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Nicotine attenuates β-amyloid peptide-induced neurotoxicity, free radical and calcium accumulation in hippocampal neuronal cultures

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1 Recent studies indicate that neuronal loss in Alzheimer's disease (AD) is accompanied by the deposition of β-amyloid protein (Aβ) in senile plaques. Nicotine as a major component of cigarette smoke has been suggested to have a protective effect for neurons against Aβ neurotoxicity.

2 Our present study demonstrates that nicotine protected cultured hippocampal neurons against the Aβ-induced apoptosis. Nicotine effectively inhibits apoptosis in hippocampal cultures caused by $A\beta_{25-35}$ or $A\beta_{1-40}$ treatment and increase of caspase activity induced by $A\beta_{25-35}$ or $A\beta_{1-40}$.

3 Measurements of cellular oxidation and intracellular free Ca^{2+} showed that nicotine suppressed Aβ-induced accumulation of free radical and increase of intracellular free Ca^{2+}.

4 Cholinergic antagonist mecamylamine inhibited nicotine-induced protection against Aβ-induced caspase-3 activation and ROS accumulation.

5 The data show that the protection of nicotine is partly *via* nicotinic receptors. Our results suggest that nicotine may be beneficial in retarding the neurodegenerative diseases such as AD.
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Keywords: Alzheimer's disease; apoptosis; Elisa; hippocampal neuronal cells; neuroprotection

Abbreviations: Aβ, amyloid β-peptide; AD, Alzheimer's disease; AFC, fluorochrome 7-amino-4-trifluoromethyl coumarin; β-APP, β-amyloid precursor protein; CDDE, The cell death detection ELISA^{plus}; DCF-DA, 2,7-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminotetraacetic acid; FBS, fetal bovine serum; fluo-3 AM, fluo-3 acetoxymethyl ester; HBSS, Hanks balanced salt solution; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl; PD, Parkinson's disease; ROS, reactive oxygen species

Introduction

Alzheimer's disease (AD) is one of the most common forms of dementia, and one of the neuropathological hallmarks of AD is the neuronal degeneration associated to senile plaques (Vickers *et al*., 2000). Such plaques are composed of compacted amyloid β-peptide (Aβ), which is a 40–43 amino-acid peptide (Soto *et al*., 1994; 1995; Selkoe, 1998). Aβ is originated by the proteolytic processing of a transmembrane glycoprotein called β-amyloid precursor protein (β-APP), which can be secreted (Saitoh *et al*., 1989) or cleaved releasing Aβ by the action of β- and γ-secretases (Hass & de Strooper, 1999). The deposition of soluble Aβ produces the aggregation of the peptide-forming amyloid fibrils, which have been reported to be neurotoxic *in vitro* (Yankner, 1996; Alvarez *et al*., 1998; Munoz & Inestrosa, 1999) and *in vivo* (Inestrosa & Reyes, 1998; Soto *et al*., 1998; Inestrosa & Larrondo, 2000). A shorter hydrophobic fragment of the protein $A\beta_{25-35}$, though not present in biological systems, is widely used together with, or instead of, the endogenous fragment $A\beta_{1-42(43)}$ by a number of investigators, and is found to be at least as toxic as the full-length fragment (Yankner *et al*., 1989).

It is true that fibrillar materials have toxic effects on a range of cells (Pike *et al*., 1993; 1997; Cribbs *et al*., 1997; Lorenzo *et al*., 1994; Pollard *et al*., 1995; Mark *et al*., 1996). The intrinsic toxicity of high levels of fibrils themselves could result from generation of oxygen-free radicals by Aβ fibrils (in the absence of any cellular elements) (Hensley *et al*., 1994), or their destabilization of membranes, resulting in changed intracellular calcium homeostasis and eventual cell death (Mark *et al*., 1996).

Numerous epidemiological studies have reported a highly significant negative association between cigarette smoking and neurodegenerative disorders, especially in Parkinson's disease (PD) (Morens *et al*., 1995; Ramon & Estefanýa *et al*., 1998) and AD (Brenner *et al*., 1993; Hillier *et al*., 1997; Ulrich *et al*., 1997). Nicotine is a predominant component of cigarette smoke, and is currently being used in pilot clinical studies for the treatment of AD (Wilson *et al*., 1995; Emilien *et al*., 2000). α4β2 nicotinic receptor activation plays an important role in neuroprotection against Aβ cytotoxicity (Kihara *et al*., 1997). Garrido *et al*. (2000; 2001) reported that nicotine could exert potent neuroprotective effects by inhibiting arachidonic acid-induced apoptotic cascades (caspase-3 activation and cytochrome *c* release) of spinal cord neurons. The α7 nicotinic acetylcholine receptor subtype mediates nicotine protection against NMDA excitotoxicity in primary hippocampal cultures through a Ca^{2+}-dependent mechanism (Dajas-Bailador *et al*., 2000). Also, we have studied the scavenging effect of nicotine on the oxygen-free radicals and found that it could effectively scavenge superoxide and hydroxyl radicals, and even could scavenge the free radicals in the gas phase of cigarette smoke (Liu *et al*., 2003).

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The key pathological change in the brain linked to the emergence and progressive development of dementia is the gradual degeneration of nerve cells and the related loss of specific synaptic connections. Only a highly specific subset of nerve cells shows vulnerability to degeneration, especially in hippocampal and cortical neurons. So, for the purpose of this reason, here we studied the protective effect of nicotine on Aβ-induced apoptosis in hippocampal neuronal cultures. We also try to explore whether changes of Ca^{2+} and reactive oxygen species (ROS) is involved in Aβ-induced neurotoxicity and whether nicotine can reverse this effect.

