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Activation of nicotinic acetylcholine receptor prevents the production of reactive oxygen species in fibrillar β amyloid peptide (1-42)-stimulated microglia

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Abbreviations: ABC, ATP binding cassette; Aβ, β amyloid peptide; AD, Alzheimer's disease; α-Bgt, α-bungarotoxin; CREB, cAMP response element binding protein; DCF-DA, dihydrodichlorofluorescein diacetate; fAβ, fibrillar Aβ; Mec, mecamylamine; nAChR, nicotinic acetylcholine receptors; oATP, oxidized ATP; PI3-K, PI3 kinase; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate; $P2X_7R$, $P2X_7$ receptor; ROS, reactive oxygen species

Abstract

Recent studies have reported that the "cholinergic anti-inflammatory pathway" regulates peripheral inflammatory responses via α7 nicotinic acetylcholine receptors (α7 nAChRs) and that acetylcholine and nicotine regulate the expression of proinflammatory mediators such as TNF-α and prostaglandin E_2 in microglial cultures. In a previous study we showed that ATP released by β-amyloid-stimulated microglia induced reactive oxygen species (ROS) production, in a process involving the $P2X_7$ receptor ($P2X_7R$), in an autocrine fashion. These observations led us to investigate whether stimulation by nicotine could regulate fibrillar β amyloid peptide (1-42) ($fA\beta_{1-42}$)-induced ROS production by modulating ATP efflux-mediated Ca^{2+} influx through $P2X_7R$. Nicotine inhibited ROS generation in $fA\beta_{1-42}$- stimulated microglial cells, and this inhibition was blocked by mecamylamine, a non-selective nAChR antagonist, and α-bungarotoxin, a selective α7 nAChR antagonist. Nicotine inhibited NADPH oxidase activation and completely blocked Ca^{2+} influx in $fA\beta_{1-42}$-stimulated microglia. Moreover, ATP release from $fA\beta_{1-42}$-stimulated microglia was significantly suppressed by nicotine treatment. In contrast, nicotine did not inhibit 2',3'-O-(4-benzoyl)-benzoyl ATP (BzATP)-induced Ca^{2+} influx, but inhibited ROS generation in BzATP-stimulated microglia, indicating an inhibitory effect of nicotine on a signaling process downstream of $P2X_7R$. Taken together, these results suggest that the inhibitory effect of nicotine on ROS production in $fA\beta_{1-42}$-stimulated microglia is mediated by indirect blockage of ATP release and by directly altering the signaling process downstream from $P2X_7R$.

Keywords: acetylcholine; adenosine triphosphate; amyloid β-protein; microglia; NADPH oxidase; nicotine; reactive oxygen species; receptors, nicotinic; purinoceptor P2Z

Introduction

The neuropathological hallmarks of Alzheimer's disease (AD) include extracellular deposition of the beta amyloid peptide (Aβ) in the form of senile plaques and the appearance of intracellular neurofibrillary tangles composed of hyperphosphorylated tau (Akiyama *et al.,* 2000). Another neuropathological feature of AD is the loss of both cholinergic neurons (Davies and Maloney, 1976; McGeer *et al.,* 1984; Muir, 1997) and nicotinic acetylcholine receptors (nAChRs) (Burghaus *et al.,* 2000; Mousavi *et al.,* 2003) in the basal forebrain, which contributes to cognitive dysfunction. The nAChR is a ligand-gated ion channel consisting of five subunits with eight different α (α2-α9) subunits and three different β (β2-β4) components (Gotti and Clementi, 2004). Of these nAChRs, α7 and α4β2 are the most abundant subunits in the brain (Buisson and Bertrand, 2002). The administration of nAChR agonists in aging animals and humans induced cognitive improvement (Newhouse *et al.,* 1997; Terry and Buccafuso, 2003) and prevented neuronal death induced by Aβ (O'Neill *et al.,* 2002).

A recent study suggests that α7 nAChRs, expressed in peripheral macrophages, are essential for operation of the so-called "cholinergic anti-

inflammatory pathway" that regulates systemic inflammatory responses in the peripheral nervous system (Wang *et al.,* 2003). Recent studies have revealed that the α7 nAChRs are expressed in murine microglia in addition to neurons and peripheral macrophages, and are involved in the suppression of neuroinflammation (Shytle *et al.,* 2004; De Simone *et al.,* 2005). Such unique receptors should play an important role in neuroprotection, because of the activation of α7 nAChRs in capable of modulating the activity of microglia, changing the microglial cell from an overactive inflammatory cell to a protective cell type (Suzuki *et al.,* 2006).

