Effects of Nicotinic Receptor Agonists on Beta-Amyloid Beta-Sheet Formation

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ABSTRACT—Previously we demonstrated that nicotinic acetylcholine receptor stimulation protects neurons against beta-amyloid (A β)-induced cytotoxicity. In the present study, the effects of nicotinic receptor agonists on the β -sheet formation were investigated using a thioflavin T (ThT)-based fluorescence assay. Nicotine, cytosine (an α 4 β 2 agonist), and 3-(2,4)-dimethoxybenzylidene anabaseine (DMXB, an α 7 agonist) did not reduce fluorescence intensity when these agents were added to the β -sheet-formed A β . Simultaneous incubation of A β with nicotinic agonists also did not cause a reduction in fluorescence intensity. This data suggests that nicotinic receptor agonists do not influence the formation of the β -sheet structure.

Keywords: Beta-amyloid, Beta-sheet, Nicotine

Beta-amyloid (A β) is a major component of senile plaques that are found in the brains of Alzheimer's disease (AD) patients. The pathogenesis of AD remains unknown, although recent reports have indicated that A β plays an important role in the neuronal death observed in AD. In previous studies, we also demonstrated that A β exerted cytotoxic effects on rat cortical neurons (1, 2). Although the precise mechanism of its toxic effect is not clear, accumulating data supports the hypothesis that A β toxicity is mediated via oxidative stress or free radicals (3). Oxidative stress might cause lipid peroxidation and sulfhydryl oxidation, which lead to cell death. This phenomenon is found when A β exhibits the β -sheet conformation (3). In another words, the extent of β -sheet formation could influence A β toxicity, so that the toxic effect of A β could be predicted by measuring the extent of β -sheet formation, and the β -sheet structure can be quantified using a fluorometric method based on the characteristics of thioflavin T (ThT) (4–6).

Nicotine, an acetylcholine receptor agonist, has been shown to exert a neuroprotective effect against glutamate- and A β -induced cell death on primary rat cortical neurons (1, 2, 7). In our studies, this protective effect was shown to be receptor-mediated because specific nicotinic receptor (α 7 and α 4 β 2 nicotinic receptors (8)) antagonists inhibited the protective effect of nicotine. In addition,

selective agonists of these receptors, 3-(2,4)-dimethoxybenzylidene anabaseine (DMXB) (9), a selective α 7 agonist, and cytosine (10), a selective α 4 β 2 agonist, protected neurons from glutamate- and A β -induced neuronal death. However, a recent study suggested that nicotine could reduce amyloidogenesis by inhibition of the formation of the β -sheet structure by A β peptides (11). Therefore, in the present study, we investigated the effect of nicotine and its related substances on the formation of the β -sheet structure using the ThT fluorometric method.

Fragment 1–40 of the A β peptide was synthesized by the Peptide Institute, Inc. (Osaka) and Bachem (Bubendorf, Switzerland). A β 1–42 and 25–35 were obtained from Bachem. ThT and cytosine were purchased from Sigma (St. Louis, MO, USA). (–)-Nicotine was purchased from Research Biochemicals International (Natick, MA, USA), and DMXB was obtained from Taiho Pharmaceutical Co. (Tokushima). Solutions of peptides were prepared by dissolving them in phosphate-buffered saline (PBS). This condition was chosen because PBS approximates physiological extracellular conditions and does not influence the fluorescence intensity. In our preliminary experiments, pH was shown to be an important factor for β -sheet formation; that is, physiological pH is the optimal condition for β -sheet formation in contrast to acidic or alkaline conditions in which the β -sheet structure was not detected (data not shown).

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β -Sheet quantification was performed by the ThT-based fluorescence intensity method with a few modifications (4, 5). ThT was dissolved in double distilled water, adjusting its concentration to 300 μ M, and then diluted with glycine (pH 8.5 adjusted with NaOH) to 6 μ M. Equal volumes of peptide samples and ThT were mixed, and the fluorescence intensity was read by Fluoroskan Ascent (LabSystems, Helsinki, Finland) under excitation and emission wavelengths of 444 and 485 nm, respectively. ThT fluorescence brightens maximally under this condition, when ThT binds to the β -sheet structure under our assay conditions.

Peptides were incubated at 37°C for various lengths of time and the fluorescence intensity measured using the ThT method. We selected a concentration of 20 μ M since A β 25–35 was cytotoxic at this concentration in our previous study (1, 2). Figure 1 shows the time course of β -sheet formation by A β 1–40. RFU (relative fluorescence units) are the values obtained from the fluorescence spectrometer. The RFU values increased with time and reached a plateau at 96-hr incubation. The RFU of peptide incubated for 96 hr is statistically significantly higher compared with that of peptide just after dissolution (one-way ANOVA followed by Fisher's PLSD test). Therefore, we chose a 96-hr incubation of 20 μ M peptides as the optimal conditions for the following experiments.

