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Mechanisms of Chronic Nicotine Treatment–Induced Enhancement of the Sensitivity of Cortical Neurons to the Neuroprotective Effect of Donepezil in Cortical Neurons

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Abstract. We have previously shown that chronic donepezil treatment induces nicotinic acetylcholine receptor up-regulation and enhances the sensitivity of the neurons to the neuroprotective effect of donepezil. Further analyses revealed that the nicotinic receptor is involved in this enhancement. In this study, we examined whether nicotinic receptor stimulation is sufficient to make neurons more sensitive to donepezil. We treated primary cultures of rat cortical neurons with nicotine and confirmed that chronic nicotine treatment induced nicotinic receptor up-regulation and made the neurons more sensitive to the neuroprotective effects of donepezil. Analyses with receptor antagonists and kinase inhibitors revealed that the effects of chronic nicotine treatment are mediated by nicotinic receptors and their downstream effectors including phosphatidylinositol 3-kinase. In contrast to chronic donepezil treatment that enhanced the level of nicotine-induced Ca^{2+} influx, chronic nicotine treatment did not significantly alter the level of Ca^{2+} influx.

Keywords: neuroprotection, Alzheimer's disease, nicotine, nicotinic acetylcholine receptor up-regulation, phosphatidylinositol 3-kinase

Introduction

Alzheimer's disease is a progressive neurodegenerative disorder characterized by the depletion of high-affinity nicotinic acetylcholine receptors and marked loss of cholinergic neurons (1, 2). The roles of cholinergic deficits in the pathogenesis of Alzheimer's disease have been suggested from several lines of neurochemical evidence, including selective depletion of cholinergic neurons in the basal ganglia of Meynert (2), decreased choline acetyltransferase and acetylcholinesterase activity, and the down-regulation of neuronal nicotinic acetylcholine receptors (3 – 7). Several drugs have been developed with the aim of targeting these deficits, one of them being donepezil, a potent and reversible acetylcholinesterase inhibitor that causes moderate improvement of cognitive function with

minimal side effects (8). While substantial evidence suggests that donepezil decreases acetylcholinesterase activity in Alzheimer's disease patient brains, there have also been numerous studies demonstrating that donepezil possesses other effects, such as neuroprotective effects, that may be involved in its therapeutic effects and that nicotinic acetylcholine receptors are involved in mediating these effects (9 – 11).

Neuronal nicotinic acetylcholine receptors belong to the family of ligand-gated channels and are assembled as a pentamer from α and β subunits, of which 12 types, nine α ($\alpha 2 - \alpha 10$) and three β ($\beta 2 - \beta 4$), are known to exist (12, 13). The effects of exposure to nicotine, such as receptor desensitization, have been widely studied in relation to nicotinic addiction. Previous studies have demonstrated that treatment with nicotine induces post-translational up-regulation of $\alpha 4$ - and $\alpha 7$ -nicotinic receptors in primary cultures of neuronal cells and cell lines expressing nicotinic receptors and that nicotinic receptors themselves are involved in mediating the effect of

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nicotine (14–16).

Previously, using primary cultures of rat cortical neurons, we have shown that treatment with donepezil enhances the sensitivity of the neurons to the neuroprotective effect of donepezil and induces nicotinic receptor up-regulation (17). After chronic treatment with donepezil, neurons can be protected from glutamate neurotoxicity by treatment with donepezil at a low concentration (1 nM, 24 h), which is not sufficient to protect neurons on its own, that is, without chronic treatment. Chronic donepezil treatment also increased the expression levels of nicotinic receptors, but did not desensitize the nicotinic receptors but rather enhanced their function. Interestingly, it was suggested through analyses using nicotinic receptor antagonists and inhibitors of intracellular signaling pathway kinases that nicotinic receptor stimulation by donepezil is important for the up-regulation of nicotinic receptors and to enhance the neuron's sensitivity to the neuroprotective effect of donepezil (18).

