Effects of Nicotine on APP Secretion and Aβ- or CT105-Induced Toxicity

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Several lines of evidence indicated that overexpression or aberrant processing of amyloid precursor protein (APP) is causally related to Alzheimer’s disease (AD). Amyloid precursor protein is principally cleaved within the amyloid β protein domain to release a large soluble ectodomain (APPs), known to have a wide range of trophic functions. The central hypothesis guiding this review is that nicotine may play an important role in APP secretion and protection against toxicity induced by APP metabolic fragments (β-amyloid [Aβ], carboxyl terminal [CT]). Findings from our experiments have shown that nicotine enhances the release of APPs, which has neurotrophic and neuroprotective activities in concentration-dependent (>50 μmol/L) and time-dependent (>2 hours) manners. In addition, pretreatment of nicotine (>10 μmol/L for 24 hours) partially prevented Aβ or CT105-induced cytotoxicity in primary cultured neuron cells, and the effects of nicotine-induced protection were inhibited by the pretreatment with a nicotine α-bungarotoxin. Nicotine (>10 μmol/L for 24 hours) partially inhibited CT105-induced cytotoxicity when PC12 cells was transfected with CT105. From these results, we proposed that nicotine or nicotinic receptor agonist treatment might improve the cognitive functions not only by supplementation of cholinergic neurotransmission, but also by protecting Aβ- or CT105-induced neurotoxicity probably through the increased release of APPs and the activation of nicotinic receptors. Biol Psychiatry 2001;49:240–247 © 2001 Society of Biological Psychiatry

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Introduction

Alzheimer’s disease (AD) is characterized by distinct neuropathologic lesions, including intracellular neuritic plaque made of amyloid β protein (Aβ), neurofibrillary tangles, and cerebrovascular amyloid deposits (Selkoe 1994). It seems that Aβ is derived from amyloid precursor protein (APP), which are 695 to 770 amino acids long and large membrane-spanning glycoproteins. Amyloid precursor protein is processed with at least two kinds of pathways (Checler 1995). In a constitutive secretory pathway, an unidentified enzyme, “α-secretase,” makes a cleavage of large ectodomain (secreted form of APP [APPs]) directly in the outer edge of the membrane and a smaller membrane-associated carboxyl-terminal (CT) fragment. The form of APP secreted by cleavage of α-secretase is known to have neurotrophic activity through lowering of the intraneuronal calcium concentration and neuroprotective activity against glucose deprivation and glutamate toxicity (Mattson et al 1993). A second pathway of APP metabolism has been identified in the endosomal–lysosomal system, resulting in larger potentially amyloidogenic CT fragments of APP by β-secretase (Estus et al 1992; Golde et al 1992; Haass et al 1992a, 1992b; Shoji et al 1992; Tamaoka et al 1992; Kozlowski et al 1992) and subsequently possibly cleaved by γ-secretase to release soluble Aβ (Koo and Squazzo 1994).

Many studies have shown that Aβ is toxic to neurons in vitro (Yankner et al 1990) and in vivo (Fukuchi et al 1994). However, a relatively high concentration (20 μmol/L) of Aβ is needed to induce toxicity, and some studies still failed to demonstrate the toxicity of Aβ in vivo (Clemens and Stephenson 1992). Amyloid β protein deposition has been found without accompanying neurodegeneration, and neurodegeneration could occur in areas with no Aβ deposition. Furthermore, it has been reported that under certain culture conditions Aβ promotes neurite outgrowth (Yankner et al 1990) instead of exerting toxic action. Thus Aβ may not be the sole fragment in the neurotoxicity associated with AD. Consequently the possible effects of other cleaved products of APP need to be explored.
Recently it has been reported that CT fragments of APP are found in media and cytosol of lymphoblastoid cells obtained from patients with early- or late-onset familial AD (Matsumoto 1994; Matsumoto and Matsumoto 1994) and Down’s syndrome (Kametani et al 1994). Transgenic mice that overexpressed the CT105 peptide showed extensive neuronal degeneration in the hippocampal area, with cognitive impairments (Kammesheidt et al 1992) and impairment of long-term potentiation (LTP) (Nalbantoglu et al 1997). Lu and colleagues very recently reported that cytotoxic properties of CT may be due to the generation and release of CT31 and its subsequent amplification effect on the cell death program (Lu et al 2000). In addition, we previously have reported that a recombinant 105-amino acid CT (CT105) itself caused direct neurotoxicity in PC12 cells and primary cortical neurons (Kim and Suh 1996; Suh et al 1996), induced strong nonspecific inward currents in Xenopus oocytes (Fraser et al 1996; Suh et al 1996), planar lipid bilayers (Kim et al 1999a), and Purkinje cells (Hartell and Suh 2000) and blocked the later phase of LTP in the rat hippocampus in vivo (Cullen et al 1997). CT105 impaired calcium homeostasis by inhibiting microsomal calcium uptake by Mg$^{2+}$:Ca$^{2+}$ adenosine triphosphatase in the rat brain microsome and Na$^{+}$:Ca$^{2+}$ exchanger activity in SK-N-SH cells, but Aβ did not (Kim et al 1998, 1999b). In addition, we found that intracerebroventricular injection of CT105 impaired learning and memory and was toxic to animals (Song et al 1998). These lines of evidence postulate that CT105 is an alternative toxic element important in the generation of the symptoms of AD.

