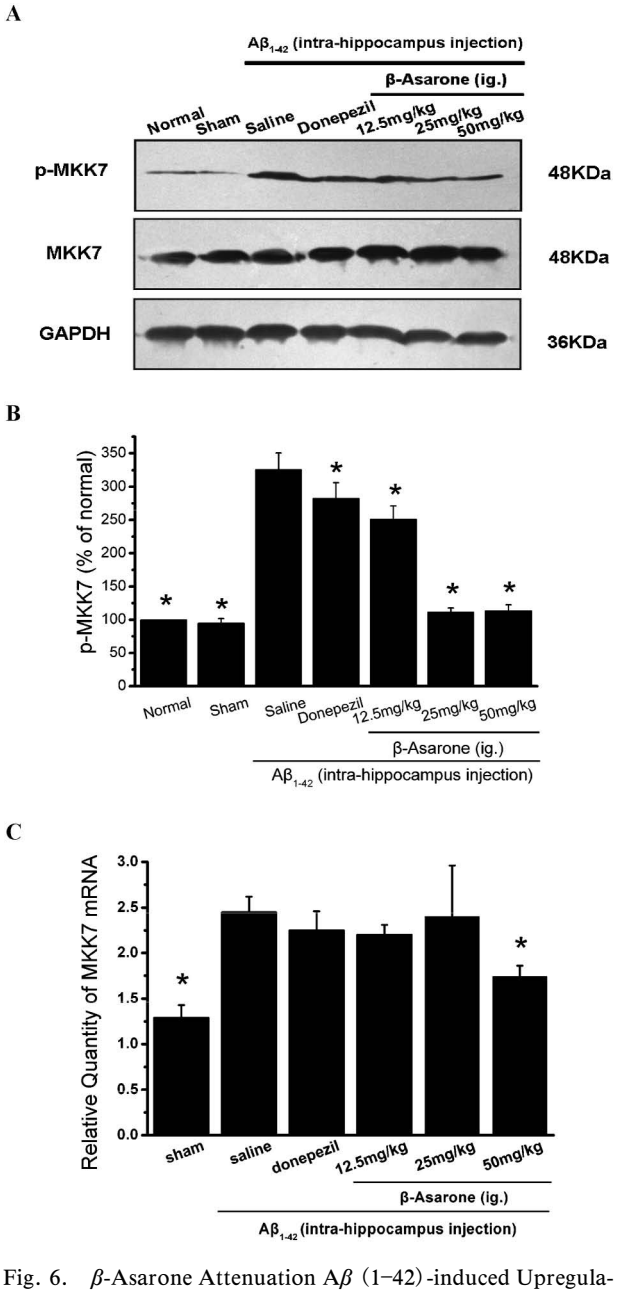

Fig. 6. β-Asarone Attenuation Aβ (1–42)-induced Upregulation of p-MKK7

Treatment with β-asarone (12.5, 25, or 50 mg/kg) or donepezil (0.33 mg/kg) given *p.o.* was initiated 3 days after the Aβ (1–42) intrahippocampal injections into SD rat for 28 days. A) MKK7 and P-MKK7 levels were determined by western blot analysis wth antibodies to MKK7 and p-MKK7. The loading of the lanes was normalized to levels of GAPDH. B) Quantitated results of p-MKK7 are presented relative to sham. Densitometric analysis of western blot obtained from 5 rats per group. C) Total RNA was isolated from hippocampus using RNAiso reagent and used for cDNA synthesis. The mRNA levels of MKK7 were detected by real-time PCR. $2^{-\Delta\Delta Ct}$ analysis of PCR obtained from 5 rats per group, and data are expressed as means±S.D.; $^{*}p<0.05$, $^{\S}p<0.05$, *vs.* Aβ (1–42)-injected rats.

