Nicotine exerts neuroprotective effects against β-amyloid-induced neurotoxicity in SH-SY5Y cells through the Erk1/2-p38-JNK-dependent signaling pathway

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Abstract. Epidemiological data have indicated that smoking tobacco can decrease the risk of developing Alzheimer's disease (AD). Nicotine, a main component of tobacco, has been shown to have therapeutic effects in AD. The aim of the present study was to assess the neuroprotective effects of nicotine against toxicity induced by β-amyloid (Aβ) in relation to cell apoptosis, and to elucidate the role of the activation of the Erk1/2-p38-JNK pathway and the modulation of anti-apoptotic proteins in the nicotine-induced neuroprotective effects. We performed *in vitro* and *in vivo* experiments using SH-SY5Y cells and C57BL/6 mice, respectively. The effects of nicotine on cell apoptosis were determined by flow cytmetry and microscopic observation. The effects of nicotine on the expression of anti-apoptotic proteins were also determined by western blot analysis. Our results demonstrated that nicotine protected the SH-SY5Y cells against $A\beta_{25-35}$-induced toxicity by inhibiting apoptosis and upregulating the expression of anti-apoptotic proteins. As shown by our *in vivo* experiments, nicotine effectively ameliorated the impairment in spatial working memory induced by $A\beta_{25-35}$; this was confirmed by a Morris water maze navigation test and further supported by the upregulation of Bcl-2 in the hippocampus of $A\beta_{25-35}$-injected mice treated with nicotine. The phosphorylation of Erk1/2, p38 and JNK increased following treatment with nicotine in the SH-SY5Y cells, whereas caspase-3 activation was inhibited by treatment with nicotine prior to exposure to $A\beta_{25-35}$. Of note, these effects of nicotine against $A\beta_{25-35}$-induced damage were abolished by inhibitors of Erk1/2, p38 and JNK phosphorylation. These findings suggest that nicotine prevents $A\beta_{25-35}$-induced neurotoxicity through the inhibition of neuronal apoptosis, and may thus prove to be a potential preventive or therapeutic agent for AD.

Introduction

Alzheimer's disease (AD), the most common form of dementia in elder persons, is a neurodegenerative disease which is characterized by the progressive loss of memory, deterioration of language, as well as defects in visual and motor coordination (1). Documented data have shown that the pathological hallmarks of AD include the accumulation of intracellular neurofibrillary tangles rich in Tau protein and extracellular plaques containing β-amyloid protein (Aβ) (2). Despite the fact that the precise cause of AD remains unknown, toxic Aβ accumulation-induced neuronal loss and Aβ, the constituent of extracellular plaques have been observed in patients with AD. This indicates that Aβ may play an important role in the development of AD (3).

Oxidative stress, an imbalance toward the pro-oxidant side of pro-oxidant/antioxidant homeostasis, occurs in some brain neurodegenerative disorders. Although it has been reported that nicotine possibly promotes lung cancer development and reduces the efficacy of chemotherapeutic agents (4), the rapid synaptic transmission in key regions controlling behavior mediated by nicotine via nicotine acetylcholine receptors (nAChRs) has also reported (5). Notably, nicotine has been confirmed to improve memory function and reduce amyloid plaque burden in a transgenic mouse model of AD (6), which indicates that nicotine may be a survival agonist against apoptosis induced by various types of stress (7). However, the exact mechanisms of action of nicotine and its role in the improvement of AD remain unclear; elucidating these mechanisms pay prove useful in the treatment of AD.

Bcl-2, Bcl-xL and Mcl-1, which belong to the Bcl-2 family, are localized in the outer mitochondrial membrane and protect cells against a variety of apoptotic stimuli (8). Despite the fact that nicotine increases Bcl-2, Bcl-xL and Mcl-1 expression and facilitates multiple drug resistance in lung cancer (9), the exact effects of the nicotine-induced upregulation of anti-apoptotic proteins in the treatment of AD have not yet been elucidated. Mitogen-activated protein kinase (MAPK) signaling pathways, which included Erk, SAPK/JNK and p38, play important roles in growth, differentiation, development and cell survival (10-12). Nicotine has been reported to activate the MAPK pathway in

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various tissues and cell types (13,14). Other studies have also shown that the inhibition of MAPK pathways is involved in the anti-apoptotic effects and mediates neuroprotection (15). Hence, the exact roles of Erk, SAPK/JNK and p38 in nicotine-mediated neuroprotection remain unelucidated.

In the present study, we aimed to investigate the protective effects of nicotine on Aβ-induced neurotoxicity. The effects of nicotine on Aβ-induced SH-SY5Y cell apoptosis were first determined by flow cytometry and microscopic observation. The effects of nicotine on Bcl-2, Bcl-xL and Mcl-1 expression, as well as MAPK kinase activation were further explored by western blot analysis. Using kinase inhibitors, the levels of Erk, SAPK/JNK and p38 phosphorylation, as well as caspase-3 activation, Bcl-xL expression and cell viability were further investigated. Importantly, the neuroprotective effects of nicotine on the impairment of spatial working memory by $A\beta_{25-35}$ and Bcl-2 expression were investigated by a Morris water maze navigation test and immunohistochemical analysis of hippocampal sections using a mouse model of Aβ-induced AD. The results revealed the following: firstly, Aβ markedly augmented SH-SY5Y cell apoptosis, the release of cytochrome *c* and caspase-3 activation in a time- and dose-dependent manner. Secondly, pre-treatment with nicotine attenuated the Aβ-induced cell apoptosis, inhibited caspase-3 activation and increased cell viability. Thirdly, treatment with nicotine markedly upregulated Bcl-2, Bcl-xL and Mcl-1 expression and MAPK kinase phosphorylation. Notably, when the activities of Erk, SAPK/JNK and p38 were inhibited, the nicotine-induced Bcl-xL upregulation and anti-apoptotic effects were reversed accordingly. Importantly, *in vivo* nicotine administration greatly ameliorated the impairment in spatial working memory induced by $A\beta_{25-35}$ and upregulated Bcl-2 expression in the hippocampus. The data presented in this study indicate that nicotine exerts neuroprotective effects against Aβ-induced neurotoxicity by activating the MAPK pathway and upregulating the expression of anti-apoptotic proteins.