One of the mechanisms explaining Aβ neurotoxicity is that microglia-mediated oxidative stress, via NADPH oxidase activation, plays a critical role in the pathogenesis of AD. Fibrillar Aβ (fAβ) stimulates reactive oxygen species (ROS) production from cultured microglial cells via activation of NADPH oxidase (Bianca *et al.,* 1999). In addition, fAβ mediated neurotoxicity in mixed neuron-microglia cultures by causing the production of ROS (Qin *et al.,* 2002). Furthermore, NADPH oxidase activation has been identified in AD brains (Shimohama *et al.,* 2000). Our previous study demonstrated that one of the mechanisms of ROS production in Aβ-stimulated microglia involved $fA\beta_{1\text{-}42}$-induced ATP release, which in turn activated NADPH oxidase in a process involving the $P2X_7$ receptor ($P2X_7R$), in an autocrine manner (Kim *et al.,* 2007). Therefore, to determine whether the activation of nAChRs affects ROS generation in $fA\beta_{1\text{-}42}$-stimulated microglia, we examined the inhibitory effects of nicotine on ROS production and NADPH oxidase activation in such cells, and then investigated the effects of nicotine on ATP efflux and Ca^{2+} influx.

Materials and Methods

Reagents

Medium and supplement for cell culture were purchased from JBI (Daegu, Korea). Chemicals were purchased from the following companies: Amyloid-$\beta_{1\text{-}42}$ ($A\beta_{1\text{-}42}$) was purchased from the American Peptide (Sunnyvale, CA). Mecamylamine hydrochloride, α-bungarotoxin, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS), adenosine 5'-triphosphate 2',3'-acylic dialcohol (oxidized ATP; oATP), apyrase (an ATP-hydrolyzing enzyme), 2',7'-dihydrodichlorofluorescein diacetate (DCF-DA), pluronic F-127, DNase I, an ATP bioluminescence assay kit, and a protease inhibitor mixture, were all purchased from Sigma (St. Louis, MO). Nicotine ([S]-3-[1-methyl-2-pyrrolindinyl] pyridine, di-*d*-Tartrate) was from Calbiochem (San Diego, CA), Fluo-3/AM was from Molecular probes (Eugene, OR). The anti-mouse and anti-rabbit HRP-conjugated secondary anti-bodies were purchased from Amersham Pharmacia (Buckinghamshire, UK). The polyclonal antibody against $p47^{phox}$, $P67^{phox}$, and Rac 1 was provided by BD Biosciences (San Diego, CA).

Microglial cell culture

Microglial cultures were prepared from the brains of 3 day-old Sprague-Dawley rats as described previously (Kim *et al.,* 2002). Briefly, whole brains were dissected into small cubes, incubated in D-PBS containing 0.1% trypsin and 40 μg/ml DNase I for 15 min at 37ºC, and dissociated into single cells by gentle pipetting. Dissociated cells were suspended in DMEM (JBI) containing 5% horse serum, 5 mg/ml glucose, 100 U/ml penicillin and 100 μg/ml streptomycin, and plated on poly-D-lysine-coated T-75 culture flasks, and incubated at 37ºC in incubator with 5% CO_2/95% air atmosphere. After 2-4 weeks of growth in flasks, microglia floating in the medium were collected and grown in separate 6-, 96-well plates or on coverslips.

Measurement of intracellular ROS

Intracellular ROS levels were measured using the fluorescent dye, dihydrodichlorofluorescein diacetate (DCF-DA), which is readily converted to a fluorescent product in the presence of ROS in cells. In brief, cells were preincubated with nicotine (1-100 μM) for 30 min in the presence or absence of mecamylamine (10 μM), α-bungarotoxin (10 nM), and then treated with 0.5 μM $fA\beta_{1\text{-}42}$ or 300 μM BzATP. $fA\beta_{1\text{-}42}$-stimulated cells were incubated with 10 μM DCF-DA in HBSS (145 mM NaCl, 2.5 mM KCl, 1.8 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose and 20 mM HEPES; pH 7.4) for 30 min. The cells were then washed extensively with D-PBS to remove extracellular DCF-DA, and fluorescence images were taken using an IX71 confocal laser scanning microscope (Olympus; Tokyo, Japan).