We examined the effect of nicotine and its related substances on the β -sheet structure. Peptides were incubated for 96 hr at 37°C to form β -sheets in advance, and then

Fig. 1. Time-dependent increase in β -sheet structure. β -Sheet formation by A β 1–40 (Peptide Institute, Inc.) was quantified using the thioflavin T fluorometric method as described in the text. The level of RFU (relative fluorescence units) increased with time and reached a plateau at 96-hr incubation. The RFU of 96-hr-incubated peptide was significantly increased compared with that of peptide measured just after dissolution. * $P < 0.05$, compared with the 0 hr-incubated peptide. Each value represents the mean, $N = 4$.

nicotine and nicotinic receptor agonists were added. Samples were incubated at room temperature for 15 min, and the fluorescence intensity was measured. Statistical significance was determined by one-way ANOVA followed by post hoc tests (Fisher's PLSD, Scheffe and Bonferroni/Dunn test). In our experiments, 10 μ M nico-

Fig. 2. Effect of nicotine and its related substances on the β -sheet conformation. ■: A β alone, □: A β + nicotine, ▨: A β + cytosine, ▩: A β + DMXB. Statistically, nicotine and its related substances did not significantly reduce the extent of the β -sheet structure by any of the A β fragments. Each value represents the mean $\pm$ S.E.M., $N = 4$. Pep. Inst. 1–40: A β fragment 1–40 synthesized by Peptide Institute, Inc.; Bachem 1–40, 1–42, 25–35: A β fragment 1–40, 1–42, 25–35, respectively, synthesized by Bachem.

Fig. 3. Effect of nicotine and its related substances on the formation of the β -sheet structure. ■: A β alone, □: A β + nicotine, ▨: A β + cytosine, ▩: A β + DMXB. Statistically, nicotine and its related substances did not significantly reduce the formation of the β -sheet structure by any of the A β fragments. Each value represents the mean $\pm$ S.E.M., N=4. Pep.Inst. 1-40: A β fragment 1-40 synthesized by Peptide Institute, Inc.; Bachem 1-40, 1-42, 25-35: A β fragment 1-40, 1-42, 25-35, respectively, synthesized by Bachem.

tine did not significantly reduce the fluorescence intensity of any of the types of peptides (Fig. 2), although this dosage of nicotine was shown to prevent neurons from A β -induced cell death in our previous reports (1, 2). Likewise, neither cytosine (a selective $\alpha 4\beta 2$ nicotinic receptor agonist) nor DMXB (a selective $\alpha 7$ nicotinic receptor agonist) significantly reduced β -sheet formation when administered at the effective dosage for neuroprotection (10 μ M). This data indicates that nicotine and nicotinic receptor agonists did not reduce or disrupt the β -sheet structure.

The effect of simultaneous application of nicotine and its related substances on β -sheet formation was examined. In another words, we investigated whether each agent could interfere with the β -sheet-forming process. As shown in Fig. 3, nicotine did not significantly influence β -sheet formation. Furthermore, the fluorescence intensity was not significantly altered by either cytosine or DMXB. This data indicates that nicotine and nicotinic receptor agonists are not capable of interrupting β -sheet formation.

We have previously shown that nicotinic agonists protect neurons from A β -induced neuronal death (1, 2). Combined with these results, it is clear that the protective effect of nicotinic agonists is not mediated via the destruction of the β -sheet structure but via receptor-mediated signal transduction.

Our present study is not consistent with the report that nicotine could inhibit amyloid formation through the inhibition of an α -helical structure - β -sheet conversion

during amyloidogenesis (11). However, our previous study clearly indicated that nicotinic receptor stimulation protects against A β -induced cell death in cultured cortical neurons (1, 2). Although the precise mechanism of A β -induced cell death remains unknown, accumulating data suggests that free radicals are involved in the events leading to cell death because radical scavengers could inhibit the cell death induced by A β (12). Furthermore, A β itself could act as a radical (13, 14) or could increase intracellular hydrogen peroxide (15). Our preliminary data also indicated that A β increased dichlorofluorescein-based fluorescence intensity, implying that A β itself is a radical (data not shown). It is thought that the β -sheet structure is important for A β to act as a radical since it was the incubated peptide, rather than the recently dissolved peptide, that induced cell death, and our results indicated that β -sheet formation was increased with the duration of incubation.

In our previous studies, nicotine and its related substances protected neuronal cells from death induced by A β . This protective effect was inhibited by nicotinic receptor antagonists. Therefore, we suggested that the neuroprotective effects of nicotine and its related substances are mediated via nicotinic receptors. In the present study, nicotine and nicotinic agonists did not inhibit the formation of the β -sheet structure. These data indirectly support the suggestion that nicotine and nicotinic agonists exert their neuroprotective effects via receptors, rather than via interference with β -sheet formation.

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