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Nicotine Reduces TNF-α Expression Through a α7 nAChR/MyD88/NF-κB Pathway in HBE16 Airway Epithelial Cells

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Key Words

Nicotine • TNF-α • Nicotine acetylcholine receptors • MyD88 • NF-κB

Abstract

Aims: To explore the signaling mechanism associated with the inhibitory effect of nicotine on tumor necrosis factor (TNF)-α expression in human airway epithelial cells. Methods: HBE16 airway epithelial cells were cultured and incubated with either nicotine or cigarette smoke extract (CE). Cells were then transfected with α1, α5, or α7 nicotinic acetylcholine receptor (nAChR)-specific small interfering RNAs (siRNAs). The effects of nicotine on the production of proinflammatory factors TNF-α, in transfected cells were analyzed. Furthermore, we assayed the expression levels of myeloid differentiation primary response gene 88 (MyD88) protein, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 protein, NF-κB activity and NF-κB inhibitor alpha (I-κBα) expression in cells after treatment with nicotine or α7 nAChR inhibitor, α -bungarotoxin (α-BTX). Results: The production of TNF-α was lower in cells pretreated with nicotine before lipopolysaccharide (LPS) stimulation, compared with LPS-only-treated cells. In contrast, in α7 siRNA-transfected cells incubated with nicotine and LPS, TNF-α expression was higher than that in non-transfected cells or in α1 or α5 siRNA-transfected cells. Addition of MyD88 siRNA or the NF-κB inhibitor pyridine-2,6-dithiocarboxylic acid (PDTC) also reduced TNF-α expression. Furthermore, we found that nicotine decreased MyD88 protein, NF-κB p65 protein, NF-κB activity and phospho-I-κBα expression induced by CE or LPS. The inhibitor α-BTX could reverse these effects. Conclusion: Nicotine reduces TNF-α expression in HBE16 airway epithelial cells, mainly through an α7 nAChR/MyD88/NF-κB pathway.

Introduction

Nicotine is a low lipoid molecular protein and exerts its cellular functions mainly through nicotinic acetylcholine receptors (nAChRs). Previous research has revealed that nAChRs contain the major binding sites for nicotine [1, 2]. It has been demonstrated that nicotine binds to the α7 nAChR subunit on macrophages and T cells, which suggests that these receptors may be involved in a regulatory mechanism of the inflammatory response [3, 4]. It

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has been shown previously that in α7 nAChR subtype gene knockout mice, the production of systemic proinflammatory cytokines or tumor necrosis factor (TNF)-α was elevated, and the inflammatory response to lipopolysaccharide (LPS) assault was more severe than that found in wild-type mice [5].

Whilst the existence of a cholinergic anti-inflammatory system in epidermal keratinocytes, in intestinal ischemia-reperfusion and in toxic shock organs has been fully documented in animal models, there are only a few studies of this process in the respiratory system. As the airway has a structure and immunocyte distribution similar to that of the intestinal tract, and studies have shown that they both have a similarly wide distribution of nAChRs subunits, it is reasonable to speculate that nicotine may regulate airway inflammation in smokers. α7 nAChR is expressed ubiquitously in normal lung cells and in a series of human lung cancer cells, and nicotine-derived nitrosamine ketone (NNK) could increase its protein level [6, 7]. In our previous study, we demonstrated that nicotine had an anti-inflammatory effect on the presence of LPS-stimulated human airway epithelial cells [8], but the exact mechanism or the probable receptor involved in this process has not been well characterized. Cigarette smoke, which contains substantial amounts of nicotine, is a well known pro-inflammatory agent. Therefore, we decided to investigate whether the reported harmful or beneficial effects of nicotine on airway inflammation are mediated by a specific nAChRs subunit present in the airway cells and to characterize the potential signaling factors involved in this process.

In the current study, we compared the different effects of pure nicotine and cigarette smoke extract (CE) on the expression of inflammatory factor TNF-α in a human epithelial cell line, HBE16. Different subunits of nAChRs were investigated for any nicotine-mediated effects by use of nAChR subunit small interfering RNAs (siRNAs). Furthermore, we assayed the expression levels of myeloid differentiation primary response gene 88 (MyD88), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and NF-κB inhibitor alpha (I-κBα) proteins, to confirm whether they were involved in this process.