Western blot analysis

Microglial cells treated with $fA\beta_{1\text{-}42}$ were lysed with lysis buffer (10 mM Na_2HPO_4, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% NP 40; pH 7.5). Lysates were centrifuged at 13,000 × *g* for 10 min at 4ºC and supernatants were collected. An aliquot of each sample containing 20 μg total protein was loaded onto a 10% acrylamide gel, and then transferred to a PVDF membrane. The

blots were incubated with blocking buffer [5% skim milk in TBST (20 mM Tris-HCl, 500 mM NaCl, 0.05% Tween 20, pH7.5)] at room temperature for 1 h, and incubated with primary antibody overnight at 4ºC. The bands were recognized by HRP conjugated anti-rabbit secondary antibody (1 : 1,000). For detecting the translocation of NADPH oxidase components, primary monoclonal antibodies against the $p47^{phox}$ (1 : 500), $p67^{phox}$ (1 : 500), Rac 1 (1 : 500), and HRP conjugated anti-mouse secondary antibody (1 : 1,000) were used.

Cell fractionation

Microglial cells were harvested and resuspended in a cold hypotonic solution (0.25 M sucrose, 10 mM Tris-HCl, and 5 mM $MgCl_2$; pH 7.4) including a protease inhibitor mixture, and centrifuged at 600 $\times$ *g* for 10 min. The supernatant was ultracentrifuged at 100,000 $\times$ *g* for 1.5 h at 4°C. The resulting supernatant was removed and saved as the cytosolic fraction, and the membrane pellet was resuspended in hypotonic solution containing 1% Triton X-100. Samples were analyzed by Western blotting using antibodies against the NADPH oxidase components $p47^{phox}$, $p67^{phox}$, and Rac 1 as described above.

Measurement of intracellular calcium

Intracellular Ca^{2+} concentration was monitored by loading cells with the fluorescent Ca^{2+} indicator Fluo-3/AM, convertible to Fluo-3 in the presence of Ca^{2+}. Cultured microglia plated onto poly-D-lysine-coated 25 mm glass coverslips were incubated with 2 μM of the acetoxymethyl ester of Fluo-3 (Fluo-3/AM) and 0.02% pluronic F-127 in HBSS for 30 min at 37°C, and then washed with HBSS. Fluo-3-loaded cells were placed in a perfusion chamber mounted on the stage of a confocal laser-scanning microscope and stimulated with 0.5 μM $fA\beta_{1\text{-}42}$. To measure the intracellular calcium concentration, a confocal laser-scanning microscope (IX71, Olympus) equipped with an Argon/Keron laser (15 mW; Coherent, Santa Clara, CA) was used. Fluo-3 was excited by the 488 nm line of an argon laser and the fluorescence was measured at an emission wavelength above 510 nm.

ATP efflux measurement

Microglial cells (3×10^4 cells/well) were plated in 96-well, and preincubated with nicotine (1-100 μM) for 30 min, and then treated with 0.5 μM $fA\beta_{1\text{-}42}$ for 1 h. At the end of this incubation, the supernatant fluids of individual wells was transferred into sterile tubes and heated at 95°C for 3 min. Extracellular ATP in the supernatants was immediately measured by luminometer (TD2020, Turner Designs, Sunnyvale, CA), using a luciferase-luciferin assay (ATP bioluminescent assay kit from Sigma) following the instructions of the manufacturer.

Statistical analysis

All statistical comparisons in this study were done using one-way ANOVA with Tukey-Kramer multiple comparisons test and data were expressed as mean $\pm$ SEM. A value of $P < 0.05$ was considered statistically significant.

Results

Nicotine inhibits $fA\beta_{1\text{-}42}$-induced ROS production in microglia

We examined the effects of nicotine on ROS production in $fA\beta_{1\text{-}42}$-stimulated microglia by measuring fluorescence signals from DCF-DA. Microglial cells were pre-treated with 1 μM, 10 μM, or 100 μM nicotine for 30 min and then stimulated with 0.5 μM $fA\beta_{1\text{-}42}$ for 2 h. Nicotine significantly decreased DCF fluorescence signals, in a dose-dependent manner (Figure 1). These results indicated that neuroprotective functions of nicotine might be mediated by suppressing $fA\beta_{1\text{-}42}$-induced ROS production in microglia.