Methods

Materials

Chemicals were obtained from the following companies: Dulbecco's modified Eagle's medium (DMEM) from GIBCO (Grand Island, NY, U.S.A.); fetal bovine serum (FBS) from Hyclone (logan, UT, U.S.A.); nicotine from Zhengzhou Tobacco Academy of China. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl (MTT), 2,7-dichlorofluorescein diacetate (DCF-DA), DNase I, ethylenediaminotetraacetic acid (EDTA), trypsin, penicillin, and streptomycin from Sigma Chemical Co. (St Louis, MO, U.S.A.); Fluo-3 acetoxymethyl ester (fluo-3 AM) from Calbiochem (San Diego, CA, U.S.A.); The Cell Death Detection ELISA^{plus} (CDDE) from Roche Molecular Biochemicals (Germany, Cat # 1 544 675); ApoAlert Caspase Fluorescent Assay Kit from Clontech (U.S.A.).

Aβ_{25-35} and Aβ_{1-40} from Bachem (Torrance, CA, U.S.A.) was stored dry at –20°C, and was dissolved in double-distilled water at a concentration of 1 mg ml^{-1} stock solution, and stored at –20°C until use. The stock solution was then stored for 2–4 days at 37°C and used for the aged Aβ condition. The Reverse sequence of Aβ_{1-40}, Aβ_{40-1}, was obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.).

Hippocampal cell cultures

Primary neuronal cultures were prepared from the hippocampal tissue of newborn Sprague–Dawley rats, as previously described (Farinelli *et al*., 1998), with a few modifications. Briefly, the hippocampus of newborn animals was dissected out in DMEM, dissociated mechanically, digested with trypsin and, after stopping the reaction with FBS, filtered through nylon net. After centrifugation, the suspension was distributed in 96-well or 6-well poly-L-lysine-coated tissue culture plates (Costar). Cells were grown in DMEM supplemented with heat-inactivated 10% FBS (HyClone), 20 mM sodium bicarbonate, 1 mM sodium pyruvate, 20 mM HEPES, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 20 mM potassium chloride. The cells were seeded at a density of 2×10^5 cells cm^{-2} and maintained at 37°C in humidified 5% CO_2, 95% air at 37°C. After 48 h, nonneuronal cell division was inhibited by a 24-h exposure to 10^{-5} M cytosine arabinoside. The culture media was refreshed every 3 days. Most experiments were performed using cultures at 7–9 days.

Mitochondrial function MTT conversion assay (determination of cell survival)

The mitochondrial function of cultured hippocampal neurons was measured by MTT conversion assay. This assay may also serve as a general indicator of cell viability. MTT conversion was performed as described earlier (Mosmann, 1983). This assay takes advantage of the conversion of the yellow MTT to purple formazan crystals by mitochondrial succinate dehydrogenase in viable cells. Briefly, MTT was diluted in water and added to cells grown in 96-well plates at a final concentration of 0.5 mg ml^{-1}. Following a 4-h incubation to allow its conversion into formazan crystals, the media was removed and cells were lysed with DMSO to allow the crystals to dissolve. Absorbance was read at 595 nm using a Bio-RAD 3350 microplate reader, and the results were expressed as a percentage of control (no nicotine and no Aβ_{25-35} treated).

Intracellular calcium concentration $[Ca^{2+}]_i$

The concentration of intracellular Ca^{2+} was measured as described elsewhere (Aoshima *et al*., 1997) with fluo-3 AM, a calcium fluorescent ester chelator. After Aβ_{25-35} or nicotine incubation, cells were harvested by gentle scraping, washed, and resuspended in a standard medium (containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 1.5 mM $CaCl_2$, and 20 mM HEPES-Na (final pH 7.4)), which was used for loading and for the subsequent fluorescence measurements.

Fluo-3 AM (5 mM) was added to the cell suspension, which was subsequently incubated for 30 min at 37°C. The cells were then washed three times, resuspended in the standard medium and transferred to thermostatically controlled cuvette equipped with a magnetic stirrer. The fluorescence intensity of fluo-3 was quantified by a fluorescence spectrophotometer (Hitachi F-4500), with a single excitation wavelength set at 490 nm and an emission wavelength monitored at 526 nm. $[Ca^{2+}]$ was calculated from the fluo-3 fluoresce intensity using the equation

$$[Ca^{2+}]_i = Kd(F - (F_{min}) \div (F_{max} - F) \ (nmol\,l^{-1})$$

For the purpose of calculation of $[Ca^{2+}]_i$, the Kd was assumed to remain constant between 10 and 25°C, and increase linearly up to 42°C and Kd = 400 nmol l^{-1} at 25°C.

The maximal Fluo-3 fluorescence intensity (F_{max}) was determined by adding 0.1% Triton X-100, and the minimal fluorescence (F_{min}) was determined by quenching Fluo-3 fluorescence with 5 mM EGTA. F is the fluorescence measured without adding Triton-X-100 or EGTA.