Materials and methods

Reagents. Nicotine and the $A\beta_{25-35}$ peptide were purchased from Sigma-Aldrich (St. Louis, MO, USA). $A\beta_{25-35}$ was dissolved in deionized distilled water at a concentration of 5 mg/ml. The stock solution was diluted to the desired concentrations immediately before use. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from HyClone (Logan, USA). The Annexin V/PI Apoptosis detection kit was obtained from Promega (Madison, WI, USA). Erk1/2 inhibitor (U0126), p38 inhibitor (SB203580) and JNK inhibitor (SP600125), as well as antibodies to tubulin, Bcl-2, Bc-xL, Mcl-1, cleaved caspase-3, cytochrome *c*, phospho-p38, phospho-Erk1/2, phospho-Mek1/2, phospho-p90Rsk, phospho-Msk, phospho-c-Jun, phospho-SAPK/JNK and phospho-MKK3/6 were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA).

Animals. Pathogen-free C57BL/6 mice (male, 4 weeks old, weighing 18-22 g) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China) and kept at the Animal Center of Xiamen University (Xiamen, China). All animal experiments were approved by the Review Board of the Medical College of Xiamen University.

Cell lines. Human SH-SY5Y neuroblastoma cells were obtained from the Shanghai Cell Bank (Shanghai, China). Cells were cultured in DMEM with 10% FBS at 37˚C in 5% CO_2 and passaged every 1-2 days to maintain logarithmic growth. Cells were synchronized by serum starvation for at least 12 h prior to treatment with nicotine or $A\beta_{25-35}$ for the indicated periods of time and concentrations.

Cell apoptosis assay. Cell apoptosis assay was determined by flow cytometry according to a previously described method (16). Briefly, $8x10^4$ SH-SY5Y cells seeded in 24-well plates were pre-treated with 0.1 μM nicotine and further treated with $A\beta_{25-35}$. The cells were then removed by trypsinization, rinsed with PBS and re-suspended in binding buffer containing Annexin V and propidium iodide (PI) for 20 min at room temperature. The samples were analyzed on a FACSCalibur flow cytometer and data were analyzed using CellQuest software.

Western blot analysis. Proteins were obtained in lysis buffer as previously described (13). Proteins were loaded onto SDS-PAGE gels for electrophoresis and transferred onto PVDF membranes. After blocking in 5% fat-free milk in TBST for 90 min, the membranes were incubated with primary antibodies at 4˚C overnight. Subsequently, the membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 90 min. After washing 6 times with TBST (for 10 min each), the bound antibodies were visualized using enhanced chemiluminescence (ECL). Tubulin was used as a loading control.

Grouping of mice and establishment of animal model of AD. To investigate the effects of nicotine on AD symptoms, a mouse model of AD induced by Aβ was established according to previously described method (17). Briefly, 4-week-old male C57BL/6 mice were randomly divided into the control group, the AD group and AD group pre-treated with nicotine (n=7 per group). The mice in the AD group pre-treated with nicotine were administered nicotine (0.1125 mg/kg) subcutaneously twice a day. Animals that were injected with the vehicle served as the controls. After 2 weeks of nicotine administration, the mice were anesthetized with 4% chloral hydrate and the scalps were incised and retracted to expose the skull. The lambda and bregma were aligned in the same horizontal plane. The AD group and AD group pre-treated with nicotine were injected with 5 μl (5 mg/ml) $A\beta_{25-35}$ using a stereotaxic instrument (RWD Life Science Co., Ltd., Shenzhen, China) and a microinjector (Kd Scientific, Holliston, MA, USA) into the hippocampal CA1 region of the right hemisphere (coordinates: anteroposterior, -2.0 mm from the bregma; lateral, -2.0 mm; dorsovental, -3.5 mm) with a 10 μl Hamilton syringe driven by a microinjector at a speed of 1 μl/min. After the injection, the needle was kept in the injection site for a further 10 min and then slowly withdrawn in 5 min; simultaneously, mice in the control group were injected with identical doses of sodium chloride into the same area. On the 17th day after the microinjection, the mice were subjected to a Morris water maze navigation test. On

the 21st day after the microinjection, all mice in the 3 groups were firstly anesthetized, and then the mouse brain tissues were removed and fixed in 4% paraformaldehyde in phosphate buffer for 24 h, and embedded in paraffin for H&E staining, Congo red staining and immunohistochemical staining.

H&E staining and Congo red staining. Mice were sacrificed by CO_2 asphyxiation, and the brains were fixed for 48 h in 4% paraformaldehyde in PBS. Free-floating sections (50 μM) were obtained using a vibratome slicing system. Sections were deparaffinized with various concentrations of ethanol. For H&E staining, the sections were stained with hematoxylin for 15 min and washed in running tap water for 20 min. Counterstaining was performed with eosin. For Congo red staining, the sections were stained with methanol Congo red for 10-20 min and following by 0.2% alkaline ethanol staining. Finally, the sections were dehydrated in 95% and absolute alcohols for 2 changes of 2 min each and observed under a microscope.

Immunohistochemitry. Mice were sacrificed by CO_2 asphyxiation, and the brains were fixed for 48 h in 4% paraformaldehyde in PBS. Free-floating sections (50 μm) were obtained using a vibratome slicing system. Bcl-2 expression in the hippocampus was determined by immunohistochemitry according to a previously described method (18). Briefly, endogenous peroxidase activity was quenched for 30 min in H_2O_2, and the sections were subsequently incubated in 90% formic acid for 7 min to expose the epitope. The primary Bcl-2 antibody was applied, and the sections were incubated overnight at 4˚C. The sections were subsequently washed in TBS to remove the excess primary antibody. The sections were then incubated in biotinylated secondary antibody for 1 h at 20˚C. After a final wash of 20 min, slides were developed with diaminobenzidine substrate by using the avidin-biotin HRP system (Vector Laboratories, Burlingame, CA, USA).