Nicotine modulates ROS production via activation of nAChRs

To determine whether nAChRs are involved in the nicotine inhibition of ROS production in microglia, we examined the effects of mecamylamine, a non-selective nAChR antagonist, and α-bungarotoxin, a selective α7 nAChR antagonist on the nicotine-induced decrease in ROS production. Nicotine treatment caused a marked reduction in $fA\beta_{1\text{-}42}$-induced ROS production. On the other hand, treatment with mecamylamine (10 μM) or α-bungarotoxin (10 nM) significantly eliminated this inhibitory activity of nicotine (Figure 2).

$fA\beta_{1\text{-}42}$-induced NADPH oxidase activation is inhibited by nicotine

Because nicotine showed potent negative effects on ROS production, we sought to determine whether nicotine might inhibit NADPH oxidase activation by preventing the translocation of the NADPH oxidase cytoplasmic subunits $p47^{phox}$, $p67^{phox}$, and Rac 1 from the cytosol to the cell membrane after $fA\beta_{1\text{-}42}$ stimulation. Following $fA\beta_{1\text{-}42}$ treatment, the

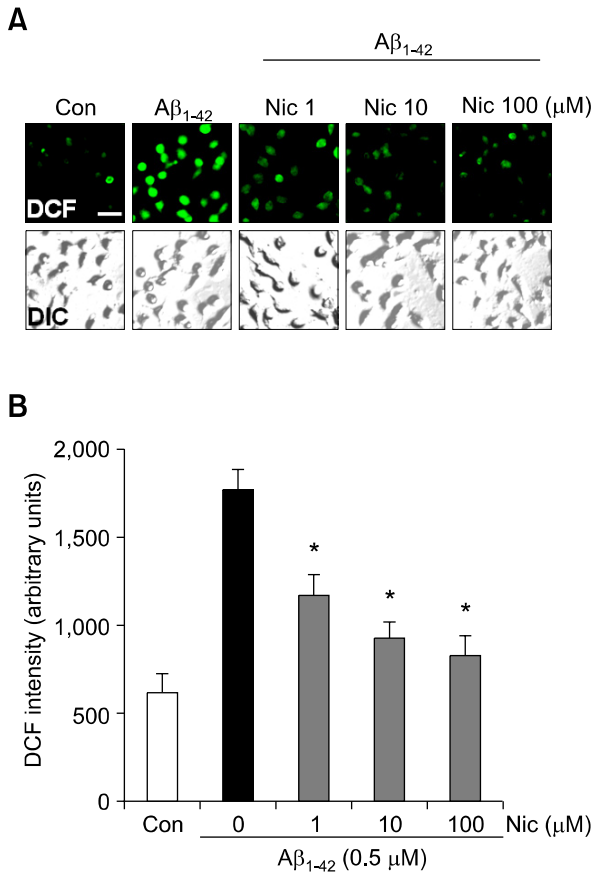

Figure 1. Effects of nicotine on reactive oxygen species production in fA$\beta_{1\text{-}42}$-stimulated microglia. (A) Primary rat microglia were plated onto coverslips (3×10^4 cells/coverslip), and then microglia were pretreated with nicotine for 30 min and stimulated with fA$\beta_{1\text{-}42}$ (0.5 μM) for 2 h. The intracellular ROS production in microglial cells was determinded using 10 μM DCF as described in Materials and Methods. (B) DCF intensities of cells were counted using Imagegage 4.0 (Fujifilm). Fluorescence (DCF) images and differential interference contrast (DIC) images were taken using an IX71 confocal microscope (Olympus). Values are mean ± SEM of 40-50 cells. *$P < 0.01$ compared with A$\beta_{1\text{-}42}$ alone. Scale bar, 20 μm.