The neuronal nicotinic acetylcholine receptor (nAChR) is a ligand-gated ion channel that consists of at least eight α-like subunit isoforms (α2–α8) and three β-like subunit isoforms (β2–β4) that exhibit distinct temporal and tissue-specific patterns of expression. Neuronal nAChR channels characteristically have a greater Ca$^{2+}$ permeability than muscle nAChRs (Levin 1992; McGehee and Role 1995) and have been found to elicit diverse behavioral effects including arousal, attention, anxiolytic activity, analgesia, and cognitive enhancement. Moreover, many studies have indicated a substantial loss of nicotinic receptor population in the brains of AD patients (Kellar and Wonnacott 1990), and the degree of cognitive impairments in AD has been reported to correlate well with the central cholinergic deficits (Bierer et al 1995). In addition, there are epidemiologic data showing a negative correlation between smoking and the onset of AD (Smith and Giacobini 1992), and pilot clinical data indicated that acutely administered nicotine might be beneficial for the treatment of the deficits in attention and information processing associated with AD (Jones et al 1992; Smith and Giacobini 1992). Thus our recent research has focused on nicotine’s effect on APP processing and the neuronal cytotoxicity induced by APP metabolites to get additional benefits of nicotinic receptor agonists as therapeutic agents in AD, and we previously reported that the release of APPs was enhanced by nicotine in PC12 cells (Kim et al 1997). In addition, our recent findings suggest that nicotine or nicotinic receptor agonist treatment might improve the cognitive functions not only by supplementation of cholinergic neurotransmission but also by protecting Aβ- or CT105-induced neurotoxicity, probably through the increased release of APPs.

**Enhanced Release of APPs by Nicotine**

*Time- and Dose-Dependent Release of APPs*

We employed PC12 cells, a rat pheochromocytoma cell line, as a model system. They have been shown to constitutively express APP and contain functional nAChRs (Sands and Barish 1992) and express α3, α5, α7, β2, and β4 subunit isoforms that are similar to those in the sympathetic ganglion (Henderson et al 1994). As shown in Figure 1, nicotine increased the release of APPs in a concentration-dependent manner. The levels of APPs after treatment with 50 μmol/L and 100 μmol/L nicotine were significantly different from those of a control group (p < .05, Duncan analysis of variance [ANOVA]). The amount of APPs in the conditioned media began to increase at 30 min following application of nicotine (100 μmol/L), reached a maximal level at 3 hours, and tended to decrease thereafter (Figure 2). The maximal stimulation of APPs release by nicotine was 2.9 times the basal level. The levels of APPs after 1- and 2-hour treatment with 100 μmol/L nicotine were significantly different from that of the control group (p < .05, Duncan ANOVA). The effect of nicotine on the secretion of APPs is attenuated by cotreatment with mecamylamine, a noncompetitive antagonist of nAChRs, especially the ganglionic-type nAChR. These results indicated nicotine could enhance the release of APPs through the specific interaction with nAChRs.