Morris water maze navigation test. To investigate the effects of nicotine administration on spatial learning and memory following exposure to Aβ, the Morris water maze navigation test was performed according as previously described (19). Briefly, the water maze consisted of a circular water tank (120 cm in diameter) filled with water (26˚C) in which an escape platform (10 cm in diameter) was hidden 0.5 cm below the surface of the 30-cm-deep water. The water was made opaque by addition of milk powder, thereby rendering the platform invisible. A video camera was placed above the centre of the pool to capture images of the swimming animal. Mice were trained in a 4-trial per day task for 5 consecutive days. Each mouse was allowed a maximum of 60 sec to find the hidden platform and allowed to remain on it for 15 sec. If a mouse failed to find the platform within 60 sec, the mouse was placed on the platform for 30 sec by the investigator. The time required to find the hidden platform during these 4 acquisition trials was averaged. The navigation of the mice was tracked by a video camera. The escape latency of the mice was recorded. The experiment lasted 5 days, and each day was divided into morning and afternoon blocks, 4 trials in each block.

Statistical analysis. Each experiment was repeated at least 3 times and similar data were obtained. All data are presented as the means ± standard error of the means (SEM). Statistical significance was examined using one-way or two-way ANOVA with a post hoc Newman-Keuls test. A value of p<0.05 was considered to indicate a statistically significant difference.

Results

Aβ$_{25-35}$ induces apoptosis of SH-SY5Y cells in a dose- and time-dependent manner. Aβ-induced neurotoxicity is thought to be a critical event in the pathogenesis of AD (2). In this study, to assess the neurotoxicity of Aβ, SH-SY5Y cells were treated with Aβ$_{25-35}$ and cell viability was determined by flow cytometry, microscopic observation. Western blot analysis was also performed to determine the release of cytochrome *c* and the levels of cleaved caspase-3. The results revealed that treatment with Aβ$_{25-35}$ (20 and 50 μM) induced approximately 7.27 and 13.8% SH-SY5Y cell apoptosis (Fig. 1A and B, p<0.05, Aβ$_{25-35}$ 20 μM vs. control; p<0.01, Aβ$_{25-35}$ 50 μM vs. control, one-way ANOVA with post hoc Newman-Keuls test) indicating that Aβ$_{25-35}$ induced apoptosis in a dose-dependent manner. Microscopic observation also revealed similar results (Fig. 1C). Importantly, cleaved caspase-3 and the release of cytochrome *c* were increased following treatment with Aβ$_{25-35}$ in a time-dependent manner (Fig. 1D). As the activation of caspases, in particular that of caspase-3, plays a prominent role in the initiation of apoptosis, the augmented cleaved caspase-3 levels and the release of cytochrome *c* induced by treatment with Aβ$_{25-35}$ indicated that Aβ exerts neurotoxic effects on SH-SY5Y cells and may contribute to the development of AD.

Pre-treatment with nicotine attenuates Aβ$_{25-35}$-induced neurotoxicity in SH-SY5Y cells. To explore the potential neuroprotective effects of nicotine against Aβ-induced neurotoxicity, the SH-SY5Y cells were treated with nicotine prior to Aβ$_{25-35}$ stimulation and cell apoptosis was determined by flow cytometry and microscopic observation. Western blot analysis was also performed to measure the levels of cleaved caspase-3. The results revealed that although treatment with Aβ$_{25-35}$ induced approximately 79% SH-SY5Y cell apoptosis, pre-treatment with nicotine markedly abolished the neurotoxic effects of Aβ$_{25-35}$ on SH-SY5Y cells (Fig. 2A and B, p<0.001, Aβ$_{25-35}$ vs. control; p<0.001, Aβ$_{25-35}$ vs. nicotine 0.01 μM/Aβ$_{25-35}$; p<0.001, Aβ$_{25-35}$ vs. nicotine 0.1 μM/Aβ$_{25-35}$, one-way ANOVA with post hoc Newman-Keuls test). Microscopic observation also revealed similar results (Fig. 2C). Importantly, pre-treatment with nicotine markedly attenuated the Aβ$_{25-35}$-induced activation of caspase-3 (Fig. 2D). These data indicate that treatment with nicotine exerts marked neuroprotective effects against Aβ-induced neurotoxicity.