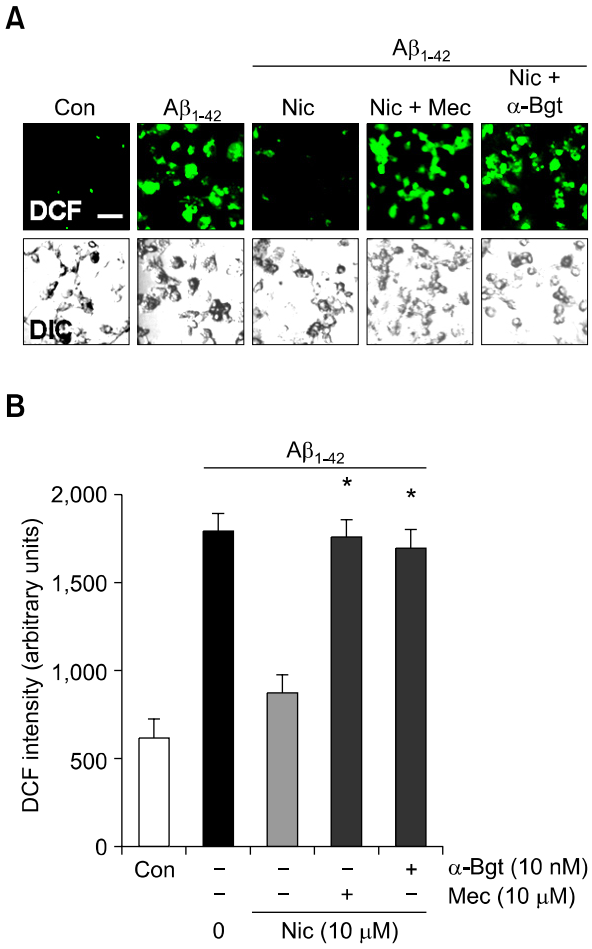

Figure 2. Effects of nAChRs antagonists on fA$\beta_{1\text{-}42}$-induced ROS production from nicotine-treated microglia. (A) The cells were preincubated with nicotine (10 μM) for 30 min in the presence or absence of 10 μM mecamylamine (Mec) or 10 nM α-bungarotoxin (α-Bgt), and then treated with 0.5 μM fA$\beta_{1\text{-}42}$ for 2 h. The intracellular ROS production in microglial cells was determined using 10 μM DCF as described in Materials and Methods. (B) DCF intensities of cells were counted using Imagegage 4.0 (Fujifilm). Fluorescence (DCF) images and differential interference contrast (DIC) images were taken using an IX71 confocal microscope (Olympus). Values are mean ± SEM of 40-50 cells. *$P < 0.01$ compared with A$\beta_{1\text{-}42}$ plus nicotine. Scale bar, 20 μm.

amounts of $p47^{phox}$, $p67^{phox}$, and Rac 1 in the membrane fraction increased, but the translocation of the cytosolic factors $p47^{phox}$, $p67^{phox}$, and Rac 1 to the plasma membrane was prevented by nicotine treatment (10 μM) (Figure 3). These results indicate that nicotine reduced fA$\beta_{1\text{-}42}$-induced ROS production through the inhibition of NADPH oxidase activation.

Nicotine inhibits fA$\beta_{1\text{-}42}$-induced ROS production in microglia by inhibition of ATP release from microglia, and not by blockade of $P2X_7R$

Our previous study demonstrated that fA$\beta_{1\text{-}42}$-stimulated ROS generation in microglial cells is regulated by ATP release-mediated Ca^{2+} influx through $P2X_7R$, in an autocrine manner (Kim *et al.,* 2007). Therefore, we investigated the effects of nicotine on ATP efflux and Ca^{2+} influx in fA$\beta_{1\text{-}42}$-stimulated microglia. Surprisingly, pretreatment of microglia with nicotine (10 μM) reduced fA$\beta_{1\text{-}42}$-induced Ca^{2+} influx to baseline levels in consistent with our previous study which showed the blockade of Ca^{2+} influx by pretreatment with apyrase (an ATP-hydrolyzing enzyme; 5 U/ml), or oATP (a $P2X_7R$-specific antagonist; 100 μM) in fA$\beta_{1\text{-}42}$-stimulated microglia (Figure 4A). Moreover, ATP release from fA$\beta_{1\text{-}42}$-stimulated microglia was significantly suppressed by nicotine treatment (Figure 4B). At this point, because a previous study had