**Mechanism of the Nicotine-Induced APPs Release**

To determine whether the increase of APPs by nicotine was due to enhanced transcription of APP, we extracted total RNA from the PC12 cells treated with nicotine and performed reverse transcription polymerase chain reaction. However, there were no significant changes in the expression levels of three major isoforms of APP (APP695, APP751, and APP770) relative to β-actin from 30 min to 4 hours after nicotine treatment (data not shown). Therefore, the enhanced release of APPs by nicotine probably arises from an accelerated proteolytic processing rather than an increased transcription of APP. Cotreatment of mecamylamine, a specific nicotinic recep-
tor antagonist, significantly attenuated the release of APPs induced by nicotine (p < .05, Wilcoxon rank sum test). Thus it was thought that the effect of nicotine on APP processing was specifically mediated by nAChRs. In addition, ethyleneglycoltetra-acetic acid (EGTA), a calcium chelator, almost completely abolished the enhancing effect of nicotine on APPs release (p < .01, Student t test) (Figure 3), implying that Ca$^{2+}$ entry through the nAChR is essential in the enhanced release of APPs by nicotine. Mecamylamine or EGTA itself had little effect on APP processing. Then we examined whether the increase in APPs release by nicotine is accompanied by the decrease in the secretion of Aß in the conditioned media to an easily detectable level. We transiently transfected Swedish mutant APP695 to PC12 cells; however, nicotine (100 μmol/L) treatment did not significantly change the amount of Aß production in the transfected cells. The mechanism of enhancement of APPs release by nicotine is not clear at present. However, with the fact that the effect of nicotine on APP processing was almost completely abolished by the calcium chelator, EGTA, it is probably related to calcium entry through nAChRs. Neuronal nAChR channels have a greater Ca$^{2+}$ permeability (P_{Ca}/P_{Na} = 20 for the α7 homomeric channel and 1–1.5 for other neuronal heteromeric channels) than muscle nAChRs (P_{Ca}/P_{Na} = 0.2) (McGehee and Role 1995). In PC12 cells, P_{Ca}/P_{Na} is approximately 2.5 (Sands and Barish 1992). Calcium entry
through the neuronal nAChR channel is sufficient to activate various Ca\(^{2+}\)-dependent cellular processes (Vernino et al 1992) such as neurotransmitter release. Depolarization induced by nAChR stimulation further increases the Ca\(^{2+}\) influx through voltage-sensitive Ca\(^{2+}\) channels. Several studies have indicated that calcium can also regulate APP processing. Buxbaum et al (1994) demonstrated that thapsigargin and cyclopiazonic acid, which inhibit intracellular Ca\(^{2+}\) uptake into the endoplasmic reticulum, increased APPs release in a protein kinase C-independent manner. Furthermore, calcium ionophore A23187 was also shown to enhance the release of APPs in differentiated PC12 cells (Loefler and Huber 1993). Electric depolarization, which also raises the intracellular Ca\(^{2+}\) concentration, enhances the APPs release from the hippocampal slices (Vernino et al 1992). The exact molecular mechanism by which calcium modulates APP processing still remains unclear. One possibility is that calcium-sensitive proteases might be directly involved in APP processing. The other possibility is that calcium might indirectly influence the activities of other proteases responsible for APP processing. Although little is known about the identity of \(\alpha\) secretase(s), several proteases have been suggested as potential candidates. One of them is the calcium-activated, dithiothreitol-sensitive metalloprotease present in the rat brain (Allsop et al 1991). However, the exact identity of \(\alpha\) secretase(s) and the role of Ca\(^{2+}\) in regulating the activity of the enzyme(s) need to be elucidated further. It is of considerable interest that APPs can stabilize the intracellular Ca\(^{2+}\) concentration (Mattson et al 1993) by activating high-conductance K\(^{+}\) channels. These results raise the possibility that APPs induced by increased intracellular Ca\(^{2+}\) may act as a negative regulator to control the intracellular level of Ca\(^{2+}\), an important signaling molecule in the neuron. Although an increased release of APPs has been expected to be accompanied by a decrease in A\(\beta\) secretion, this is not always true. Several studies demonstrated a dissociation between APPs release and A\(\beta\) generation (Dyrks et al 1994; Loefler and Huber 1993; Querfurth and Selkoe 1994). In the present study, nicotine could not lower the A\(\beta\) production from the Swedish mutant APP transfectants, whereas it could stimulate the release of APPs. Thus there might be a complex regulatory mechanism for these two processing events of APP. However, since we only examined the effects of nicotine on the pathologically high production of A\(\beta\), the modulation of the physiologic A\(\beta\) production by nicotine needs to be established in future studies.