Nicotine increases Bcl-2, Bcl-xL and Mcl-1 expression in SH-SY5Y cells. Given that Bcl-2 family proteins are important modulators of cell apoptosis, we determined the effects of nicotine on Bcl-2, Bcl-xL and Mcl-1 protein expression in the SH-SY5Y cells treated with Aβ$_{25-35}$ by western blot analysis. The results revealed that nicotine markedly increased Bcl-2, Bcl-xL and Mcl-1 expression in a dose- and time-dependent manner (Fig. 3). The results from western blot analysis (Fig. 3A) revealed that pre-treatment with nicotine (0.01-100 μM for 12 h) markedly increased the Bcl-2, Bcl-xL and Mcl-1 protein levels in
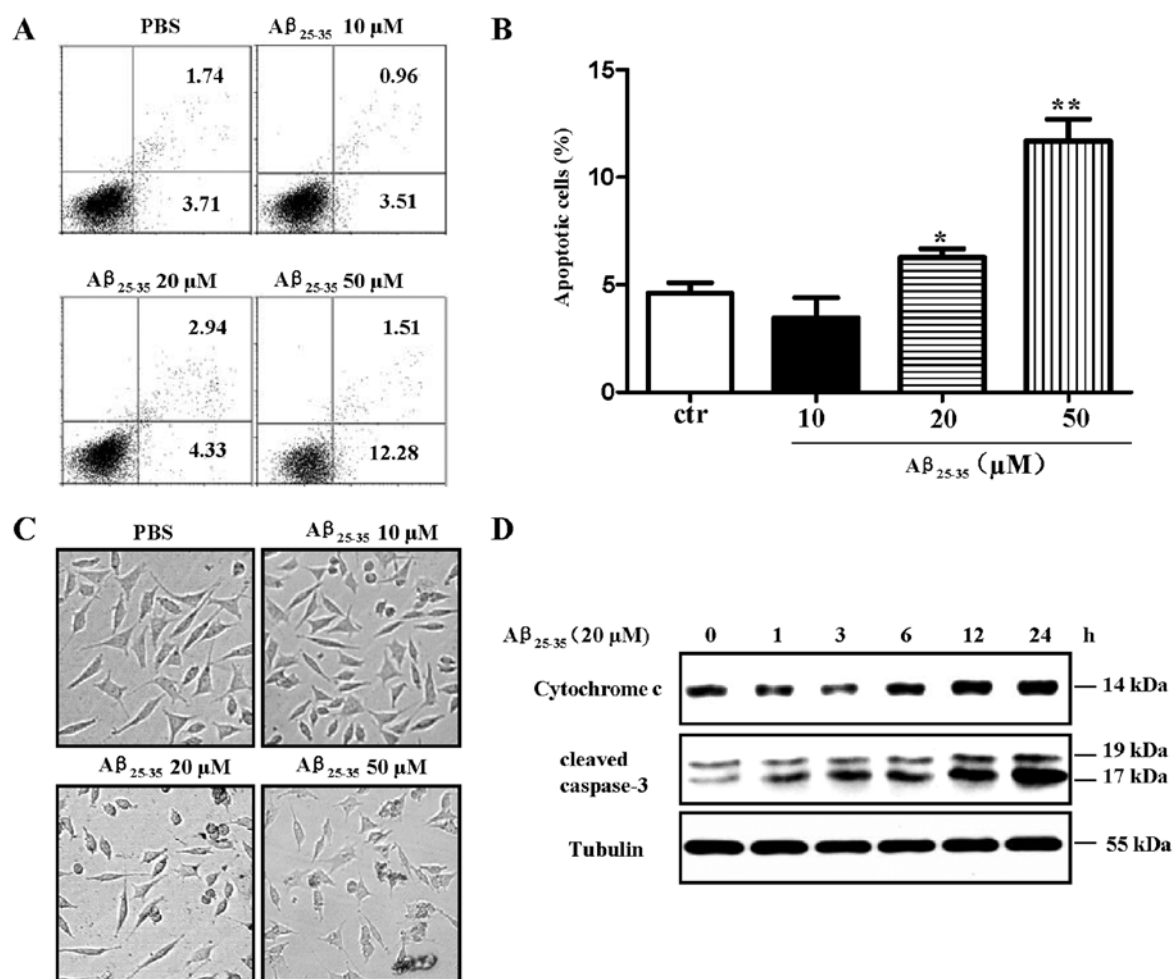
Figure 1. Aβ$_{25-35}$ induces apoptosis in SH-SY5Y cells. To investigate the effects of Aβ on cell apoptosis, SH-SY5Y cells were pre-treated with 10-50 μM Aβ$_{25-35}$ for 24 h and cell viability was determined by (A and B) flow cytometry; (C) microscopic observation; and (D) western blot analysis. (A) The percentage of apoptotic cells was determined by flow cytometry with Annexin V and PI staining. The numbers in the histogram indicate the percentage of apoptotic cells. (B) Bar graph of results in (A). Data are presented as the means ± SEM. Aβ$_{25-35}$ 20 μM vs. control, *p<0.05; Aβ$_{25-35}$ 50 μM vs. control, **p<0.001. One-way ANOVA with post hoc Newman-Keuls test. (C) Cell morphology was observed under a microscope. (D) Aβ$_{25-35}$ treatment increased the release of cytochrome *c* and caspase-3 activation. Tubulin was used as an internal control. A representative out of 3 independent experiments is shown.

the SH-SY5Y cells. Pre-treatment with 0.1 μM nicotine induced the most marked upregulation of Bcl-2, Bcl-xL and Mcl-1 expression. Treatment with 0.1 μM nicotine increased Bcl-2, Bcl-xL and Mcl-1 expression in a time-dependent manner. As the Bcl-2 family play an important role in regulating apoptosis, as well as the mitochondrial-initiated release of cytochrome *c* and activation of caspases (20), the fact that nicotine attenuated the Aβ-induced activation of caspase-3, indicated that nicotine may affect Bcl-2 family protein expression (21).

Nicotine increases Erk1/2, p38 and SAPK/JNK phosphorylation in SH-SY5Y cells. The Erk1/2, p38 and SAPK/JNK pathways are involved in regulating the expression of anti-apoptotic proteins (22-24). To investigate the role of the Erk1/2, p38 and SAPK/JNK pathways in the nicotine-induced increase in Bcl-2, Bcl-xL and Mcl-1 expression, the effects of nicotine on Erk1/2, p38 and SAPK/JNK MAPK kinase activation were determined. In initial experiments, SH-SY5Y cells were stimulated with a range of nicotine concentrations (0.01-100 μM) for 5 min, and the phosphorylation levels of Erk1/2, p38 and c-Jun MAPKs were determined by western blot analysis. Nicotine

(0.1-100 μM) induced a significant enhancement of Erk1/2, p38 and c-Jun MAPK phosphorylation in a dose-dependent manner (Fig. 4A, C and E), approximately 100% above basal levels. Importantly, Mek1/2, p90Rsk, Msk1, SAPK/JNK and MKK3/6 were also activated following treatment with nicotine (Fig. 4A, C and E).

The time course of the activation of Erk1/2, p38 and c-Jun MAPKs by 0.1 μM nicotine in the SH-SY5Y cells indicated that Erk1/2, p38 and c-Jun MAPK phosphorylation was increased at 5 min and persisted over 30 min of nicotine stimulation (Fig. 4B, D and F). Importantly, MeK1/2, p90Rsk, Msk1, SAPK/JNK and MKK3/6 were activated at 5 to 30 min following treatment with 0.1 μM nicotine (Fig. 4B, D and F). These results indicate that nicotine activates MAPK pathways and this may play an important role in the nicotine-mediated induction of anti-apoptotic proteins.