Materials and Methods

Materials

DMEM/Ham's F12 medium, HEPES, fetal bovine serum (FBS), anti-β-actin, α-tubulin monoclonal antibody, and α-Bungarotoxin (α-BTX) were purchased from Sigma (St. Louis, MO, USA). Lipofectamine^{2000} and OPTI-MEM reduced serum medium were purchased from Invitrogen (San Diego, CA, USA). α1, α5, α7 nAChR siRNA, MyD88 siRNA, rabbit anti-MyD88 antibody, and mouse anti-NF-κB p65 antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-IκBα polyclonal antibody, rabbit anti-p-IκBα polyclonal antibody, pyridine-2,6-dithiocarboxylic acid (PDTC), pNF-κB-Luc luciferase reporter vector, and double luciferase reporter gene assay kit were obtained from BioTime Technology (Beijing, China). pRL-TK Renilla luciferase reporter vector was purchased from Promega (Madison, WI, USA). TNF-α enzyme-linked immunosorbant assay (ELISA) kits were bought from Boster Biological Technology (Wuhan, China). Bioteke high-purity total RNA extraction kit was purchased from Bioteke. Biotechnology (Beijing, China) PrimeScript RT reagent kit was purchased from Takara (Dalian, China).

Preparation of cigarette smoke extract

Cigarette smoke extract (CE) was prepared by bubbling smoke from burning "Camel" brand cigarettes into ethanol or chloroform solvents. Extracts were collected as previously described [9]. Smoke from 10 "Camel" brand cigarettes was aspirated into a polypropylene syringe (30 ml) at a rate of 1 puff/min and bubbled slowly into 20 ml of 50 mM HEPES buffer (hereafter called "cigarette smoke" solution), with 10 puffs per cigarette. The cigarette smoke solution was then sterilized by filtration through a 0.22-μm cellulose acetate sterilizing system, aliquoted, and stored at –20°C. Aliquots of the cigarette smoke solution were thawed at 4°C, and various dilutions were prepared with DMEM/Ham's F12 medium immediately before incubation with cells. The chemicals identified in the chloroform extract (CE) mainly comprised of terpenoids, phenolic compounds, hydrocarbons, but no nicotine was identified.

Cell culture

The HBE16 human airway epithelial cell line, which was obtained previously from a segmental human airway and immortalized using the origin-defective SV40 virus [10], were plated in six-well plates at a concentration of $5\text{-}6\times10^5$ cells per well in 2 ml DMEM/Ham's F12 medium that contained 10% fetal bovine serum (FBS). Cells were then incubated at 37°C in 5% CO_2 in air in a humidified incubator. The culture medium was changed to growth factor-free medium prior to the start of the experiment. Confluent HBE16 cells were incubated with 100 μg/ml cigarette smoke chloroform extract (CE) or 20 μM nicotine, respectively, for 24 h. Doses were selected in accordance with our previous experiments [8]. The subunits of nAChRs present in HBE16 cells were analyzed after CE and nicotine stimulation. Cell supernatants and lysates were collected, and assays were performed as described below.

siRNA transfection

To study if the effects of nicotine were mediated via nAChRs, epithelial cells were transfected with α1, α5, or α7 nAChR siRNA carried by Lipofectamine^{2000} before nicotine stimulation, in accordance with the manufacturer's instructions. Cell viability was measured by the 3-(4,5-dimethyl-thiazol-2,5-diphenyltetrazolium bromide (MTT) reduction method [11].

RNA isolation and real-time PCR for TNF-α

Transfected HBE16 cells were cultured in 10% FBS DMEM/Ham's F12 medium, and incubated with nicotine, or LPS, for an additional 12 h. Cells were harvested and total RNA was extracted from HBE16 cells in each group, using Bioteke high-purity total RNA extraction kit, verified by 1.5 % agarose gel electrophoresis. The absorbance A260/280 was in the range of 1.8-2.0. RNA was primed with Oligo(dT) and reverse transcribed with the PrimeScript RT reagent kit. Real-time PCR was performed with SYBR Premix EX TaqTM II real time PCR kit. The sequences of primers used for real-time PCR were: TNF-α (NM000594) forward primer: 5′-TTT GAT CCC TGA CAT CTG GA-3′, and reverse primer: 5'-GGC CTA AGG TCC ACT TGT GT-3′; and GAPDH (BC026907) forward primer: 5′-GAA GGT GAA GGT CGG AGT-3′, and reverse primer: 5′-GAA GAT GGT GAT GGG ATT TC-3′ GAPDH was used as a loading control. Relative mRNA expression was determined by comparison with a standard curve.ELISA for TNF-α protein

Cell culture supernatants were collected from each group. TNF-α protein was measured by specific ELISA kits in accordance with the manufacturer's instructions. Briefly, 100 μl of assay diluent and 50 μl of sample were added to each well and incubated for 2 h at room temperature. Next, 200 μl of conjugate was added to each well for another 2 h incubation at room temperature. Following a wash step, 200 μl of substrate reaction solution was added to each well and the plate was incubated for 30 min. Finally, 50 μl of stop solution was added to quench the reaction. Absorbance was read at 450 nm.