**Effect of Nicotine on the CT\(_{105}\) or A\(\beta\)-Induced Cytotoxicity**

We previously reported CT\(_{105}\)-induced cytotoxicity in various neuronal cells including primary neuronal cells (Kim and Suh 1996). In summary, CT\(_{105}\) peptide induced a significant lactic dehydrogenase (LDH) release from cultured rat cortical and hippocampal neurons, PC12 cells, and SHSY5Y cells in a concentration- and time-dependent manner but did not affect the viability of U251 cells originating from human glioblastoma. Moreover, when PC12 cells were induced to differentiate into neurons by pretreatment with nerve growth factor (NGF), the cells became much more sensitive to CT\(_{105}\). The toxic effect of CT\(_{105}\) was more potent than any fragments of APP. These results indicate that the toxic effect of CT\(_{105}\) is nonspecific. In contrast to CT\(_{105}\), A\(\beta\) increased LDH release only slightly, even at 50 \(\mu\)mol/L, but inhibited 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction at submicromolar concentrations (Kim and Suh 1996; Suh 1997).

Recently there has been extensive evidence indicating that nicotine modulates the neurotoxic effect of A\(\beta\) (Zamani et al 1997). For example, nicotine receptor stimulation protects neurons against A\(\beta\) toxicity (Kihara et al 1997, 1998) and \(\beta\)-amyloid peptide binds to the \(\alpha7\) nicotinic receptor (Wang et al 2000). It has been reported that nicotine inhibits amyloid formation by the A\(\beta\) peptide.
a with nicotine and the nicotine-induced protection were prevented by cotreatment of the effects of nicotine on the CT105-octa peptide (Singh and colleagues reported that nicotine inhibits phospholipases A2 and D activation by Aβ (Kihara et al 1999; Salomon et al 1996). Additionally, Singh and colleagues reported that nicotine inhibits phospholipases A2 and D activation by Aβ (Kihara et al 1999; Salomon et al 1996). These findings strongly suggest that nicotine inhibits Aβ peptide-induced cytotoxicity and that nicotine might inhibit the cytotoxicity caused by another important APP-derived fragment, CT105. Therefore, we investigated the effects of nicotine on the CT105- or Aβ-induced cytotoxicity. Pretreatment of nicotine (>5 μmol/L for 24 hours) partially inhibited CT105-induced LDH release in primary neuronal cells (55%), and the effects of nicotine-induced protection were prevented by cotreatment with nicotine and the α7 neuronal receptor antagonist α-bungarotoxin (αBTX) (50%) (Figure 4). Pretreatment of nicotine (>10 μmol/L for 24 hours) partially increased Aβ-induced MTT reduction in primary neuron cells, and the effects of nicotine-induced protection were prevented by the cotreatment with nicotine and αBTX, but there was no significance (Figure 5).

Among the various nicotinic receptor subtypes (Elgoyhen et al 1994; McGeehe and Role 1995), α7 neuronal receptors (homo-oligomers made up exclusively of the α7 subunits) and neuronal central nervous system receptors (hetero-oligomers made up of α4 and α2 subunits) are thought to be major subtypes in the central nervous system (Watson et al 1996). In our study, the subtype-specific antagonist (the α7 receptor-selective antagonist αBTX) antagonized the nicotine-induced protection against Aβ-induced cytotoxicity, though its antagonistic effects were not complete. These results suggest that the marked loss of both cholinergic innervation and nAChRs in the cerebral cortex and hippocampus seen in the AD brain may exacerbate the toxic effects of the Aβ peptide or CT105 peptide because nicotine and its receptor activation appear to play a neuroprotective role.

Our recent studies using CT105 transfection in PC12 cells supported the finding that nicotine inhibited the CT105 peptide–induced cytotoxicity. In line with the experiment using extracellularly administered CT105, nicotine (>10 μmol/L) pretreatment partially protected cytotoxicity induced by CT105 in CT105-transfected PC12 cells, and the protective effect was blocked by the cotreatment of the nicotinic receptor antagonist αBTX (data not shown). Therefore these results (that the cytotoxicity induced by either extracellular or intracellular CT105 is partially blocked by nicotine and reappears with αBTX) indicated that this effect might be mediated by the nicotinic receptor.