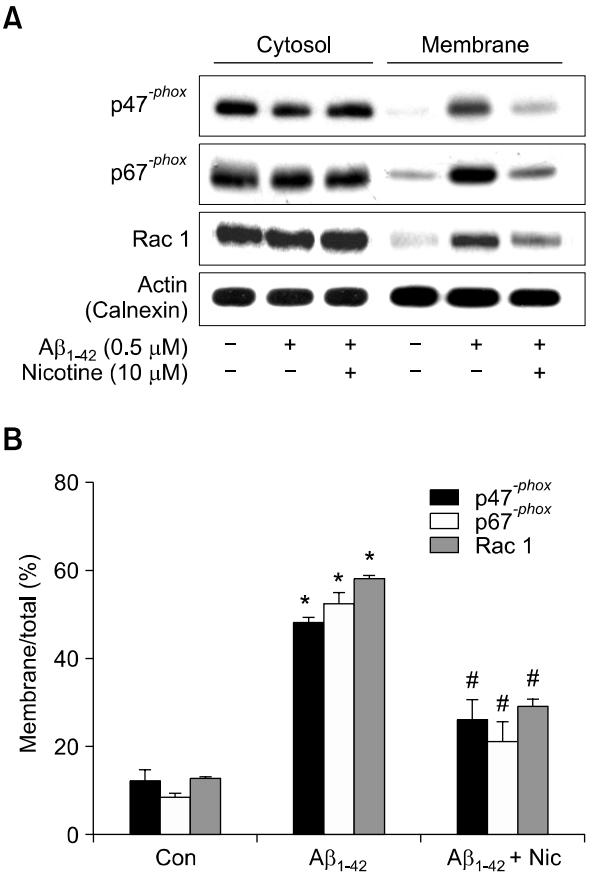

Figure 3. Nicotine inhibits fA$\beta_{1\text{-}42}$-induced NADPH oxidase activation. NADPH oxidase was activated by fA$\beta_{1\text{-}42}$, as evidenced by the translocation of the $p47^{phox}$, $p67^{phox}$, Rac 1 subunits from the cytosol to the membrane; this translocation was inhibited by nicotine treatment. (A) The cells were treated with 10 μM nicotine for 30 min and stimulated with 0.5 μM fA$\beta_{1\text{-}42}$ for 90 min. Fractionated proteins were analyzed by SDS-PAGE and subjected to immunoblotting with anti-$p47^{phox}$, anti-$p67^{phox}$, anti-Rac 1 antibody. The blots were reprobed with antibodies against the calnexin membrane protein as loading controls to exhibit fractionation efficiency. (B) The histogram shows quantitation of $p67^{phox}$, $p47^{phox}$, Rac 1 levels expressed as the ratio of membrane fraction to total. The results represent the mean ± SEM of four to five separate experiments. $^{*}P < 0.01$ compared with control, $^{\#}P < 0.01$ compared with A$\beta_{1\text{-}42}$ alone.

reported superoxide generation after Ca^{2+} influx through $P2X_7R$ in microglia (Parvathenani *et al.,* 2003), we investigated the effects of nicotine on the activation of $P2X_7R$ by examining the effects of the drug on Ca^{2+} influx in BzATP-stimulated microglia. Pretreatment of microglia with nicotine (10 μM) did not inhibit BzATP-induced Ca^{2+} influx (Figure 4C), but inhibited BzATP-induced ROS generation (Figure 4D), indicating that an inhibitory effect of nicotine lies downstream of signaling initiated from $P2X_7R$. Taken together, these results suggest that the inhibitory effects of nicotine on fA$\beta_{1\text{-}42}$-induced ROS production are mediated by inhibition of ATP efflux from microglia, resulting in blockade of Ca^{2+} influx, and by prevention of $P2X_7R$ intracellular signaling.

Discussion

The present study demonstrates the neuroprotective effects of nicotine against fA$\beta_{1\text{-}42}$-induced ROS production in rat microglial cultures. First, nicotine inhibits fA$\beta_{1\text{-}42}$-induced ROS production via activation of nAChRs and activation of fA$\beta_{1\text{-}42}$-induced microglial NADPH oxidase. Second, nicotine inhibits fA$\beta_{1\text{-}42}$-induced ROS production by blocking the Ca^{2+} influx that follows inhibition of ATP efflux, and by prevention of $P2X_7R$ downstream signaling.

fA$\beta_{1\text{-}42}$ has a direct toxic effect on neurons, but the accumulation of activated microglia at sites of fA$\beta_{1\text{-}42}$ deposits in AD indicates that activated microglia may also contribute to the progression of the disease (Akiyama *et al.,* 2000). Recently, several lines of evidence have shown that oxidative stress plays an important role in inflammation-mediated neurodegeneration in AD (de la Monte and Wands, 2006; Sultana *et al.,* 2006). Aβ stimulates ROS generation from cultured microglial cells via activation of NADPH oxidase (Bianca *et al.,* 1999) and mediates neurotoxicity by stimulating production of ROS in mixed neuron-microglia cultures (Qin *et al.,* 2002). Besides Aβ neurotoxicity, another feature of AD is the loss of cholinergic projections and decline of nAChRs from the early stage of AD (Oddo and LaFerla, 2006). In this regard, recent studies have reported the existence of a cholinergic control of microglial activation by showing that nicotine reduced LPS-induced production of TNF-α and IL-18, indicating that nicotine has immunosuppressive effects (Shytle *et al.,* 2004; De Simone *et al.,* 2005; Suzuki *et al.,* 2006; Takahashi *et al.,* 2006). On the other hand, nicotine treatment significantly increased the expression of COX-2 and the synthesis of PGE_2 in LPS-stimulated microglia, and even enhanced TNF-α production in BzATP-stimulated microglia, suggesting a neuroprotective effect of nicotine at low concentration (De Simone *et al.,* 2005). In this paper we expand the neuroprotective role of nicotine by showing that nicotine inhibits fA$\beta_{1\text{-}42}$-induced ROS production and NADPH oxidase activation in microglia. In line with our results, a recent study reported a neuroprotective effect of nicotine on dopaminergic neurons using LPS-induced *in vitro* and *in vivo* inflammation models (Park *et al.,* 2007).