NF-κB activity analyzed using pNF-κB luciferase reporter assay system

pNF-κB-luc plasmid and pRL-TK Renilla luciferase reporter plasmid were transfected into HBE16 cells using Lipofectamine. After transfection for 18 h, cells were incubated with 20 μM nicotine for 1 h, then followed by 100 μg/ml CE and 10 μg/ml LPS stimulation. The activity of NF-κB was assayed in accordance with the manufacturer's instructions given in the luciferase reporter gene assay kit. Results were expressed as detected luciferase relative light unit (RLU)/Renilla luciferase RLU.

Western blotting for MyD88, NF-κB p65, I-κBα and phosphorylated (p)-I-κBα protein

The nuclear protein extract in each group HBE16 cells was prepared for NF-κB p65 protein assay. Cells in each group were harvested, the cell pellets were suspended in nuclear extract buffer I (10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.5% Nonidet P-40, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin), incubated on ice for 15 min and then with vigorous shaking on a vortex, the precipitate was collected and then resuspended in nuclear extract buffer II (20 mM HEPES (pH 7.9), 20% glycerin, 420 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin), vigorously mixed on a vortex and then centrifuged at 12 000 r/min for 15 min at 4°C. The supernatant was collected for further assay. Protein in each group of whole cells lysates was extracted for MyD88, IκBα, p-IκBα protein assay. All the samples were measured by bicinchoninic acid (BCA) protein assay reagent. Samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with 5% skimmed milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 for 1h and then incubated with mouse anti-NF-κB p65 antibody, rabbit anti-MyD88 polyclonal antibody, rabbit anti-IκBα polyclonal antibody or rabbit anti-phospho-IκBα polyclonal antibody (1:1000), respectively, for 30 min at room temperature, then overnight at 4°C The membranes were washed three times and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:1000), respectively, for 2 h at room temperature. Blots were developed with an enhanced chemiluminescence (ECL) reagent in accordance with the manufacturer's instructions.

Statistical analysis

Results are expressed as mean±SE. All *in vitro* experiments that used cell lines were carried out with materials collected from at least six separate cell cultures in duplicate or triplicate. More than two groups were compared using Student's *t*-test or one-way analysis of variance (ANOVA), followed by Bonferoni analysis with a significance value defined as $P < 0.05$.

Results

Effects of α7 nAChR siRNA on nicotine-induced TNF-α expression in HBE16 cells

Cells were pretreated with nicotine prior to LPS stimulation. The TNF-α mRNA expression in cells and TNF-α protein release in culture supernatants from nicotine-treated cells were lower than from non-nicotine-treated LPS-stimulated cells, $P<0.05$. TNF-α expression was increased in cells that had been transfected with α7 nAChR siRNA, then co-incubated with nicotine and LPS, compared with nicotine and LPS co-treated cells without α7 nAChR siRNA transfection ($P<0.05$). TNF-α expression was not significantly different in cells transfected with α1 or α5 nAChR siRNA compared with non-transfected cells (Fig. 1).

Effects of MyD88 siRNA and PDTC on TNF-α expression in HBE16 cells

TNF-α expression induced by CE or LPS was lower in HBE16 cells transfected with MyD88 siRNA compared with non-transfected cells ($P<0.05$). Pretreatment with the NF-κB inhibitor PDTC for 30 min before CE or LPS stimulation, also reduced the expression of TNF-α compared with induction by CE or LPS only. In MyD88 siRNA transfected cells, pretreatment with PDTC for 30 min before incubation with CE or LPS, significantly decreased TNF-α expression compared with CE- or LPS-stimulated non-transfected cells ($P<0.01$) (Fig. 2).