Nicotine upregulates Bcl-xL expression by activating the Erk1/2, p38 and SAPK/JNK pathways. To explore the role of nicotine-activated MAPK pathways in the nicotine-induced increase in the expression of anti-apoptotic proteins, the
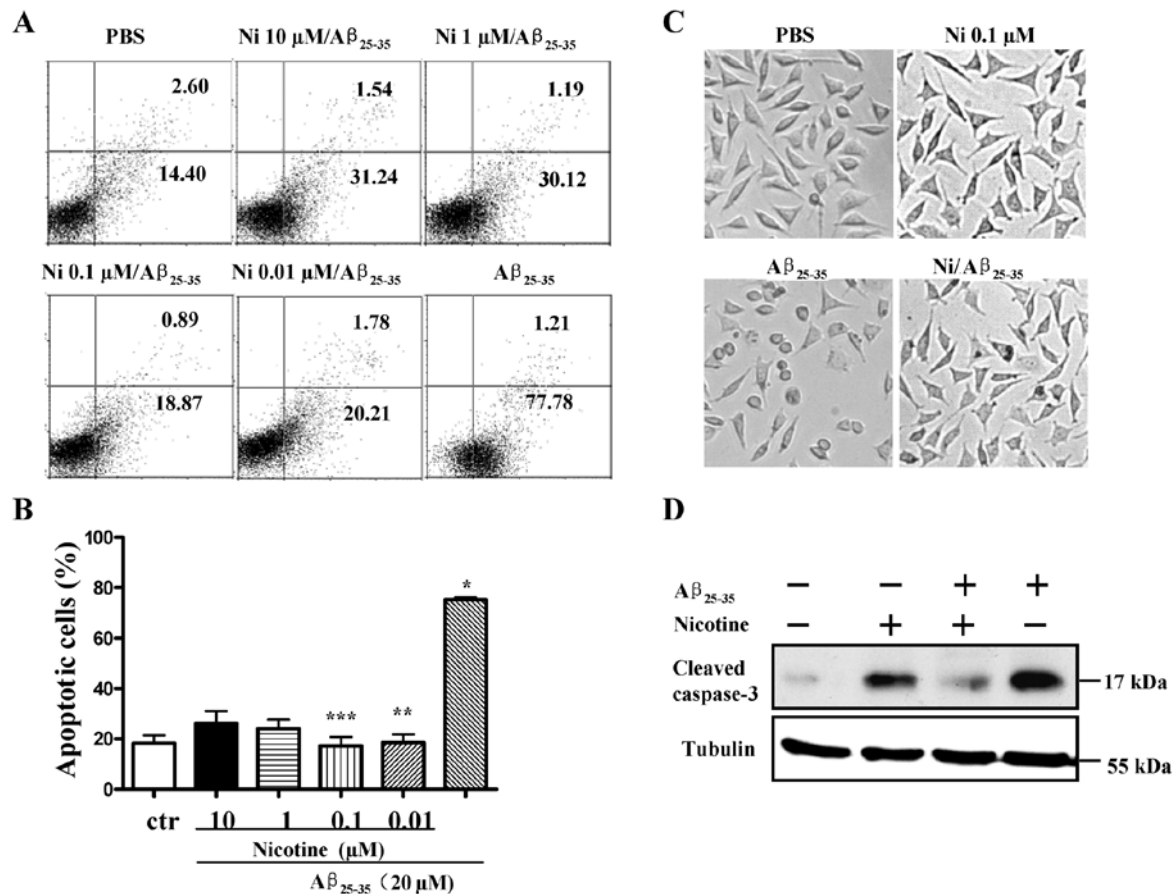
Figure 2. Prevention of Aβ$_{25-35}$-induced cell death by nicotine. To investigate the neuroprotective effects of nicotine against Aβ-induced apoptosis, SH-SY5Y cells were treated with nicotine (0.01-10 μM) prior to 20 μM Aβ$_{25-35}$ stimulation for 24 h and cell apoptosis was determined by (A and B) flow cytometry; (C) microscopic observation and (D) western blot analysis. (A) The percentage of apoptotic cells were determined by flow cytometry with Annexin V and PI staining. The numbers in histogram indicate the percentage of apoptotic cells. (B) Bar graph of results in (A). Data shown are the means ± SEM. Aβ$_{25-35}$ 20 μM vs. control, *p<0.001; Aβ$_{25-35}$ 20 μM vs. nicotine (0.01 μM)/Aβ$_{25-35}$ 20 μM, **p<0.001; Aβ$_{25-35}$ 20 μM vs. nicotine (0.1 μM)/Aβ$_{25-35}$ 20 μM, ***p<0.001. One-way ANOVA with post hoc Newman-Keuls test. (C) Cell morphology was observed under a microscope. (D) Nicotine treatment abrogated Aβ$_{25-35}$-induced caspase-3 activation. Tubulin was used as an internal control. A representative out of 3 independent experiments is shown. Ni, nicotine.
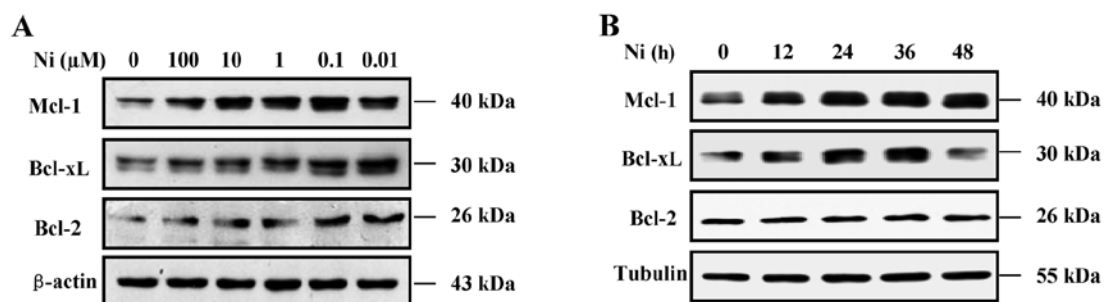

Figure 3. Nicotine treatment increases Bcl-2, Bcl-xL and Mcl-1 expression. To explore the effects of nicotine on Bcl-2, Bcl-xL and Mcl-1 expression, SH-SY5Y cells were treated with (A) various concentrations of nicotine for 12 h or (B) 0.1 μM nicotine for the indicated periods of time. Whole cellular protein was extracted and Bcl-2, Bcl-xL and Mcl-1 expression was determined by western blot analysis. A representative out of 3 independent experiments is shown. Tubulin was used as an internal control.