Our previous study showed that ROS generation in fA$\beta_{1\text{-}42}$-stimulated microglia is mediated by ATP

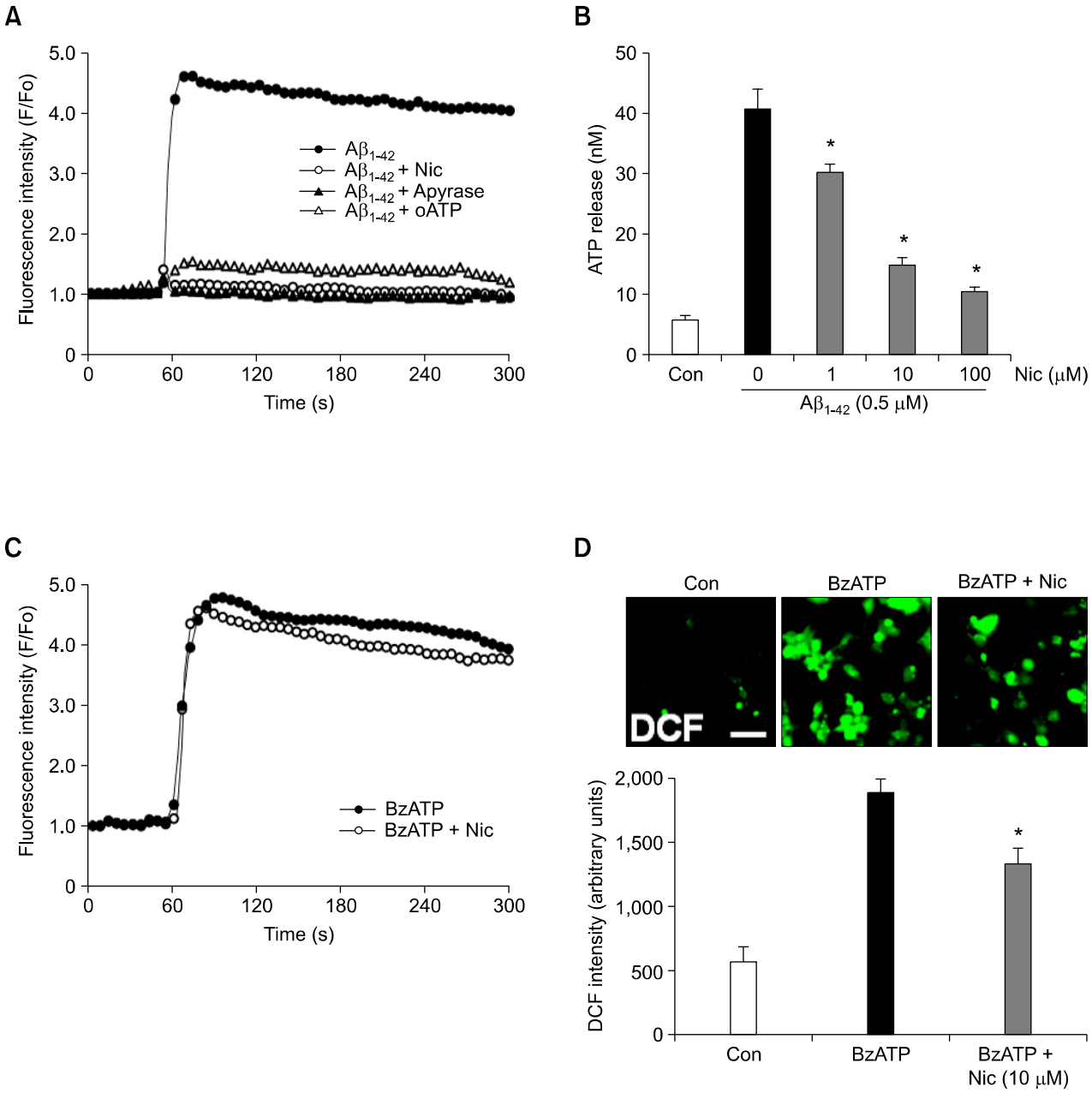

Figure 4. Effects of nicotine on Ca^{2+} influx in fAβ_{1-42}- or BzATP-stimulated microglia and ATP efflux in fAβ_{1-42}-stimulated microglia. Microglial cells were plated onto coverslips (3×10^4 cells/coverslip), and pretreated with nicotine (10 μM) (A-D), apyrase (5 U/ml), or oATP (100 μM) for 30 min (A), and then treated with 0.5 μM fAβ_{1-42} (A and B) or 300 μM BzATP (C and D). (A and C) Intracellular Ca^{2+} concentration was measured by Fluo-3 as described in Materials and Methods, and represented by the ratio between the fluorescence intensity after treatment (F) and fluorescence in the resting state (F_0). (B) Microglial cells (3×10^4 cells/well) were plated into 96 well plate, and pretreated with nicotine (10 μM) for 30 min, and then treated with 0.5 μM fAβ_{1-42}. ATP concentrations in the culture supernatants were determined at 1 h after fAβ_{1-42} stimulation. Values are mean ± SEM of triplicate samples. *$P < 0.01$ compared with Aβ_{1-42}. (D) Microglial cells were plated onto coverslips (3×10^4 cells/coverslip), and pretreated with nicotine (10 μM) for 30 min, and then treated with 300 μM BzATP. Intracellular ROS levels were assayed 2 h after BzATP stimulation using 10 μM DCF. Fluorescence (DCF) images were taken using an IX71 confocal microscope (Olympus). Scale bar, 20 μm. DCF intensities of cells were counted using Imagegage 4.0 (Fujifilm). Values are mean ± SEM of 40-50 cells. *$P < 0.01$ compared with BzATP alone.

release and subsequent Ca^{2+} influx, in a process involving the activation of $P2X_7R$, in an autocrine manner (Kim *et al.,* 2007). Interestingly, the present study demonstrated that inhibitory effects of nicotine on fAβ_{1-42}-induced ROS production are mediated by decreasing Ca^{2+} influx to a basal level