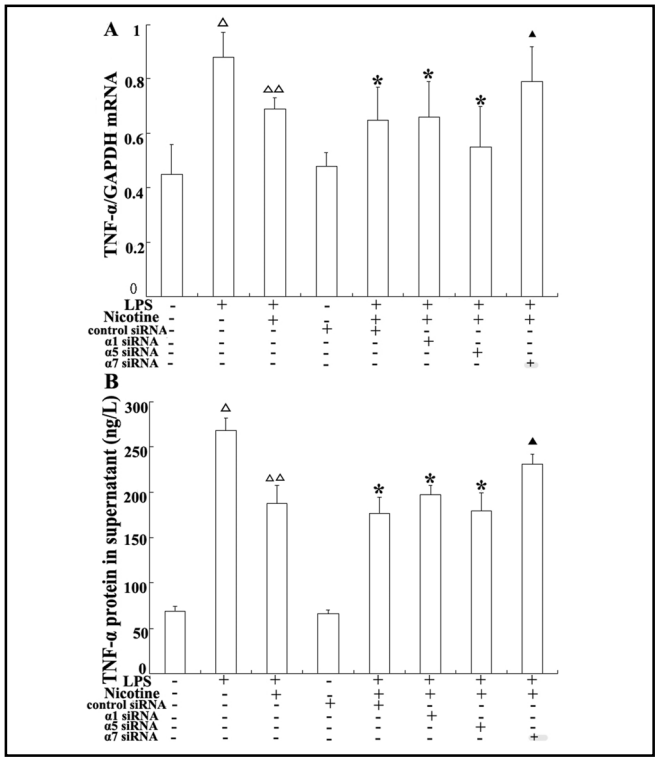

Fig. 1. Effects of α1, α5, α7 nicotinic acetylcholine receptor (nAChR) siRNA on tumor necrosis factor (TNF)-α mRNA and protein expression in HBE16 cells. Cells were transfected with α1, α5 or α7 nAChR-specific siRNA, then incubated with lipopolysaccharide (LPS) plus or minus nicotine pre-treatment. (A) The expression of TNF-αmRNA was analyzed by real-time polymerase chain reaction (PCR). (B) TNF-α protein levels were assayed by enzyme-linked immunosorbent assay (ELISA). Data are expressed as means±standard error (SE), n=6. △: compared with the control group, P<0.01; △△: compared with the LPS-treated group, P<0.05; ▲: compared with the nicotine+LPS-treated group, P<0.05; *: compared wih the nicotine+LPS+ α7 siRNA group, P<0.05.

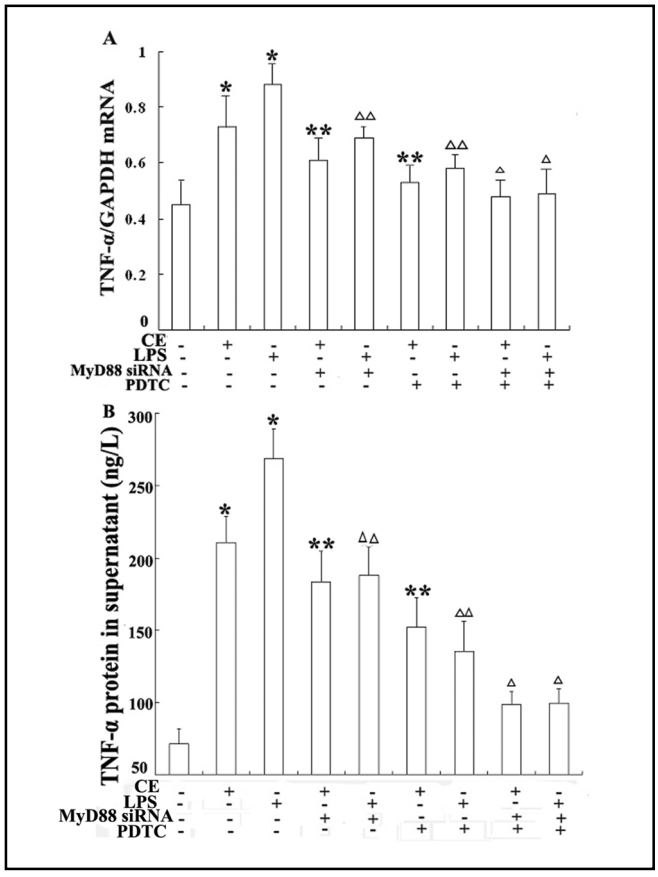

Fig. 2. Effects of myeloid differentiation primary response gene 88 (MyD88) siRNA and pyridine-2,6-dithiocarboxylic acid (PDTC) on tumor necrosis factor (TNF)-α mRNA and protein expression in HBE16 cells. Cells were transfected with MyD88 siRNA, then incubated with lipopolysaccharide (LPS) or cigarette smoke extract (CE) alone or combined with PDTC. (A) The expression of TNF-α mRNA was analyzed by real-time polymerase chain reaction (PCR). (B) TNF-α protein levels were assayed by enzyme-linked immunosorbent assay (ELISA). Data are expressed as means±standard error (SE), n=6. *: Compared with the control group, P<0.01; **: compared with the CE-treated group, P<0.05; △△: compared with the LPS-treated group, P<0.05; △: compared with CE-treated only group or the LPS-treated only group, P<0.01.