Measurement of intracellular ROS

The level of intracellular ROS was quantified by fluorescence with 2′, 7′-dichlorofluorescein diacetate (2′, 7′-DCF-DA). DCF-DA, a nonfluorescent compound, is deacetylated by viable cells to 2′, 7′-dichlorofluorescin (DCF) by H_2O_2. Hanks balanced salt solution (HBSS) (0.25 ml) and 10 ml of 0.5 mM DCF-DA dissolved in *N*,*N*-dimethyl-formamide were added to six-well plates. Cells were loaded with DCF-DA by incubating them for 50 min in the presence of 100 μM DCF-DA. At the end of the incubation period, cells were washed once and the relative levels of fluorescence were quantified using a

fluorescence spectrophotometer (485-nm excitation and 535-nm emission, Blanc *et al*., 1997). The measured fluorescence values were expressed as a percentage of fluorescence in control cultures (no nicotine and no Aβ_{25-35} treated).

Measurement of caspase-3 proteolytic activity

Caspase-3 activity was quantified by means of the ApoAlert Caspase Fluorescent Assay Kits (BD Biosciences Clontech), according to the manufacturer's instructions. This assay is based on the release of the fluorochrome 7-amino-4-trifluoromethyl coumarin (AFC), when the provided pseudosubstrate acetyl-Asp-Glu-Val-Asp-aldehyde-AFC is cleaved by caspase-3. Free AFC produces fluorescence that can be monitored and quantified to estimate caspase-3 activity. Briefly, the cultured hippocampal cells were lysed and cell extracts were centrifuged to eliminate cellular debris. Aliquots (50 μl) of the cell extracts were incubated for 1 h at 37°C in the presence of the substrate. Generation of free AFC was quantified using a fluorescence spectrophotometer (400-nm excitation and 505-nm emission).

Cellular DNA fragmentation measured by ELISA

This assay is based on assessment of accumulation of DNA fragments in the cytoplasm of apoptotic cells. The enrichment of mono- and oligo-nucleosomes in the cytoplasm is due to the fact that DNA degradation occurs several hours before plasma membrane breakdown (Duke *et al*., 1986). In this assay, the accumulation of cytosolic histone-bound DNA fragments was quantified using a commercial ELISA kit (Cat #: 1 544 675, Roche Molecular Biochemicals). The measurement of apoptosis by this assay is sensitive and consistent with other morphometric indices of apoptosis (Ye *et al*., 1999). Briefly, the hippocampal cells were plated into poly-L-lysine-coated 96-well plates at a density of $3.2 \times 10^4\,cm^{-2}$. Cells were exposed to aged Aβ_{25-35} or nicotine. After aged Aβ_{25-35} or nicotine treatment, the cultures were washed twice with 0.01 M PBS, and cultured cells were lysed. A volume of 20 μl of cell lysate from each well was mixed with 80 μl of antibody solution in the coated wells. The loaded wells were incubated at room temperature for 2 h. The substrate was added to each well after it was washed three times in incubation buffer. After incubation at room temperature for 10–20 min, the optical density was measured using a Bio-RAD 3350 microplate reader with a light filter of 405 nm. The readings were used to measure the degree of apoptosis. The DNA fragmentation was expressed in the enrichment of histone-associated mono- and oligonucleosomes released into the cytoplasm. The enrichment factor was calculated according to absorption at 405 nm, which represented the enrichment of histone-associated DNA fragmentation and accounted for apoptosis of hippocampal cells.

Statistical analysis

Treatment groups were compared using one-way analysis of variance (ANOVA). Differences in which $P<0.05$ were considered statistically significant. In cases where significant differences were detected, specific *post hoc* comparisons between treatment groups were examined using the Student–Newman–Keuls tests.

Result

Nicotine prevents Aβ (Aβ$_{25-35}$, Aβ$_{1-40}$ or Aβ$_{40-1}$)-induced loss of hippocampal neuronal cell viability

The effects of different concentrations of nicotine on Aβ (Aβ_{25-35}, Aβ_{1-40} or Aβ_{40-1})-induced alterations of mitochondrial function of cultured hippocampal neurons are shown in Figure 1. Mitochondrial function was measured by the MTT conversion assay, which may also serve as a general indicator of cell viability. As indicated in Figure 1, treatment with 50 μM Aβ_{25-35} or 20 μM Aβ_{1-40} for 24 h markedly decreased MTT conversion in cultured hippocampal neuronal cells. In addition, treatment with 10 μM of nicotine significantly attenuated Aβ_{25-35} or Aβ_{1-40}-induced decrease in MTT conversion. Lower doses of nicotine, such as 1 μM, also decreased the alterations in cell viability induced by aged Aβ_{25-35}. Even for low concentrations of Aβ_{25-35}, this trend could be found. It can be found that there is no any effect on the cell viability for nicotine alone at 1 or 10 μM. Since marked protection against aged Aβ_{25-35}-induced neurotoxicity was observed with 10 μM nicotine, nicotine at this concentration was used in the remaining experiments. Aβ_{40-1} has no effect on the cell viability in the presence or absence of nicotine.