These results prompted us to question whether nicotinic receptor stimulation is sufficient to enhance the neuron's sensitivity to the neuroprotective effect of donepezil. We chronically treated primary cultures of rat cortical neurons with nicotine and examined whether nicotine treatment enhances the neuron's sensitivity to the neuroprotective effect of donepezil. We also examined whether nicotinic receptors are up-regulated and desensitized in primary cultures under the nicotine treatment conditions we used and examined the mechanism of nicotine treatment-induced enhancement of the sensitivity of neurons to the neuroprotective effect of donepezil.

Materials and Methods

Materials

Eagle's minimal essential medium was purchased from Nissui Pharmaceutical Co. (Tokyo). Fetal bovine serum and horse serum were purchased from JRH Biosciences (Lenexa, KS, USA). Drugs and sources were as follows: (–)-nicotine tartrate (ICN Biomedicals, Inc., Costa Mesa, CA, USA); methyllycaconitine citrate and dihydro- β -erythroidine-HBr (Research Biochemicals International, Natick, MA, USA); mecamylamine-HCl and (–)-scopolamine-HBr (Sigma Chemical Co., St. Louis, MO, USA); LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one], PP2 [4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine], and AG490 [(*E*)-*N*-benzyl-2-cyano-3-(3,4-dihydroxyphenyl)acrylamide] (Calbiochem, Darmstadt, Germany); fura 2-acetoxymethylester (Dojindo Laboratories, Kumamoto); L-glutamic acid monosodium salt (Nacalai Tesque, Kyoto); and trypan blue (Wako Pure Chemical Industries,

Ltd., Osaka). Donepezil hydrochloride [(±)-2-[(1-benzylpiperidin-4-yl)methyl]-5,6-dimethoxy-indan-1-one monohydrochloride; E2020] was kindly given to us by Eisai Co., Ltd. (Ibaragi).

Cell cultures

Primary cultures were obtained from the cerebral cortex of fetal Wistar rats (17–19 days of gestation) according to previously described procedures (19). Briefly, single cells dissociated from the whole cerebral cortices of fetal rats were plated on 0.1% polyethyleneimine-coated plastic or glass coverslips placed in Falcon 60- or 35-mm dishes, 12- or 24-well plates ($1.8–4.8 \times 10^5$ cells/cm²). The cultures were incubated in Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum (1–7 days after plating) or 10% heat-inactivated horse serum (8–13 days after plating), glutamine (2 mM), glucose (total 11 mM), NaHCO₃ (24 mM), and HEPES (10 mM). The cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere. Six days after plating, non-neuronal cells were removed by adding cytosine arabinoside (10 μ M). Only mature cultures (10–12 days in vitro) were used for experiments.

Measurement of neurotoxicity

The neurotoxicity induced by glutamate was quantitatively assessed by examining cultures under Hoffman modulation microscopy according to the methods described previously (10, 19). All experiments were performed in Eagle's minimal essential medium at 37°C. The cells were stained in trypan blue solution for 10 min at room temperature, fixed with isotonic formalin (pH 7.0, 2°C–4°C), and rinsed with physiological saline; and then the cell viability was assessed by counting the number of trypan blue-stained cells and non-stained cells. In each experiment, cells on five coverslips were counted to obtain the mean $\pm$ S.E.M. of cell viability.

Immunoblotting

The cells were washed twice with cold Tris-buffered saline, harvested using a cell scraper, and lysed in buffer containing Tris (20 mM), β -glycerophosphate (25 mM), EGTA (2 mM), Triton X-100 (1%), phenylmethylsulfonyl fluoride (1 mM), aprotinin (1%), dithiothreitol (2 mM), and vanadate (1 mM) on ice. The lysates were then sonicated and centrifuged at 15,000 rpm at 4°C for 30 min and boiled for 5 min. An aliquot (approx. 30 μ g of protein) of the supernatant was loaded onto a sodium dodecyl sulfate (SDS) polyacrylamide gel, separated electrophoretically, and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). Next, the polyvinylidene difluoride mem-

brane was incubated with 10 mM Tris-buffered saline containing 0.1% Tween 20 and 5% dehydrated skimmed milk to block nonspecific protein binding. The membrane was then incubated with primary antibodies: anti-nicotinic receptor $\alpha 7$ subunit antibody (Covance Research Products, Berkeley, CA, USA) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then with secondary antibodies. Immunodetection was performed using the ECL Western blotting detection system (Amersham Biosciences, Buckinghamshire, UK). Five rounds of independent experiments were conducted to confirm the results. The captured images were quantified using NIH image software.