SH-SY5Y cells were treated with inhibitors of related kinases and the expression of Bcl-2/Bcl-xL and the activation of caspase-3 were determined by western blot analysis. The results revealed that while pre-treatment with nicotine attenuated the Aβ-induced caspase-3 activation, treatment with inhibitors of Erk1/2, p38 and SAPK/JNK markedly promoted the activation of caspase-3. The results also revealed that the nicotine-induced increase in Bcl-xL expression was dependent on Erk1/2 (U0126), p38 (SB203580) and SAPK/JNK (SP600125) phosphorylation (Fig. 5A). Notably, SAPK/JNK, but not Erk1/2 or p38, was shown to play a more important role in the attenuation of Aβ$_{25-35}$-induced caspase-3 activation and Bcl-xL expression (Fig. 5A). Microscopic observation also revealed similar results (Fig. 5B). These data indicate that the Erk1/2, p38 and SAPK/
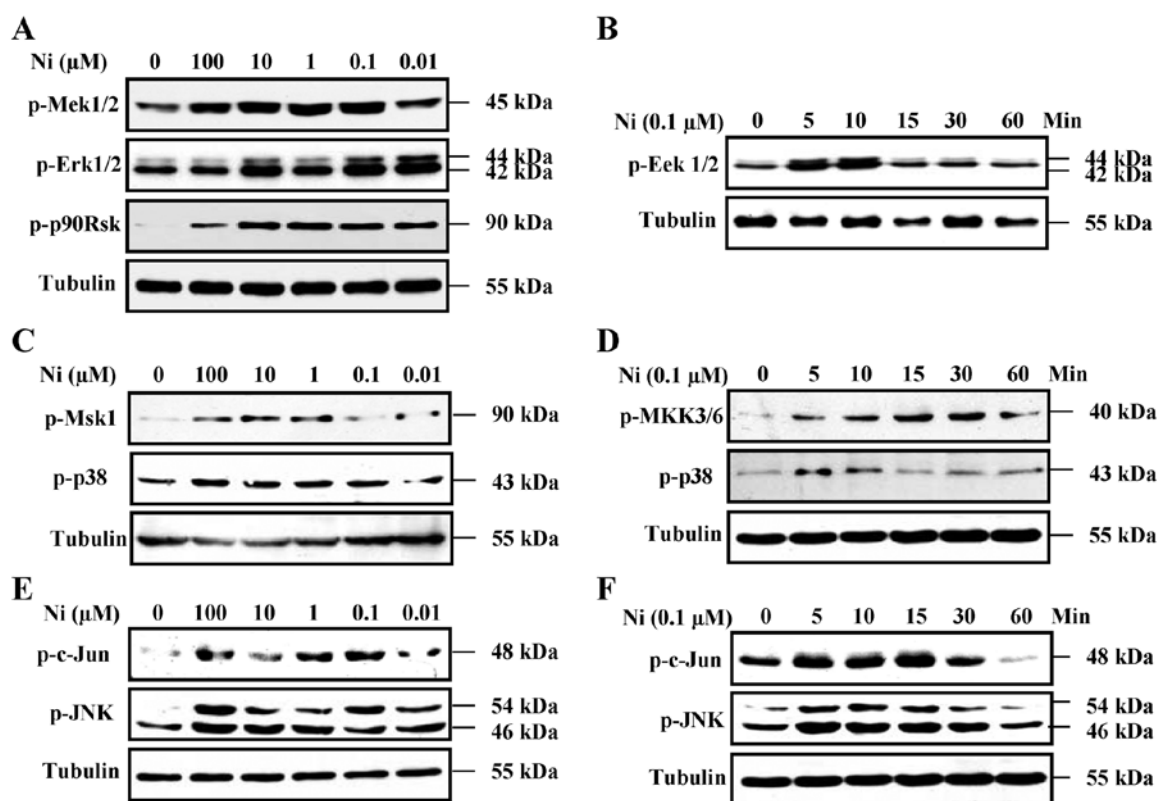
Figure 4. Nicotine treatment activates the Erk1/2, p38 and SAPK/JNK signaling pathways. To investigate the effects of nicotine on MAPK kinase activation, SH-SY5Y cells were treated with nicotine (0.1-100 μM) for 10 min (A, C and E), or 0.1 μM nicotine for the indicated periods of time (B, D and F). Whole cellular protein was extracted and the phosphorylation of MAPK kinases was determined by western blot analysis. A representative out of 3 independent experiments is shown. Tubulin was used as an internal control.
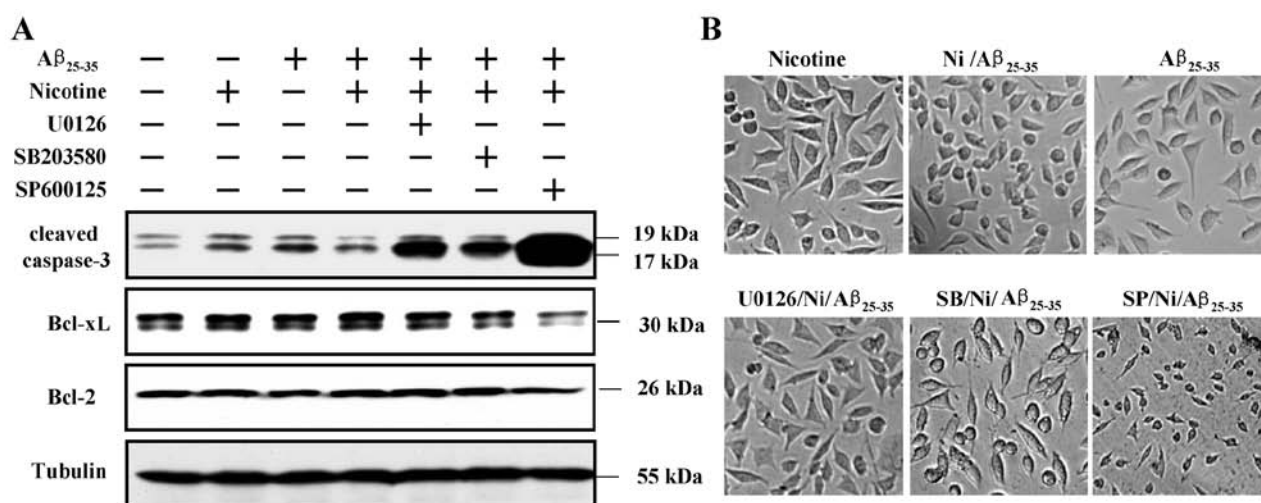