Assessment of hippocampal neuronal cell apoptosis

DNA fragmentation was a marker of cell apoptosis in most cases (Bortner *et al*., 1995; Earnshaw, 1995; Khodarev *et al*.,

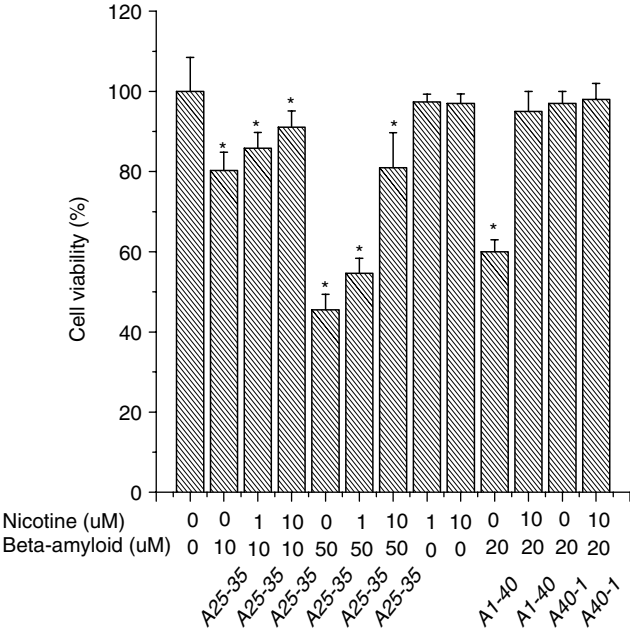

Figure 1 Effect of nicotine on Aβ (Aβ_{25-35}, Aβ_{1-40} or Aβ_{40-1})-induced MTT reduction in hippocampal neuronal cells. Cultures were incubated for 24 h with nicotine or Aβ (Aβ_{25-35}, Aβ_{1-40} or Aβ_{40-1}) as the indicated concentration. The MTT reduction assay was carried out as described in 'Method'. Data shown are expressed as percentages of control (no nicotine and no Aβ (Aβ_{25-35}, Aβ_{1-40} or Aβ_{40-1}) treated) values and each data point ($\pm$s.e.m.; bars) is the mean of five independent trials.*$P<0.05$ significantly different from control cells. A1–40: the concentration of Aβ_{1-40}; A40–1: the concentration of Aβ_{40-1}; A25–35: the concentration of Aβ_{25-35}.

1998) In order to evaluate the effects of nicotine on DNA fragmentation in Aβ_{25-35}- or Aβ_{1-40}-induced apoptosis, ELISA kit was used to analyze DNA degradation quantitatively. With anti-histone monoclonal antibody, the histone-associated DNA fragmentation (mono- and oligonucleosomes) was detected in this method. As shown in Figure 2, when the cells were incubated with Aβ (Aβ_{25-35} or Aβ_{1-40}) for 48 h, the enrichment factor (EF) values of DNA fragmentation were concentration-dependently increased significantly (10 and 50 μM). Also, if nicotine was added to the culture with Aβ_{25-35} or Aβ_{1-40}, DNA fragmentation was significantly decreased, especially when the concentration of nicotine was 10 μM. Aβ_{40-1} has no effect on DNA fragmentation in the presence or absence of nicotine. It can also be found that there is no any effect on EF for nicotine alone at 1 or 10 μM.

Nicotine attenuates Aβ (Aβ$_{25-35}$, Aβ$_{1-40}$ or Aβ$_{40-1}$)-induced increase in caspase-3 activity

Aβ_{25-35}- or Aβ_{1-40}-induced decrease in viability of hippocampal cells may be caused by induction of apoptosis. To clarify the interaction of nicotine with the intracellular downstream signaling cascade of Aβ_{25-35} and Aβ_{1-40}, the activity of caspase-3, which was an enzyme involved in the executive phase of apoptosis, was measured in hippocampal cultures exposed to Aβ (Aβ_{25-35}, Aβ_{1-40} or Aβ_{40-1}) and nicotine. As shown in Figure 3, treatment with 50 μM Aβ_{25-35} or 20 μM Aβ_{1-40} for 24 h markedly increased caspase-3 activity. However, treatment with 10 μM nicotine decreased the Aβ_{25-35} and Aβ_{1-40}-induced elevation of caspase-3 activity to the control levels. Aβ_{40-1} has no effect on the caspase-3 activity in the presence or absence of nicotine. Treatment with nicotine alone did not affect caspase-3 activity.

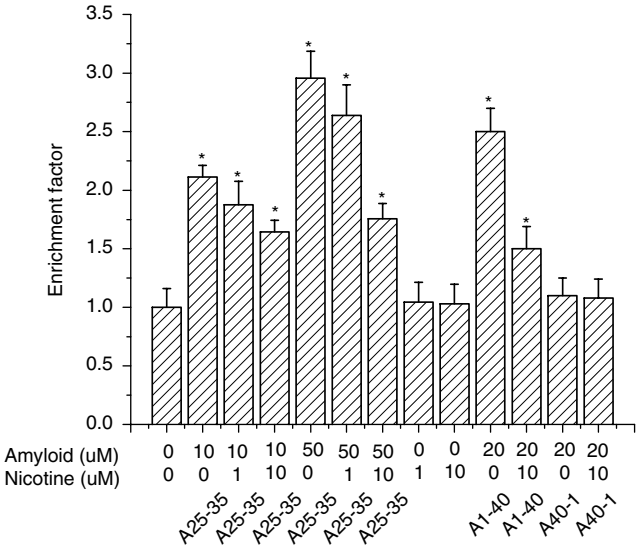

Figure 2 Quantification of apoptosis of hippocampal cells with a cellular DNA fragmentation ELISA. Cells were plated at a density of 3.2×10^4 cm^{-2}, and exposed to Aβ (Aβ_{25-35}, Aβ_{1-40} or Aβ_{40-1}) for 48 h. Apoptosis was measured with a cellular DNA fragmentation ELISA, as described in the Methods. Each data point (±s.e.m.; bars) is the mean of five independent trials.*$P<0.05$ denotes a statistically significant difference from control (no nicotine and no Aβ (Aβ_{25-35}, Aβ_{1-40} or Aβ_{40-1}) treated). A1–40: the concentration of Aβ_{1-40}; A40-1: the concentration of Aβ_{40-1}; A25–35: the concentration of Aβ_{25-35}.