In the present study, nicotine showed significant protective effects at a concentration of 10 μmol/L. But it is known that nAChRs, especially those containing α7, desensitize rapidly when exposed to nicotine at more than 10 μmol/L (Zhang et al 1994), and it is possible that the nicotine added to culture media stimulated nAChRs continuously and caused desensitization-elicited receptor dysfunction in at least some of the cells examined. However,
this possibility is not likely, since the concomitant application of nicotine and nicotinic receptor antagonists significantly reduced the neuroprotective action of nicotine and APPs release in our study. Although the protective mechanism of nicotine is still unclear, it has been reported that nicotine activation regulates c-fos transcript levels (Greenberg et al 1986). These findings suggest that persistent receptor stimulation and the increased release of APPs by nicotine are necessary to elicit its protective effect against CT\textsubscript{105} or A\textbeta-induced cytotoxicity.

**Conclusions**

It is suggested that nicotine protects cortical neurons against CT\textsubscript{105} peptide- or A\textbeta-induced neurotoxicity via nicotinic receptor stimulation. Although further study is required to determine the intracellular mechanisms underlying the neuroprotective action of nicotine, nicotine protects neurons against the cytotoxicity of CT and A\textbeta fragments. Therefore, we propose that acetylcholine acting on nicotinic cholinergic receptors can function as a putative neuroprotective factor against neurodegeneration caused by A\textbeta or CT\textsubscript{105} fragments in AD brains.

Recent studies strongly suggest that APPs has potent neurotrophic and protective activities in several cultured cell models. It can stimulate neurite outgrowth in PC12 cells, promote the proliferation of fibroblasts, and protect cultured neurons from metabolic and excitotoxic insults (Mattson et al 1993). Therefore, APPs may act as a paracrine neurotrophic and neuroprotective factor. Interestingly, nicotine was also shown to attenuate the neuronal degeneration induced by glutamate (Akaike et al 1994) and NGF deprivation (Yamashita and Nakamura 1996) in vitro. These findings were further extended to in vivo studies that demonstrated the protective effects of nicotine against neurotoxin- or mechanically induced degeneration of the nigrostriatal dopaminergic neuron (Janson et al 1989). The evidence that the release of APPs was increased by nicotine suggested that nicotine could play a neuroprotective role against neurodegeneration or neurotoxicity.

The physiologic relevance of the enhanced APPs release by nicotine is not clear. Nicotine has been shown to cause a myriad of psychopharmacologic effects such as cognitive enhancement (Levin et al 1992). The central effects of nicotine were believed to be principally mediated by neuronal nAChRs. Activation of neuronal nAChRs located in the presynaptic sites could facilitate release of neurotransmitters such as glutamate, \textgamma-aminobutyric acid, and dopamine and enhanced the synaptic transmission (McGehee and Role 1995). Our data indicate that the processing of APP can also be modulated by nicotine receptor activation. In AD brains, nicotinic neurotransmission is severely damaged (Bierer et al 1995), which may lead to an altered processing of APP. Reduced APPs release might secondarily contribute to the neuronal loss in AD. Because the degree of cognitive impairments in AD has been reported to correlate well with the deficits of cholinergic neurotransmission in the brain (Smith et al 1992), elevation of acetylcholine level was hypothesized to be helpful in improving the cognitive deficits in AD. Many groups have tried to supplement the cholinergic transmission by administration of acetylcholine precursor, muscarinic or nicotinic receptor agonists, or acetylcholinesterase inhibitors. Although most of them failed to effectively ameliorate the symptoms of AD, pilot clinical studies indicated that nicotine might be beneficial for the treatment of the deficits in attention and information processing associated with the disease (Newhouse et al 1997; Giacobini 2000; Jann 2000). However, nicotine itself has limited utility as a therapeutic agent because of its dose-limiting side effects such as hypertension, tachycardia, and abdominal pain. Thus, many groups are now trying to develop a novel nicotinic receptor agonist that is able to enhance the cognitive functions by specific interaction with neuronal nAChRs without eliciting peripheral side effects. Our results strongly imply that nicotine or nicotinic agonists might be beneficial for the treatment of AD not only by supplementation of the cholinergic neurotransmission but also by protecting A\textbeta- or CT105-induced neurotoxicity, probably through the increased release of APPs.

**References**


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