Surface biotinylation assay

Cultured cortical neurons were placed on ice, rinsed in cold PBS, and incubated in PBS containing 1.0 mg/ml Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) for 30 min at 4°C. After incubation, the neurons were rinsed three times in PBS and lysed in 150 μ l PBS containing complete proteases (Nacalai Tesque), phosphatase inhibitor cocktail (Sigma, Tokyo), 0.1% SDS, and 1% Triton X-100. To detect the total expression of $\alpha 7$ -nicotinic receptor, 10% of the cell lysate was heated at 95°C for 5 min in SDS-containing buffer with 5% β -mercaptoethanol. To detect the surface expression of $\alpha 7$ -nicotinic acetylcholine receptor, 80% of the cell lysate was incubated with streptavidin gel (Pierce).

Intracellular Ca^{2+} imaging

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured with a Ca^{2+} -sensitive fluorescent dye, fura 2-acetoxymethylester, on a fluorescence imaging system (ARGUS/HiSCA; Hamamatsu Photonics K.K., Shizuoka), according to the previously described methods (17, 20). Cortical neurons cultured on glass coverslips were incubated in Krebs-Ringer buffer (137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 25 mM *d*-(+)-glucose, and 10 mM HEPES at pH 7.4) containing 5 μ M fura 2-acetoxymethylester and 0.01% cremophor EL for 30 min at 37°C and then rinsed with the buffer. Then the coverslips were mounted on a recording chamber placed under a fluorescent microscope. The cells were alternatively illuminated with light (wavelengths of 340 and 380 nm) at intervals of 2 s, and the emission was measured at 500 nm. Fluorescence imaging was performed for 3 min at room temperature. The peak amplitude of the fluorescence ratio (340/380 nm) immediately after nicotine application was adopted as an index of the nicotine-induced increase in $[Ca^{2+}]_i$. Fluorescence values were obtained from a collection of 80 cells. Eight rounds of independent experiments were performed to confirm the

observations.

Statistics

Data are expressed as the mean $\pm$ S.E.M. The statistical significance of differences between groups was determined by one-way analysis of variance (ANOVA) followed by Dunnett's two-tailed test using the InStat software (Graph Pad Software, San Diego, USA). Statistical significance was defined as a probability value of less than 5%.

Results

Effects of chronic nicotine treatment on the neuron's sensitivity to the neuroprotective effect of donepezil

We have previously shown that chronic treatment with donepezil enhances the neuron's sensitivity to the neuroprotective effect of donepezil (17). In order to examine whether nicotinic receptor stimulation is sufficient to enhance the neuron's sensitivity to donepezil, primary cultures of fetal rat cortical neurons were chronically treated with nicotine (10 μ M) for 4 days, washed extensively to remove nicotine, pretreated with donepezil (1 nM) for 24 h, and treated with glutamate (100 μ M) for 24 h (Fig. 1). The viability of neurons were examined by the trypan blue method. The viability of the neurons treated only with glutamate was significantly lower than that of the untreated cells, showing that glutamate induces neuronal death in primary culture (Fig. 1). Donepezil treatment (1 nM) did not increase the viability of the neurons without the chronic nicotine treatment. However, when neurons were treated with nicotine before donepezil, the viability of the neurons was significantly higher than that of neurons treated only with donepezil. Treatment with nicotine alone did not protect the neurons, indicating that the neuroprotection is not due to chronic nicotine treatment.