Figure 5. Erk, p38 and SARK/JNK pathways are involved in the nicotine-mediated neuroprotective effects against Aβ$_{25-35}$-induced neurotoxicity. To explore the role of the Erk, p38 and SARK/JNK pathways in the nicotine mediated neuroprotective effects, SH-SY5Y cells were treated with 10 μM U0126 (Erk inhibitor), SB203580 (p38 inhibitor) or SP600125 (JNK inhibitor) prior to treatment with 0.1 μM nicotine and treatment with 20 μM Aβ$_{25-35}$. The expression of Bcl-2/Bcl-xL and cell viability were determined by (A) western blot analysis and (B) microscopic observation, respectively. Tubulin was used as an internal control. A representative out of 3 independent experiments is shown. Ni:nicotine; SB, SB203580; SP, SP600125.

JNK pathways, particularly SAPK/JNK, play an important role in the nicotine-mediated anti-apoptotic effects.

Treatment with nicotine markedly ameliorates cognitive deficits in mice injected with Aβ$_{25-35}$. To investigate the effects of nicotine on spatial learning deficits induced by Aβ$_{25-35}$

injection, C57BL/6 mice were administered nicotine prior to the Aβ$_{25-35}$ injection and the ability of the mice to learn and process spatial information was examined by a Morris water maze. In the hidden platform test, the mice became more efficient at finding the platform on a successive trail (Fig. 6A). Two-way ANOVA (3 groups, 5 days) with repeated measures
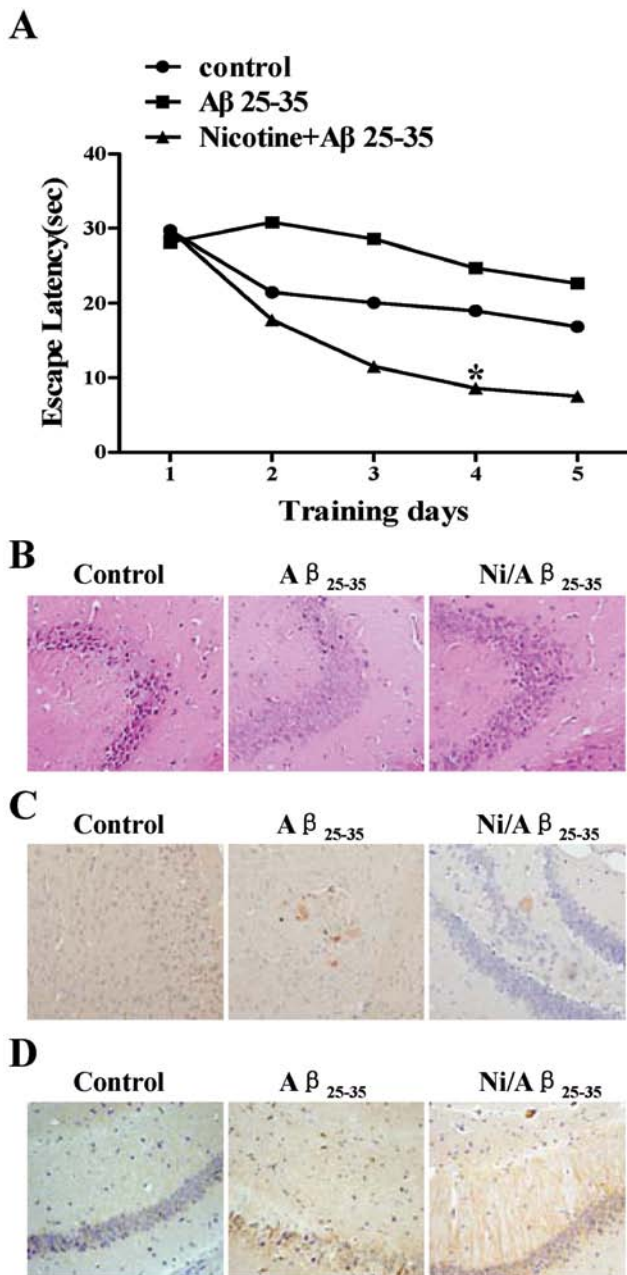
Figure 6. Nicotine attenuates $A\beta_{25-35}$-induced spatial working memory impairment by upregulating Bcl-2 expression. To investigate the effects of nicotine on spatial learning deficits induced by the $A\beta_{25-35}$-injection, C57BL/6 mice (n=7) were subcutaneously administered 0.1125 mg/kg nicotine twice a day for 2 weeks prior to 4.0 μg hippocampal $A\beta_{25-35}$ injection. The ability of the mice to learn and process spatial information was examined by performing a Morris water maze test (A) on the 17th day after the $A\beta_{25-35}$ injection. On the 21st day after the $A\beta_{25-35}$ injection, all the mice were sacrificed and brain tissues were fixed in 4% paraformaldehyde. Animals that were injected with saline served as the vehicle controls. Free-floating sections (50 μM) were obtained using a vibratome slicing system and subjected to (B) H&E staining, (C) Congo red staining or (D) immunohistochemitry. (A) Nicotine administration attenuated the $A\beta_{25-35}$-induced impairment in spatial working memory. Data shown are the means ± SEM, n=7. *p<0.01, two-way ANOVA. (B) Cell morphology was investigated by H&E staining. (C) Nicotine pre-treatment attenuated $A\beta$ deposition in the hippocampal region. (D) *In vivo* nicotine administration augmented Bcl-2 expression in the hippocampal region. Ni, nicotine.