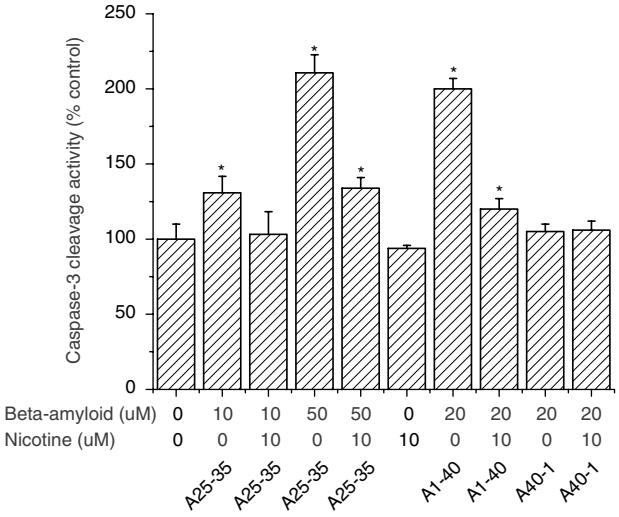

Figure 3 Inhibitory effect of nicotine on Aβ (Aβ_{25-35}, Aβ_{1-40} or Aβ_{40-1})- induced caspase-3 activation. Hippocampal neuronal cells were treated with the indicated concentrations of Aβ (Aβ_{25-35}, Aβ_{1-40} or Aβ_{40-1}) for 24 h in the presence or absence of nicotine. Each data point (±s.e.m.; bars) is the mean of four independent trials. *$P<0.05$ compared with control (no nicotine and no Aβ (Aβ_{25-35}, Aβ_{1-40} or Aβ_{40-1}) treated) denotes a statistically significant difference. A1–40: the concentration of Aβ_{1-40}; A40–1: the concentration of Aβ_{40-1}; A25–35: the concentration of Aβ_{25-35}.

Nicotine attenuates Aβ (Aβ$_{25-35}$, Aβ$_{1-40}$ or Aβ$_{40-1}$)-induced increase of ROS level

Some researchers suggested the involvement of oxidative stress in the pathogenesis of hippocampal neuronal cell death in AD (Miranda *et al*., 2000a, b). By using ROS fluorescent dye, DCF, it was found that exposure of culture hippocampal cells to 50 μM Aβ_{25-35} or 20 μM Aβ_{1-40} for 24 h resulted in a highly significant 100% increase in DCF fluorescence in the cells (Figure 4). The increase in DCF fluorescence in culture cells was essentially eliminated by cotreatment with nicotine (10 μM). Aβ_{25-35}-induced concentration-dependent ROS increase and Aβ_{1-40} (20 μM)-induced ROS increase was almost completely inhibited by 10 μM nicotine. Aβ_{40-1} has no effect on the generation of ROS in the presence or absence of nicotine. It can be found that 10 μM nicotine has no effect on the generation of ROS in the cells.

Nicotine attenuates Aβ (Aβ$_{25-35}$, Aβ$_{1-40}$ or Aβ$_{40-1}$)-induced elevation of neuronal [Ca^{2+}]$_i$

Since the mechanism of toxicity of aged Aβ_{25-35} is mediated, in part, by elevations of [Ca^{2+}]$_i$ (Mattson *et al*., 1993; Brorson *et al*., 1995), we determined whether nicotine affected Aβ (Aβ_{25-35}, Aβ_{1-40} or Aβ_{40-1})-induced increase of [Ca^{2+}]$_i$. (Figure 5). Exposure of hippocampal neuronal cells to Aβ_{25-35} for 24 h resulted in an about four-fold elevation of [Ca^{2+}]$_i$. Similarly, Aβ_{1-40} also resulted in an about 3.5-fold elevation of [Ca^{2+}]$_i$. Aβ_{40-1} has no effect on the generation of ROS in the presence or absence of nicotine. Nicotine alone had little effect on [Ca^{2+}]$_i$; however, the [Ca^{2+}]$_i$ in hippocampal neuronal cells cotreated with nicotine (10 μM) plus 50 μM Aβ_{25-35} or 20 μM Aβ_{1-40} was significantly less than that in hippocampal neuronal cells treated with 50 μM Aβ_{25-35}- or