Effects of chronic nicotine treatment on the expression level of the $\alpha 7$ -nicotinic receptor

We next examined whether chronic nicotine treatment up-regulates nicotinic receptor expression levels in primary cultures of rat cortical neurons. Previous studies have confirmed that the $\alpha 7$ -nicotinic receptor is expressed in fetal rat cortical neurons (17). The expression level of the $\alpha 7$ -nicotinic receptor was examined by Western blotting after chronic treatment with nicotine (10 μ M). Chronic incubation with nicotine (10 μ M) significantly increased the level of the $\alpha 7$ -nicotinic receptor in the total cell extract by 1.5-fold (Fig. 2). Examination of the level of nicotinic receptors at the plasma membrane revealed that the expression of surface nicotinic receptors was also significantly up-regulated by chronic nicotine treat-

Fig. 1. Effect of nicotine treatment on the neuron's sensitivity to the neuroprotective effect of donepezil. **A)** Nicotine treatment (10 μM, 4 days) enhances the sensitivity of neurons to the neuroprotective effect of donepezil (1 nM, 24 h) against neuronal death induced by glutamate (100 μM). The viability of neurons was determined by trypan blue treatment. ** $P < 0.01$, compared with sham treatment. ## $P < 0.01$, compared with donepezil or nicotine treatment alone. Data represent the means $\pm$ S.E.M. of $n = 5$ independent observations. **B)** Time course of the experiments performed to examine the enhancement of the sensitivity to the neuroprotective effect of donepezil. First, cortical cultures were chronically treated with 10 μM nicotine or co-treated with nicotine and nicotinic receptor antagonists or signaling pathway inhibitors for 4 days. Next, they were thoroughly washed with medium prior to pretreatment with 1 nM donepezil for 24 h. Thirdly, neurons were exposed to 100 μM glutamate for 24 h to induce neuronal death. Finally, cell viability was examined by trypan blue staining.

ment nearly 2-fold compared to that in control untreated neurons.

Effects of chronic nicotine treatment on nicotine-induced Ca^{2+} influx

In order to examine whether chronic nicotine treatment induces functional up-regulation of nicotinic receptors, we examined the level of Ca^{2+} influx in treated and non-treated neurons. It is known that acute exposure of cortical neurons to nicotine induces Ca^{2+} influx and that en-

hancement and down-regulation of nicotinic receptor function leads to increased and decreased Ca^{2+} influx, respectively. The neurons were exposed to nicotine (1 mM) after chronic nicotine treatment to induce Ca^{2+} influx, and the level of Ca^{2+} influx was examined by an intracellular Ca^{2+} level imaging system. Significant differences in Ca^{2+} influx levels were not observed between the levels of Ca^{2+} influx in chronically treated and non-treated control neurons (Fig. 3).

Effects of nicotinic receptor antagonists on chronic nicotine treatment-induced enhancement of sensitivity to the neuroprotective effect of donepezil

Previous studies have shown that the effects of nicotine treatment are mediated via acetylcholine receptors and intracellular signaling pathways such as the phosphatidylinositol 3-kinase (PI3K) pathway. To elucidate the mechanism that enhances the neuron's sensitivity toward the neuroprotective effect of donepezil, we exposed cortical neurons to acetylcholine receptor antagonists along with nicotine during the 4-day chronic treatment. Following the chronic treatment, the neurons were treated with donepezil for 24 h and glutamate for 24 h, and the viability of the neurons was examined by the trypan blue method. Chronic co-treatment with nicotine and methyllycaconitine (MLA, 10 μM), mecamylamine (MAA, 10 nM), or dihydro-β-erythroidine (DHβE, 10 nM), but not scopolamine (10 μM), significantly decreased the viability of the neurons (Fig. 4A), suggesting that nicotinic receptors, especially α4- and α7-nicotinic receptors, but not muscarinic receptors are involved in mediating nicotine's effect.