Effects of α7 nAChR inhibitor α-BTX on MyD88 protein expression in HBE16 cells

MyD88 protein expression in CE- or LPS-treated cells was increased, compared with the vehicle control cells (P<0.01). When cells were pretreated with nicotine before exposure to CE or LPS, MyD88 expression was attenuated, however, α7 nAChR specific inhibitor α-BTX reversed the nicotine inhibitory effect on MyD88 protein expression (Fig. 3).

Effects of α-BTX on I-κBα, phosphorylated (p)-I-κBα protein expression in HBE16 cells

In non-transfected HBE16 cells, we found that expression of p-I-κBα protein were increased after CE or LPS incubation, compared with the control group. The expression of p- I-κBα decreased markedly in cells that were incubated with nicotine before CE or LPS stimulation, compared with only CE- or LPS-treated cells; the levels of I-κBα protein showed no significant change. The expression of p- I-κBα protein was higher in cells pretreated with α-BTX, then incubated with nicotine and CE or LPS, than for non-α-BTX-treated cells (Fig. 4).

Effects of α-BTX on NF-κB p65 protein and NF-κB activity in HBE16 cells

CE or LPS increased NF-κB p65 protein expression in HBE16 cells. Cells pretreated with nicotine had reduced NF-κB p65 protein levels in CE- or LPS-treated

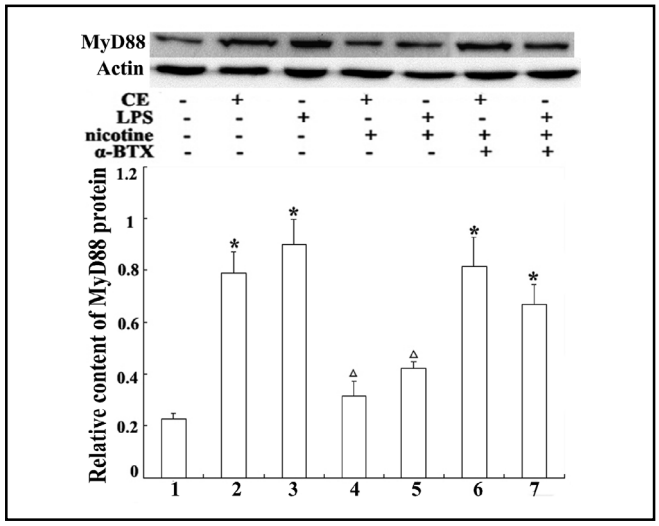

Fig. 3. Expression of myeloid differentiation primary response gene 88 (MyD88) protein in each group. Cells were divided into the following groups: (1) control group; (2) cigarette smoke extract (CE)-treated group: 100 μg/ml CE were added to the serum-free medium; (3) lipopolysaccharide (LPS)-treated group: 10 μg/ml LPS were added in the serum-free medium; (4) nicotine plus CE-treated group: 100 μg/ml CE and 20 μM nicotine were added in the medium; (5) nicotine with LPS-treated group: 10 μg/ml LPS and 20 μmol/L nicotine were added in the medium; (6) nicotine+CE+α-bungarotoxin (α-BTX) group: cells were pretreated with 10 μM α7 nicotinic acetylcholine receptor (nAChR)-specific inhibitor α-BTX for 30 min, then 100 μg/ml CE and 20 μM nicotine were added in the culture medium; (7) nicotine+LPS+ α-BTX group: cells were pretreated with 10 μM α7 nAChR specific inhibitor α-BTX for 30 min, then 10 μg/ml LPS and 20 μM nicotine were added in the culture medium. The relative content of MyD88 protein in each group cells were assayed by western blotting.*: compared with normal control group, $P<0.01$; △: compared with α-BTX group, $P<0.01$.