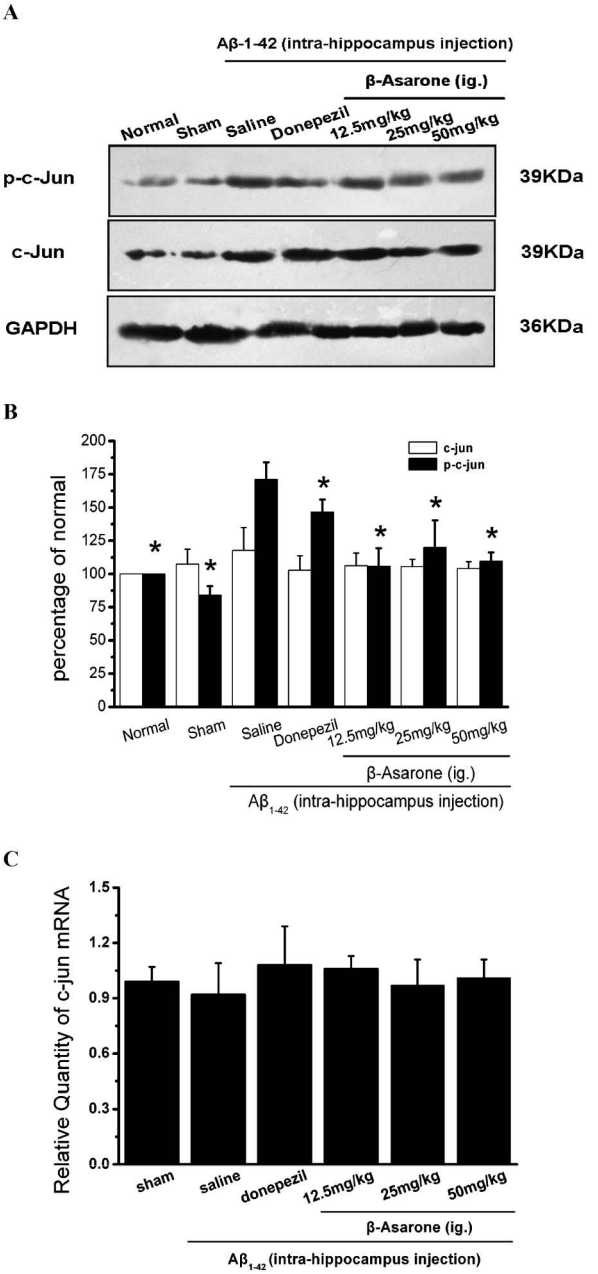

Fig. 7. β-Asarone Attenuation Aβ (1–42)-induced c-Jun Phosphorylation

Treatment with β-asarone (12.5, 25, or 50 mg/kg) or donepezil (0.33 mg/kg) given *p.o.* was initiated 3 days after the Aβ (1–42) intrahippocampal injections into SD rat for 28 days. C-Jun and p-c-Jun levels were determined by western blot analysis wth antibodies to c-Jun and p-c-Jun. The loading of the lanes was normalized to levels of GAPDH. B) Quantitated results of p-c-Jun are presented relative to sham. Densitometric analysis of western blot obtained from 5 rats per group, and data are expressed as means±S.D.; $^{*}p<0.05$, $^{\S}p<0.05$, *vs.* Aβ (1–42)-injected rats.

ous pharmacological actions on the central nervous system (CNS).[15] The rhizomes and leaves of *Acorus tatarinowii* Schott are known to contain 0.11–0.42% of essential oil consisting of 30 kinds of compounds which may affect CNS.[19] The more recent results of Wu *et al.* confirmed that β-asarone, a component isolated from essential oil of *Acorus tatarinowii* Schott, is easy to pass through blood brain barrier, and brain is an major organ of distributing of it.[20] β-asarone,

however, has not yet been evaluated for actions on the AD and its mechanism of action.

Apoptosis is a fundamental process of cell death that occurs *via* activation of distinct signaling pathways involving mitochondria, mitochondrial regulatory proteins, and activation of caspases.[21] Ultimately, cells undergo nuclear chromatin condensation, DNA fragmentation, and formation of apoptotic bodies. Several studies presently indicate that apoptosis selectively increased in primary neuronal cultures exposed to Aβ and also augmented in brain tissue derived from AD patients.[22] Caspase-9 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.[23] The cleavage of caspase-specific substrates results in the biochemical destruction of the cell and phenotypic changes associated with apoptosis.[24] Caspase-9 is thought to be initiator caspases.[25] In the present study, we used TUNEL staining to identify apoptotic cells in the hippocampus. Brain sections from the sham group showed very few TUNEL-positive nuclei, whereas there was an increase in TUNEL-positive cells in the Aβ hippocampus injection group. The increase in TUNEL-positive cells was inhibited after administration of β-asarone for 28 days. We further determined the activation of caspase-9 and show Aβ hippocampus injection increased the level of cleaved caspase-9, marker for caspase-9 activity, which is the feature of apoptosis in AD. Beta-asarone treatment attenuated the expression of activated caspase-9 induced by Aβ in the hippocampus in a dose-dependent manner.