rendered that the effect were statistically significant (p<0.01). Post hoc analysis (Student-Newman-Keuls) revealed that the $A\beta_{25-35}$-injected mice had a significantly longer escape latency (took longer to find the platform) than the sham-operated mice on days 2-5; a marked decrease in escape latency was observed following the administration of nicotine as compared with the $A\beta_{25-35}$-injected mice (Fig. 6A), indicating that the injection of $A\beta_{25-35}$ greatly impaired spatial working memory and that treatment with nicotine attenuated this impairment in memory.

To investigate the effects of nicotine on cell viability in $A\beta_{25-35}$-injected mice, $A\beta$ accumulation and Bcl-2 expression, hippocampal tissue sections from the mice were stained with H&E and Congo red, and immunohistochemitry was also performed. The results revealed that despite the fact that the $A\beta_{25-35}$ injection markedly decreased cell viability (Fig. 6B) and increased $A\beta$ deposition (Fig. 6C), nicotine administration greatly attenuated $A\beta$ accumulation and abolished the $A\beta_{25-35}$-induced neurotoxicity (Fig. 6B and C). Bcl-2 immunohistochemitry indicated that pre-treatment with nicotine markedly upregulated Bcl-2 expression (Fig. 6D). These results indicate that the neuroprotective effects of nicotine on $A\beta_{25-35}$-induced neurotoxicity may be mediated by the upregulation in the expression of anti-apoptotic proteins, such as Bcl-2.

Discussion

In recent studies, we investigated the biological role of nicotine and found that nicotine activates bone marrow-derived dendritic cells and that nicotine exerts potential anti-tumor effects on dendritic cells (13,14,25). Nicotine has also been shown to mediate synaptic transmission in regions controlling behavior (5) and to improve memory function in a transgenic mouse model of AD (6), indicating that nicotine may be a potential therapeutic agent in AD. In the present study, JNK and α7 nAchR were found to be potential molecules in the treatment of AD. Firstly, the $A\beta$-induced increase in cell apoptosis, caspase-3 activation and the release of cytochrome *c* were markedly abrogated by treatment with nicotine. Secondly, the upregulation in Bcl-2, Bcl-xL and Mcl-1 and MAPK kinase phosphorylation was achieved following treatment with nicotine. Of note, the inhibition of MAPK activity markedly promoted caspase-3 activation and the downregulated nicotine-induced increase in Bcl-xL expression; importantly, nicotine administration improvement memory function and reduced $A\beta$ accumulation in a mouse model of $A\beta$-induced AD.

MAPK signaling pathways, which include Erk, SAPK/JNK and p38, play important roles in cell survival (10-12,22-24). The Erk 1/2 signaling pathway has been found to be involved in nicotine-mediated neuroprotection in spinal cord neurons (26). The p38/JNK MAPK pathway mediates cortical neuronal apoptosis (27). In the present study, using Erk1/2, p38 and JNK kinase inhibitors, the inhibition of Erk1/2, p38 and JNK kinases markedly increased caspase-3 activation and abrogated the nicotine-mediated Bcl-xL upregulation (Fig. 5A), indicating that nicotine increased Bcl-xL expression and attenuated $A\beta$-induced neurotoxicity by activating the Erk1/2, p38 and JNK pathways in SH-SY5Y cells. The PI3K/Akt and NF-κB pathways are also involved in nicotine-mediated neuroprotection (28,29). However, the exact role of the PI3K-Akt and NF-κB pathways in nicotine-mediated neuroprotective effects against $A\beta$ stimulation remain unknown and require further investigation.

JNK/SAPK, one of the vital signal transduction pathways which transmits and converts stress signaling into apoptotis signaling (27), has been reported to induce receptor-mediated apoptosis by upregulating Fas ligand expression in T lymphocytes (30) and is necessary for irradiation-induced mitochondrial-mediated apoptosis in embryonic fibroblasts (31). JNK activation has an anti-apoptotic function in cardiac myocytes (32). In the present studies, the inhibition of JNK kinase increased the activity of caspase-3 and downregulated Bcl-xL (Fig. 5A), indicating that JNK phosphorylation facilitates the survival of SH-SY5Y cells. The anti-apoptotic function of JNK may be due to the nicotine-induced JNK activation and may be cell type-dependent, as JNK has been found to play either a pro-apoptotic or anti-apoptotic role depending on cell type (31).

Bcl-2, downstream of the SAPK/JNK cascade, plays important roles in regulating programmed cell death and has been shown to be associated with neurodegenerative disorders, including AD (33). In the present study, both JNK activation (Fig. 4) and Bcl-2 upregulation augmented by nicotine treatment were observed. As Bcl-2 is located in the outer mitochondrial membrane and protects cells against a variety of apoptotic stimuli (34), the nicotine-mediated Bcl-2 upregulation (Fig. 3) and anti-apoptotic effects (Fig. 2) indicated that the Aβ-induced apoptosis was mitochondrial-dependent. Hence, it was not surprising to find that Aβ treatment mrakedly promoted the release of cytochrome *c* and mitochondrial-dependent caspase-3 activation (Fig. 1). As nicotine treatment significantly augmented Bcl-xL and Mcl-1 expression, the exact roles of these proteins in the nicotine-mediated anti-apoptotic effects require further investigation.

Taken together, our data reveal that the anti-apoptotic effects of nicotine may be mediated by the increased expression of Bcl-2 family proteins and MAPK kinase phosphorylation, indicating that JNK may be a potential molecule in the treatment of AD.

Acknowledgements

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