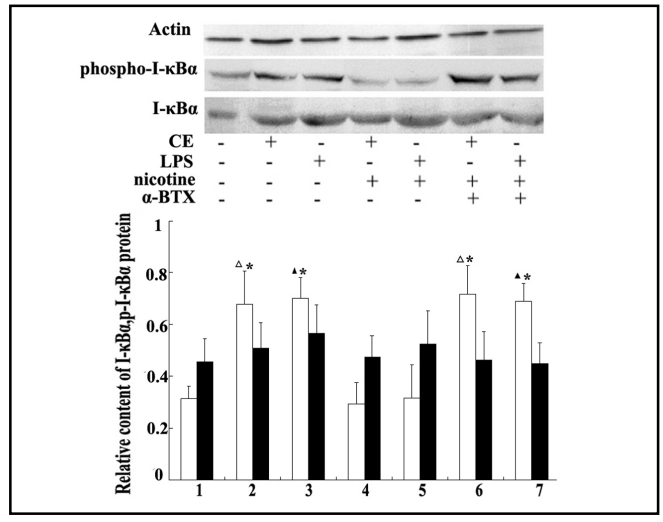

Fig. 4. Expression of NF-κB inhibitor alpha (I-κBα) and phosphorylated (p)- I-κBα protein in each group. Cells were divided into seven groups: (1) control group; (2) CE-treated group: 100 μg/ml CE were added to the serum-free medium; (3) LPS-treated group: 10 μg/ml LPS were added in the serum-free medium; (4) nicotine plus CE-treated group: 100 μg/ml CE and 20 μM nicotine were added in the medium; (5) nicotine with LPS-treated group: 10 μg/ml LPS and 20 μmol/L nicotine were added in the medium; (6) nicotine+CE+α-BTX group: cells were pretreated with 10 μM α7 nAChR-specific inhibitor α-BTX for 30 min, then 100 μg/ml CE and 20 μM nicotine were added in the culture medium; (7) nicotine+LPS+ α-BTX group: cells were pretreated with 10 μM α7 nAChR specific inhibitor α-BTX for 30 min, then 10 μg/ml LPS and 20 μM nicotine were added in the culture medium. The relative content of I-κBα and p- I-κBα protein in each group cells was assayed by western blotting.*: compared with normal control group, $P<0.01$; △: compared with nicotine+cigarette smoke extract (CE) group, $P<0.01$; ▲: compared with nicotine+LPS group, $P<0.01$.

HBE16 cells. The α7 nAChR inhibitor, α-BTX, reversed the inhibitory effect of nicotine (Fig. 5A). Cells were transfected with a pNF-κB-luc reporter plasmid and treated with various stimuli. The relative luciferase unit (RLU) levels were lower in the nicotine and CE-co-treated group, or in the nicotine and LPS-cotreated group, compared with the CE group or the LPS group. α-BTX increased the RLU levels in both the nicotine and CE-co-treated group and the nicotine and LPS-co-treated group, $P<0.01$ (Fig. 5B).

Discussion

Although nicotine is the component in cigarettes responsible for addiction, it is considered to be the least harmful tobacco component. Several studies, confirmed in mouse models, have demonstrated that the inflammatory response induced by tobacco may be offset by the anti-inflammatory effects of nicotine. Research has shown that nicotine plays a pharmacological role in many diseases including Parkinson's disease, Alzheimer's disease, ulcerative colitis, chronic obstructive pulmonary disease, and diabetes [12, 13]. The nAChR is a type cholinergic receptor that forms ligand-gated ion channels in the plasma membrane and has been studied mainly in relation to nerve signal transmission. It can be regulated by endogenous acetylcholine, choline and other members of the exogenous nicotine complex. The nAChR is sensitive to nicotine and can be classified as existing as two subtypes, "muscle derived" or "neuronal derived," based on the receptor's primary site of expression. Neuronal nAChRs are more sensitive to nicotine than muscle-derived receptors. It has been shown that there is wide-

spread distribution of nAChRs in peripheral tissues such as airway epithelial cells, and that these receptors are involved in various physiological and pathological activities [14].

The mammalian nAChR family of proteins consists of seven subunits that exist in various homomeric or heteromeric combinations of the ligand-binding site subunits (α2, α3, α4, α6, α7, α9, or α10) and the four structural subunits (α5, β2, β3, or β4). All of these combinations have unique pharmacological properties. Lung epithelial cells and vascular endothelial cells express AChR that include the nAChR α7, α3 or α5 and β2 or β4 subunits. Other potential homologous receptors composed of α9 and α10 subunits also have been confirmed to be present in the human respiratory system [15, 16]. Studies have found that human peripheral blood mononuclear cells express only the α2, α5, α6 or α7 nAChR subunits; the expression of α5 or α7 subunit mRNAs in smokers was found to be lower than that in non-smokers. Zia et al. [17] reported that rhesus monkey lung tissue and bronchial epithelial cells expressed α3, α4, α7, α9, α10, β2, β4 subunit nAChRs. Further studies have revealed that nicotine can activate α7 nAChR to increase the inward current through the channel.