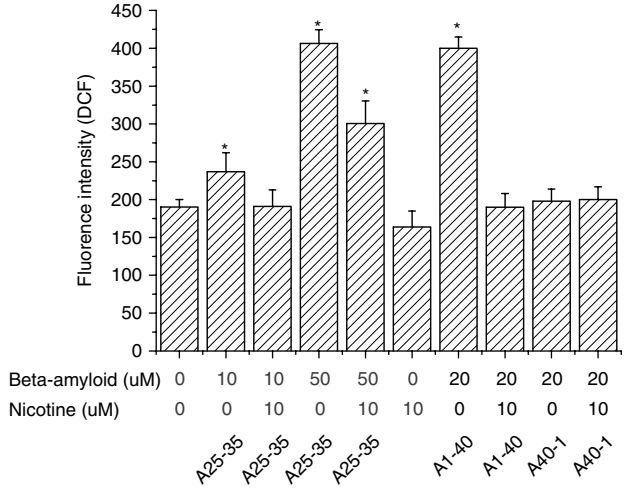

Figure 4 Nicotine attenuates Aβ ($A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{40-1}$)-induced accumulation of ROS. Culture cells were exposed to nicotine or Aβ ($A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{40-1}$) for 24 h, as indicated concentration. DCF-DA was then added to a final concentration of 2 μg ml^{-1} and DCF fluorescence was determined 40 min later. Each data point (±s.e.m.; bars) is the mean of five independent trials. *$P<0.05$ compared with control (no nicotine and no Aβ ($A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{40-1}$) treated) denotes a statistically significant difference. A1–40: the concentration of $A\beta_{1-40}$; A40-1: the concentration of $A\beta_{40-1}$; A25–35: the concentration of $A\beta_{25-35}$.

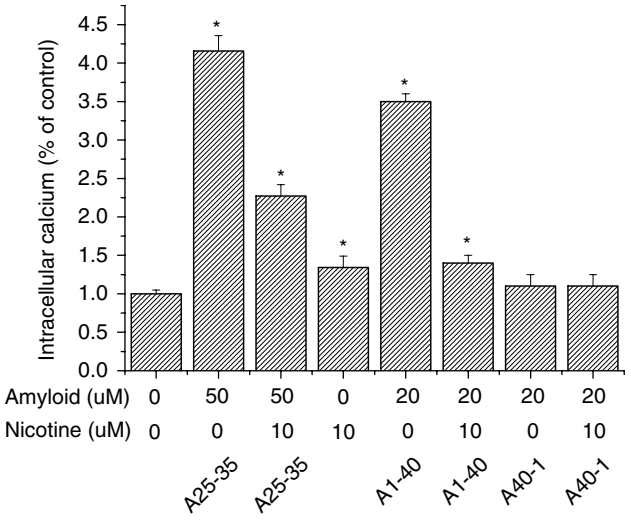

Figure 5 Nicotine attenuates Aβ ($A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{40-1}$)-induced elevation of neuronal $[Ca^{2+}]_i$. Cultured cells were exposed to Aβ ($A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{40-1}$) and (or) nicotine and $[Ca^{2+}]_i$ was measured 24 h later. Each data point (±s.e.m.; bars) is the mean of five independent trials. *$P<0.05$ compared with control (no nicotine and no Aβ ($A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{40-1}$) treated) denotes a statistically significant difference. A1–40: the concentration of $A\beta_{1-40}$; A40-1: the concentration of $A\beta_{40-1}$; A25–35: the concentration of $A\beta_{25-35}$.

20 μM $A\beta_{1-40}$ alone. Thus, nicotine attenuated $A\beta_{25-35}$ or $A\beta_{1-40}$-induced elevation of free $[Ca^{2+}]_i$.

Effects of mecamylamine on nicotine-induced protection against Aβ-induced caspase-3 activation

To investigate whether nicotine-induced neuroprotection against Aβ-induced caspase-3 activation is due to a specific effect mediated by nicotinic receptors, the effects of the cholinergic antagonist mecamylamine were examined. The concentration of mecamylamine used was same as previous reports (Kihara *et al*., 1997; Zamani *et al*., 1997). As shown in Figure 6, treatment with 10 μM nicotine for 24 h significantly decreased the $A\beta_{25-35}$- or $A\beta_{1-40}$-induced elevation of caspase-3 activity. $A\beta_{40-1}$ had no effect on the caspase-3 activity in the presence or absence of nicotine. However, mecamylamine significantly antagonized nicotine-induced protection against Aβ-induced caspase-3 activation. Mecamylamine alone had no effect on Aβ-induced caspase-3 activation.

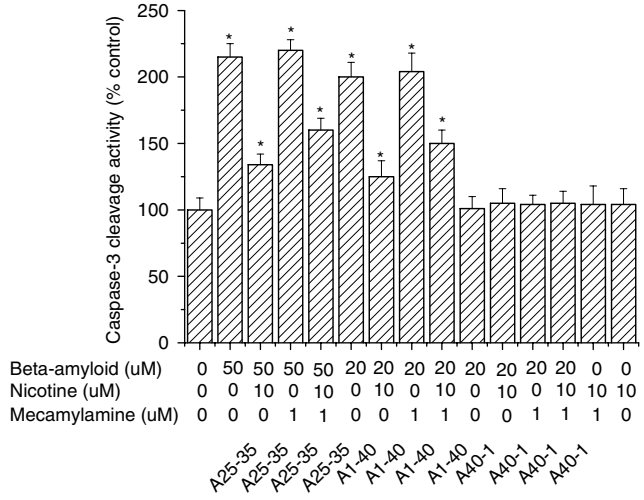

Figure 6 Effects of mecamylamine (cholinergic antagonist) on nicotine-induced protection against Aβ-induced caspase-3 activation. Nicotine was added to the medium containing Aβ ($A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{40-1}$). Mecamylamine was added simultaneously with nicotine and (or) Aβ ($A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{40-1}$) and caspase-3 activity was measured 24 h later. Each data point (±s.e.m.; bars) is the mean of five independent trials. *$P<0.05$ compared with control (no nicotine and no Aβ ($A\beta_{25-35}$, $A\beta_{1-40}$ or $A\beta_{40-1}$) treated) denotes a statistically significant difference. A1–40: the concentration of $A\beta_{1-40}$; A40–1: the concentration of $A\beta_{40-1}$; A25–35: the concentration of $A\beta_{25-35}$.