Many components of the mitochondrial apoptotic cascade appear to be involved in the neuronal toxicity of Aβ peptides.[26] Aβ can upregulate pro-apoptotic Bax expression or require Bax to mediate neurotoxicity.[27] In addition, Bax protein levels have been reported to be increased in AD brain.[28] Another proapoptotic protein Bad is located in the cytosol but translocate to the mitochondria and form a proapoptotic complex with Bcl-2.[29] Bad and Bax have been reported to directly inhibit members of the caspase family, including caspases-9 which is a potent effector of neuronal death among the identified caspases.[30] In the present study, we observed that Aβ-induced apoptosis are characterized by increased expression of the proapoptotic Bax and Bad. Furthermore, Aβ-injected rats treated with β-asarone exhibited descent of proapoptotic protein Bax and Bad. These results suggested that the mitochondrial pathway of cell death might be involved in therapeutic effect of β-asarone against Aβ toxicity. Our results are in agreement with previous findings reported by Solá *et al.* in cortical neurons.[31]

ASK1 is a ubiquitously expressed MAPKKK that activates the JNK and p38 pathways by directly phosphorylating and thereby activating their respective MAPKK7 and MAPKK4.[32] Overexpression of wild-type or constitutively active ASK1 induces apoptosis in various cells through mitochondria-dependent caspase activation.[33] ASK1 is present in neuronal cells and has been implicated in various types of neuronal cell death.[34] JNK and c-Jun are directly activated by the dual specificity MAP kinase kinases MKK4 and MKK7. The organization of the JNK/c-Jun signaling pathway is similar to that of other mammalian MAPK modules.[35] The extracellular signal-regulated kinase (ERK) group of MAPK is activated by MKK1 /MKK2 and p38 group of MAPK is activated by MKK3/MKK6.[36] *In vitro* assays demonstrate that MKK4 can activate both JNK and p38 MAPK. In contrast, MKK7 selectively activates only JNK.[37] Concerted actions of molecular signaling networks determine cell fates. In the many stress-responsive signaling pathways, JNK/c-Jun signaling cascade is crucial for the maintenance of cell homeostasis and controls many cellular processes, including cell growth, transformation, differentiation and apoptosis.[38] There is very strong evidence linking the activation of JNK to neuronal loss in response to Aβ neurotoxicity.[39] JNK/c-Jun signaling promotes apoptosis that is linked to transcriptional regulation of many genes, including Bad and Bax.[40] SP600125, JNK pharmacological inhibitor, effectively prevents Aβ-induced alterations of Bcl-2 family expression during apoptosis, indicating that this critical step in the Aβ induced-apoptosis pathway is dependent on JNK activation.[41] Thus, the suppression of c-Jun-dependent apoptosis gene expression may be an extremely effective therapeutic strategy for preventing neuronal cell apoptosis. In the present study, we observed that β-asarone significantly attenuated Aβ-induced changes in Bad and Bax expression. Notably, it also significantly reduced Aβ-induced c-Jun phosphorylation, suggesting that β-asarone attenuation of As-induced changes in Bad and Bax expression is linked to JNK/c-Jun pathway in rat hippocampus. These findings are con-

sistent with previous data from the literature indicating that c-Jun represents a key target of various neuroprotective agent, including estrogen.[41]

In conclusion, our results are consistent with the hypothesis that β-asarone potentially attenuate neuronal apoptosis in rats induced by Aβ (1–42) when taken orally. The attenuation is associated with the inhibition of p-c-Jun activation, Bad expression, Bax expression, and activation of caspase-9. Our findings suggest that β-asarone, an important active principal of *Acorus tatarinowii* Schott, might be a potential drug for the AD to suppress AD-related neuronal cell apoptosis.

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