In order to examine which subunits participate in the anti-inflammatory effects of nicotine, we transfected cells with α1, α5 or α7 nAChR siRNA. Results showed that α1 or α5 siRNA-transfected cells stimulated with LPS and nicotine had no significant change in their expression of TNF-α compared with non-transfected cells. However, TNF-α expression in α7 nAChR siRNA-transfected cells was higher than that found in non-transfected cells following LPS and nicotine stimulation. These results suggested that nicotine has lost its ability to reduce LPS-induced inflammatory cytokine protein production and gene transcription in cells in which α7 nAChR expression was repressed. These findings indicated that α7 nAChR activation is involved in the nicotine anti-inflammatory process, whilst activation of the α1 or α5 subunits had no significant effect.

It is generally considered that nAChRs regulate inflammation or cellular functions mainly through the vagus nerve, hence the term "cholinergic anti-inflammatory pathway" [18–20]. It has been demonstrated that the α7 nAChR subunit is an important anti-inflammatory component of this pathway, which is involved in regulation of the inflammatory and immune responses [21, 22]. Under normal circumstances, the vagus nerve in the peripheral tissue releases acetylcholine, which acts as a cholinergic agonist. Studies have shown that nicotine has a more pronounced effect than acetylcholine on reduction of pro-inflammatory factors and on inhibition of the inflammatory signal [23]. Nicotine can activate the α7 nAChR subunit to inhibit the expression of inducible nitric oxide synthase and nitric oxide through the mitogen-activated protein kinase (MAPK)/NF- κB signaling pathway [24]. In airway epithelial cells, nicotine can also induce granulocyte–macrophage colony-stimulating factor (GM-

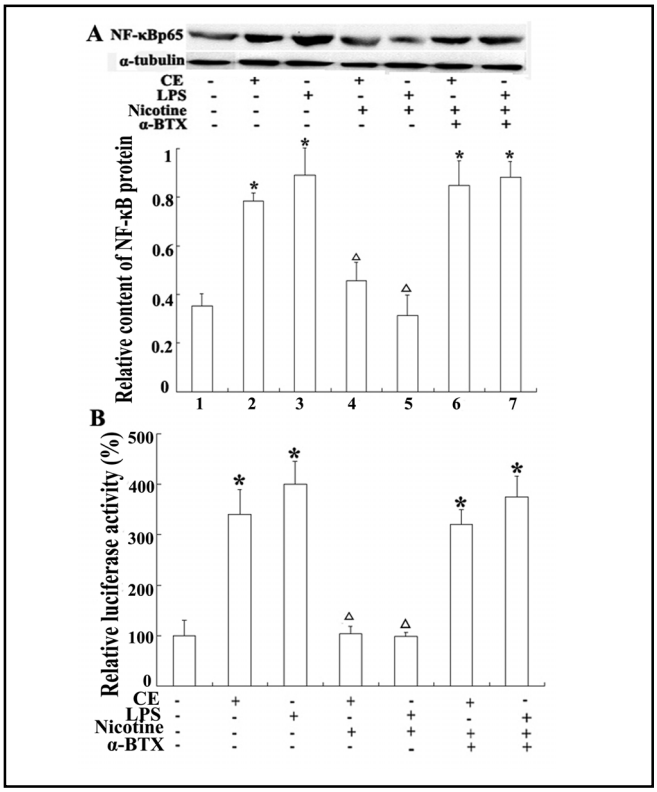

Fig. 5. (A) Expression of NF-κB p65 protein in each group HBE16 cells. Cells were divided into seven groups: (1) control group; (2) CE-treated group: 100 μg/ml CE were added to the serum-free medium; (3) LPS-treated group: 10 μg/ml LPS were added in the serum-free medium; (4) nicotine plus CE-treated group: 100 μg/ml CE and 20 μM nicotine were added in the medium; (5) nicotine with LPS-treated group: 10 μg/ml LPS and 20 μmol/L nicotine were added in the medium; (6) nicotine+CE+α-BTX group: cells were pretreated with 10 μM α7 nAChR-specific inhibitor α-BTX for 30 min, then 100 μg/ml CE and 20 μM nicotine were added in the culture medium; (7) nicotine+LPS+α-BTX group: cells were pretreated with 10 μMα7 nAChR specific inhibitor α-BTX for 30 min, then 10 μg/ml LPS and 20 μM were nicotine added in the culture medium. The relative content of NF-κB p65 protein in each group cell nucleus was assayed by western blotting. *: compared with normal control group $P<0.01$; △: compared with α-BTX-treated group, $P<0.01$. (B) Effect of nicotine on NF-κB activity. The results were expressed as relative luciferase activity. *: compared with normal control group, $P<0.01$; △: compared with α-BTX-treated group, $P<0.01$.