Effects of mecamylamine on nicotine-induced protection against Aβ-induced accumulation of ROS

To investigate whether nicotine-induced neuroprotection against Aβ-induced accumulation of ROS is due to a specific effect mediated by nicotinic receptors, the effects of the cholinergic antagonist mecamylamine were examined. As shown in Figure 7, treatment with 10 μM nicotine for 24 h significantly decreased the $A\beta_{25-35}$- or $A\beta_{1-40}$-induced accumulation of ROS. $A\beta_{40-1}$ had no effect on the accumulation of ROS in the presence or absence of nicotine. However, mecamylamine significantly antagonized nicotine-induced protection against Aβ-induced accumulation of ROS. Mecamylamine alone had no effect on Aβ-induced accumulation of ROS.

Discussion

Multiple lines of evidence have implicated that Aβ is neurotoxic and neuronal cells enter the death machinery *via*

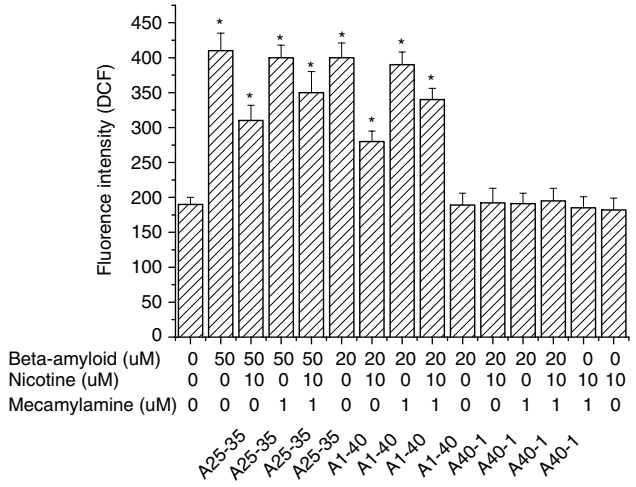

Figure 7 Effects of mecamylamine (cholinergic antagonist) on nicotine-induced protection against Aβ-induced accumulation of ROS. Nicotine was added to the medium containing Aβ ($A\beta_{25–35}$, $A\beta_{1–40}$ or $A\beta_{40–1}$). Mecamylamine was added simultaneously with nicotine and (or) Aβ ($A\beta_{25–35}$, $A\beta_{1–40}$ or $A\beta_{40–1}$) and caspase-3 activity was measured 24 h later. Each data point (±s.e.m.; bars) is the mean of five independent trials. $^*P<0.05$ compared with control (no nicotine and no Aβ ($A\beta_{25–35}$, $A\beta_{1–40}$ or $A\beta_{40–1}$) treated) denotes a statistically significant difference. A1–40: the concentration of $A\beta_{1–40}$; A40–1: the concentration of $A\beta_{40–1}$; A25–35: the concentration of $A\beta_{25–35}$.

an apoptotic process. The neurotoxicity of Aβ has been reported to be mediated with ROS and attenuated by antioxidants and free radical scavengers (Behl *et al*., 1992; Cribbs *et al*., 1997; Pike *et al*., 1997; Daniels *et al*., 1998; Miranda *et al*., 2000a, b). The present study shows the neuroprotective effect of nicotine against Aβ-induced hippocampal neuronal apoptosis. Nicotine has been demonstrated to display a potent antioxidant property (Linert *et al*., 1999). There are several studies of neuroprotective effect of nicotine against neuronal injury *in vitro* and *in vivo*. There has been extensive evidence indicating that nicotine modulates the neurotoxic effect of Aβ (Zamani *et al*., 1997; Linert *et al*., 1999). However, there is no previous report on the protective effects of nicotine against Aβ-induced neuronal injury in hippocampal cultures. This study is the first to report the protective effects of nicotine against hippocampal neuronal apoptosis induced by Aβ. In the present study, nicotine significantly reduced hippocampal neuronal cell death induced by Aβ in comparison with its vehicle (Figure 1).

In normal development and tissue homeostasis, most of the cells die through physiological or programmed cell death to remove excessive or damaged cells (Vaux *et al*., 1999). Imbalance in apoptosis leads to many pathological states including cancers and neurodegenerative disorders. DNA fragmentation, the cleavage of chromosomal DNA into oligonuclesome-sized fragments, is a very important hallmark in apoptosis. Also, we use cell death Elisa assay, detection of DNA fragmentation, to quantitate apoptosis. As shown in Figure 2, $A\beta_{25–35}$ or $A\beta_{1–40}$ treatment induces apoptosis in hippocampal cultures and nicotine effectively inhibits it. As to the intracellular death effectors, the most important family in the process of apoptosis is caspase (Pettmann *et al*., 1998). Caspase activation plays a critical role in the apoptosis of neurons (Marin *et al*., 2000; Masumura *et al*., 2000). In this study, we found that caspase activity was activated in hippocampal neurons by Aβ treatment (Figure 3). Therefore, caspases might be one of the main effector proteins in Aβ-induced neuronal cell death. Interestingly, nicotine inhibited the increase of caspase activity induced by $A\beta_{25–35}$ and $A\beta_{1–40}$. These results suggest that nicotine can attenuate Aβ-induced apoptosis in neuronal cells through caspase 3.