through inhibition of ATP release from microglia. On the other hand, pretreatment with nicotine did not inhibit BzATP-elicited Ca^{2+} influx. These results indicate that blockade of fAβ_{1-42}-elicited Ca^{2+} influx by nicotine treatment is mediated by preventing ATP release from microglia, and not by either interference with ATP binding to $P2X_7R$ or by decreasing the activity of this channel. Recently, it has been recognized that ATP can be released from LPS- or glutamate-stimulated microglial cells (Ferrari *et al.,* 1997; Seo *et al.,* 2004; Liu *et al.,* 2006), and ATP efflux from either astrocytes or microglia occurred via ATP binding cassette (ABC) proteins (Ballerini *et al.,* 2002). Future work on identifying the mechanisms involved in nicotine-mediated inhibition of ATP release will provide a better understanding of the role of nicotine under the pathological conditions of AD.

Besides blockade of Ca^{2+} influx by inhibition of ATP efflux, nicotine-induced suppression of ROS production in fAβ_{1-42}-stimulated microglia seems to also involve interference with $P2X_7R$ downstream signaling. Our present results showed that pre-treatment of microglia with nicotine did not inhibit BzATP-induced Ca^{2+} influx but did inhibit BzATP-induced ROS generation. It has been shown that p38 MAPK and PI3 kinase (PI3-K) play key roles in the production of ROS in BzATP-stimulated microglia (Parvathenani *et al.,* 2003). However, a recent study reported that nicotine did not affect the activation of p38 MAPK in BzATP-stimulated microglia (Suzuki *et al.,* 2006), and it remains unclear whether nicotine inhibits PI3-K in BzATP-stimulated microglia.

In conclusion, our study provides evidence for the first time that nicotine can downregulate ROS production in fAβ_{1-42}-stimulated microglia by inhibition of both ATP release and $P2X_7R$ signaling. The finding that nicotine prevents ROS production in microglia provides evidence for molecular links between Aβ, cholinergic dysfunction, and cognitive impairments, during the progress of AD.

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