Effects of PI3K, JAK2, and Fyn inhibitors on chronic nicotine treatment-induced enhancement of the sensitivity of neurons to the neuroprotective effect of donepezil

Previous studies have demonstrated that stimulation of the nicotinic receptor is mediated in the cell by the PI3K signaling pathway. We examined the effect of a PI3K inhibitor on the nicotine-induced enhancement of sensitivity of neurons to the neuroprotective effect of donepezil. The neurons were treated with LY294002 (10 μM), an inhibitor of PI3K, and nicotine for 4 days, donepezil (1 nM) for 24 h, and glutamate (100 μM) for 24 h, and the viability of the neurons was examined by the trypan blue method. The viability of the neurons co-treated with LY294002 was significantly lower than that of the neurons treated with nicotine (Fig. 4B). Previous studies have also suggested that α7-nicotinic receptors activate PI3K via JAK2 and Fyn, kinases that bind to α7-nicotinic receptors (20, 21). We examined the effect of AG490 and PP2, inhibitors of JAK2 and Fyn, respectively, on the nicotine-induced enhancement of the sensitivity of the

Fig. 2. Effects of chronic nicotine treatment (10 μ M, 4 days) on the surface and total expression level of the $\alpha 7$ -nicotinic receptor as examined by Western blotting. A) Surface receptor levels were determined by surface biotinylation assays. Western blotting images showing representative results from seven independent experiments. B) Summary of the results of Western blotting. The results are expressed as the percentage of detected signals obtained from each sample divided by the mean of the control, taken as 100%. Each signal was corrected using that of the corresponding GAPDH value. ** $P < 0.01$, compared with control. Data represent the means $\pm$ S.E.M. of $n = 7$ independent observations.

Fig. 3. Effects of chronic nicotine treatment on the nicotine-induced increase in $[Ca^{2+}]_i$. A) Representative charts showing the changes in the fura-2 fluorescence ratio induced by acute nicotine treatment. B) Summary of the effects of chronic nicotine treatment on nicotine-induced increase in $[Ca^{2+}]_i$. NS, not significant. Data represent the means $\pm$ S.E.M. of $n = 8$ independent observations.

Fig. 4. The effects of chronic nicotine treatment are mediated via nicotinic receptors and the PI3K pathway. A) Effects of acetylcholine-receptor antagonists on chronic nicotine treatment-induced enhancement of the sensitivity of neurons to the neuroprotective effect of donepezil. Cortical neurons were treated with nicotine (10 μ M) and MAA (10 μ M), DH β E (10 nM), MLA (10 nM), or scopolamine (10 μ M) for 4 days prior to donepezil pretreatment (1 nM, 24 h) and glutamate (100 μ M) treatment, and viability of the neurons was examined by the trypan blue method. B) Effects of intracellular signaling pathway inhibitors on chronic nicotine treatment-induced enhancement of the sensitivity of neurons to the neuroprotective effect of donepezil. Cortical neurons were treated with nicotine (10 μ M), and LY294002 (10 μ M), AG490 (10 μ M) or PP2 (10 μ M) for 4 days prior to donepezil pretreatment (1 nM, 24 h). Neuronal death was induced by glutamate treatment, and the viability of the neurons was observed by the trypan blue method. * P < 0.05, ** P < 0.01, compared with nicotine and donepezil treatment. NS, not significant. Data represent the mean $\pm$ S.E.M. of n = 5 independent observations.

neurons to the neuroprotective effect of donepezil. The neurons were treated with AG490 (10 μ M) or PP2 (10 μ M) and nicotine for 4 days, donepezil (1 nM) for 24 h, and glutamate (100 μ M) for 24 h. Co-treatment with a JAK2 or Fyn inhibitor significantly decreased the viability of the neurons (Fig. 4B).