Previous studies showed that aggregated Aβ accumulates at the plasma membrane of cultured neurons (Mattson *et al*., 1993) and that free radical peptides are produced during the process of Aβ aggregation (Hensley *et al*., 1994). Furthermore, Aβ-derived radicals damage cellular enzymes (Hensley *et al*., 1994) and induce the oxidation of membrane component, which may be responsible for the disruption of Ca^{2+} homeostasis caused by Aβ (Cribbs *et al*., 1997; Pike *et al*., 1997; Miranda *et al*., 2000a, b). Marked elevation in calcium triggers the activation of different degradative processes, such as ROS formation (Kihara *et al*., 1997; Janus *et al*., 2000), impaired energy production (Janus *et al*., 2000), and activation of several hydrolytic enzymes (Castillo *et al*., 1998; Kruman *et al*., 1999). Increased intracellular Ca^{2+} stimulates phospholipase A_2 activity and increases the levels of arachidonic acid, which lead to increased free radical production (Bonventre, 1992; Verity, 1993). Also, it was reported that oxidative stress caused rising Ca^{2+} concentrations in the cytoplasm and the nucleus. Ca^{2+} activates calmodulin binding to calcineurin and enhances calcineurin's phosphatase activity. Calcineurin causes cytochrome *c* release and caspase -3 activation, leading to apoptosis (Ermakv *et al*., 2001). It suggests that ROS mediates Aβ-induced toxicity. Nicotine increases intracellular calcium (Barrantes *et al*., 1995), but from Figure 5 it can be found that this increase of calcium was much smaller than $A\beta_{25–35}$- and $A\beta_{1–40}$-induced increase, and did not induce the apoptosis of hippocampal neuronal cells. When nicotine and $A\beta_{25–35}$ or $A\beta_{1–40}$ were both added to the culture, calcium level was much smaller than that added $A\beta_{25–35}$ alone (Figure 5). The antiamyloidogenic effect of nicotine may be used to explain the decrease (Zeng *et al*., 2001). We found that intracellular ROS and Ca^{2+} levels increase when $A\beta_{25–35}$ or $A\beta_{1–40}$ are added to the culture. Also, our present studies demonstrate that nicotine stabilized Ca^{2+} homeostasis and decrease ROS level in hippocampal neuronal cultures exposed to Aβ (Figures 4, 5), which might contribute to neuroprotection. These data implicate that keeping Ca^{2+} homeostasis and ROS level may be responsible for the inhibition of hippocampal neuronal cell apoptosis.

Many studies indicate that nicotine-induced protection against Aβ-peptide toxicity is primarily mediated by the α4β2 (Kihara *et al*., 1998) and α7 nicotinic receptors (Kihara *et al*., 2000). However, previous study also reported that cytosine (a selective α4β2 nicotinic receptor agonist) and 3-(2,4)-dimethoxybenzylidene anabaseine (DMXB, a selective α7 nicotinic receptor agonist) only partially reversed nicotine-mediated protective effects on Aβ-induced neurotoxicity in cultured neuronal cells (Kihara *et al*., 1997; 1998; 2000). Therefore, it appears that nicotine exerted effects on cultured neuronal cell viability can also be mediated in part by other nicotinic receptors, or even by yet undefined nonreceptor mechanisms. Recently, the antiamyloidogenic effect of nicotine is reported, that nicotine inhibits fibril Aβ formation probably by binding to a small, soluble β-sheet aggregate (Zeng *et al*., 2001) and nicotine can also exert its effect by the disruption of preformed

fibril Aβ (Ono *et al*., 2002). The antiamyloidogenic effect of nicotine may be used to explain the decrease of calcium. In addition, some previous studies implied that Aβ-induced cytotoxicity is mediated by glutamate toxicity (Brorson *et al*., 1995; Le *et al*., 1995) and nicotine protects neurons form glutamate-induced cytotoxicity (Kaneko *et al*., 1997; Shimohama *et al*., 1998). The protective effect of nicotine against Aβ-induced cytotoxicity is probably mediated by its effect on glutamate toxicity (Shimohama *et al*., 2001). Although the precise mechanism of nicotine neuroprotection remains unclear, all of the above proposals may be involved in the neuroprotective effect of nicotine. Application of antioxidant and nicotinic agonists may be better than just one of them. As mentioned above, nicotine not only has antioxidant property (Linert *et al*., 1999, Liu *et al*., 2003), but also has agonistic property.

To further understand the effect of nicotine in caspase activation and accumulation of ROS, we used cholinergic antagonist mecamylamine to study whether nicotinic receptors are involved in protective effect of nicotine. We found that the effect of the nicotine on Aβ-mediated caspase-3 activation and accumulation of ROS is *via* nicotinic receptors. But mecamylamine (even in higher concentration, data unshown) did not completely inhibit nicotine-induced protection against Aβ-induced caspase-3 activation and accumulation of ROS. As mentioned above, the protection of nicotine is partly *via* nicotinic receptors.

Increasing data for understanding Aβ-driven pathogenesis come from transgenic mice models for AD in which transgenes for human APP provide elevated brain levels of Aβ. Multiple strains show specific AD-like neurological deficits and strongly support a role for Aβ in the pathogenesis in AD (Hsiao *et al*., 1996; Janus *et al*., 2000). The present data indicate that nicotine can protect hippocampal neurons against Aβ toxicity and suggest that nicotine may be beneficial in retarding the neurodegenerative diseases such as AD.

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