CSF) release through nAChRs activation [25]. Pavlov [26] and Wang [27] first reported that human peripheral blood mononuclear cells pretreated with acetylcholine and nicotine had the ability to reduce LPS-induced TNF-α generation by post-transcriptional regulation. Nicotine has been also found to reduce IL-8, IL-1, and prostaglandin E2 production, which may explain to some extent the immune suppression found in smokers [28-30]. Summers [31] showed that, in HT29 epithelial cells, nicotine inhibited IL-8 release by α7 nAChR subunit activation. In contrast, other studies have shown that in the human respiratory tract, nicotine stimulated neutrophils to produce IL-8 and induced vascular endothelial cells to produce ICAM-1 and VCAM-1, both via nAChR activation, I-κBα and -β protein separation, NF-κB p65 and p50 activation, and the MAPK signaling pathway [32, 33]. These effects should be explored further.

In our experiments, the NF-κB inhibitor PDTC could reverse the inhibitory effect of nicotine on TNF-α expression., furthermore CE or LPS stimulation led to both I-κBα phosphorylation and NF-κB activation. The phosphorylation levels of IκBα, NF-κB p65 protein, and NF-κB activity were significantly decreased after cells were pre-treated with nicotine. MyD88 protein was increased after CE or LPS incubation; nicotine treatment reduced MyD88 protein expression in CE- or LPS-treated cells. A specific inhibitor of the α7 nAChR subunit, α-BTX, could reverse the effects of nicotine on these factors.

IκBα is the main regulator of NF-κB [34]. In the resting state, IκBα continues to be synthesized in the body, but most molecules are quickly degraded and leave only a small part of free protein. NF-κB nuclear translocation and transcriptional activation are inhibited when almost all NF-κB and IκBα are in a combined state [35]. Myeloid differentiation factor 88 (MyD88), a key anchor protein of the Toll-like receptor signaling pathway, can activate the IκB kinase complex, (IKK), which leads to further phosphorylation of IκB and to NF-κB activation [36, 37]. MyD88 can also activate the IL-1R associated kinase (IRAK) tumor necrosis factor receptor-associated factor 6 (TRAF6) and beta-transforming growth factor-activated protein kinase 1 (TAK1), to activate NF-κB, and to further induce inflammatory factor generation [38]. It has also been shown that, in inflammatory bowel disease, MyD88 plays a pivotal role in the TLR4/MyD88/NF-κB signaling pathway, which leads to increase in the level of inflammation [39]. Furthermore, nicotine has the ability to reduce inflammatory bowel disease, therefore this effect may be caused by inhibition of the MyD88/NF-κB signaling pathway. Greene and colleagues [40] have reported that nicotine has the ability to act against TLR2- and TLR4-induced IL-8 production in CFTE29o- airway epithelial cells, through nAChR α7 activation, which suggests an involvement of TLR/MyD88 in the process. In our study, we confirmed the existence of the MyD88/IκB/NF-κB signaling pathway in HBE16 airway epithelial cells. CE or LPS treatment increased MyD88, and NF-κB protein levels, and these effects could be attenuated by nicotine incubation. The α7 nAChR subunit-specific inhibitor, α-BTX, reversed the inhibitory effects of nicotine. LPS- or CE-regulated TNF-α production was inhibited by pre-treatment with nicotine or with α7 nAChR siRNA or MyD88 siRNA, respectively

In summary, we showed that nicotine could suppress TNF-α expression mainly through activation of the α7 nAChR subunit, which inhibited the MyD88/IκBα/NF-κB signaling pathway in HBE16 airway epithelial cells. These findings may provide new information on the potential pharmacological effects of nicotine and nAChR in the treatment of respiratory inflammatory diseases. Further research on nicotine and nAChRs may provide more evidence for the treatment of inflammatory diseases and the development of related drugs.

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