Discussion

Previously, we have shown that chronic donepezil treatment makes neurons more sensitive to the neuroprotective effect of donepezil and that nicotinic receptors are involved in mediating the effect of chronic donepezil treatment (17, 18). In this study, we addressed the question of whether nicotinic receptor stimulation is sufficient to enhance the neuron's sensitivity to the neuroprotective effect of donepezil. Our results indicate that nicotinic receptor stimulation is indeed sufficient to enhance the sensitivity and suggest that nicotinic receptors and its downstream PI3K signaling pathway are involved in mediating the effects of chronic nicotine treatment. We noted some differences between the effects of chronic donepezil and nicotine treatment. Although both donepezil and nicotine treatment up-regulated the expression level of nicotinic receptors at the cell surface, only donepezil induced the up-regulation of the level of nicotine-induced Ca^{2+} influx (17, 18). The lack of up-regulation of nicotine-induced Ca^{2+} influx suggests that nicotinic receptors were partially desensitized in neurons chronically treated with nicotine. We have previously shown that Ca^{2+} influx is involved in the neuroprotection by donepezil pretreatment (22). Therefore, it is interesting that neurons chronically treated with nicotine were protected from neurotoxic insults in spite of the lack of increase in the level of Ca^{2+} influx. One possibility is that while donepezil may enhance the neuron's sensitivity by increasing the number of nicotinic receptor and Ca^{2+} influx, nicotine may enhance the sensitivity via a different pathway, via modulation of signaling downstream of Ca^{2+} or through modulation of pathways that are activated downstream of nicotinic receptors but are independent of Ca^{2+} .

Our results show that chronic nicotine treatment up-regulates nicotinic receptor expression levels to 2-fold, higher than that of the total cell expression level. This is consistent with previous studies that showed that nicotine treatment induces increases in the cytoplasm to cell surface trafficking of nicotinic receptors (23). Although the proportion is not clear, nicotinic receptors may be partially desensitized in our studies because we observed increases in their expression level, but not Ca^{2+} influx. The mechanism of nicotinic receptor up-regulation and partial desensitization is not clear at present. Examining

the conformational change of the up-regulated nicotinic receptors, the level of receptor trafficking in and out of the surface membrane, and the level of subunit assembly and maturation may be informative in understanding the mechanism of up-regulation.

Our results suggest that PI3K, which is activated downstream of the nicotinic receptor, is involved in the enhancement of the sensitivity, but the mechanism operating downstream of PI3K is not clear. PI3K may be involved in modulating the function of nicotinic receptors or their downstream intracellular signaling factors. As some PI3K downstream factors such as Akt and Bcl-2 are involved in donepezil-induced neuroprotection (24), such modulation may accelerate the neuroprotective signaling pathway that acts downstream of donepezil, allowing a relatively low concentration of donepezil (1 nM in our study) to exert a sufficient neuroprotective effect against glutamate insults. On the other hand, modulation of nicotinic receptor function may also change how the neuroprotective effects of donepezil, which are mediated by nicotinic receptors, are transduced. These changes in nicotinic receptor-mediated signaling could be qualitative, through the modulation of nicotinic receptor functions, or quantitative, through increases in the number of nicotinic receptors at the cell surface. Modulating the channel capacities of $\alpha 7$ -nicotinic receptors, however, is not likely to be important, because we did not detect significant changes in nicotine-induced $[Ca^{2+}]$ in chronically treated neurons. The functions of other receptor subtypes that were expressed in the primary culture, such as $\alpha 4$ -nicotinic receptors may have been modulated and hence involved. We have shown that the surface expression level of the $\alpha 7$ -nicotinic receptor is significantly increased in chronically treated neurons. Since the direct binding targets of donepezil in neuroprotection have not been elucidated, we do not know whether this leads to increased numbers of donepezil binding sites, and so lead to enhancement of neuron sensitivity. Changes in gene expression may also play role in enhancing the sensitivity because the activation of PI3K signaling can lead to the activation of the Wnt/ β -catenin signaling pathway through the inactivation of GSK-3 β , a PI3K downstream factor. These are interesting points that should be addressed in future studies.

In conclusion, we have presented results suggesting that nicotinic receptor stimulation enhances the neuron's sensitivity to the neuroprotective effect of donepezil and up-regulates nicotinic receptor expression levels. Our analyses also suggest that the nicotinic receptor-PI3K pathway is involved in enhancing the sensitivity. These results are interesting in that nicotinic receptor stimulation may be used to modulate the effect of donepezil, which is extensively used in therapy. The differences

between chronic donepezil or chronic nicotine treatment-induced enhancement of the sensitivity indicate that it may be possible to devise drugs that can be utilized together with donepezil to convergently enhance the effect of donepezil. This is an interesting prospect that should be addressed in the future.

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