

Nicotine promotes cell proliferation and induces resistance to cisplatin by $\alpha 7$ nicotinic acetylcholine receptor-mediated activation in Raw264.7 and E14 cells

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Abstract. Although nicotine is a risk factor for carcinogenesis and atherosclerosis, epidemiological data indicate that nicotine has therapeutic benefits in treating Alzheimer's disease. Our previous studies also showed that nicotine-treated dendritic cells have potential antitumor effects. Hence, the precise effects of nicotine on the biological characterizations of cells are controversial. The aim of the present study was to assess the roles of $\alpha 7$ nicotinic acetylcholine receptors (nAChRs), Erk1/2-p38-JNK and PI3K-Akt pathway in nicotine-mediated proliferation and anti-apoptosis effects. The results firstly showed that nicotine treatment clearly augmented cell viability and upregulated PCNA expression in both Raw264.7 and E14 cells. Meanwhile, nicotine afforded protection against cisplatin-induced toxicity through inhibiting caspase-3 activation and upregulating anti-apoptotic protein expression. Further exploration demonstrated that nicotine efficiently abolished cisplatin-promoted mitochondria translocation of Bax and the release of cytochrome *c*. The pretreatment of α -bungarotoxin and tubocurarine chloride significantly attenuated nicotine-augmented cell viability, abolished caspase-3 activation and $\alpha 7$ nAChR upregulation. Both Erk-JNK-p38 and PI3K-Akt signaling pathways could be activated by nicotine treatment in Raw264.7 and E14 cells. Notably, when Erk-JNK and PI3K-Akt activities were inhibited, nicotine-augmented cell proliferation and anti-apoptotic effects were abolished accordingly. The results presented here indicate that nicotine could achieve $\alpha 7$ nAChR-mediated proliferation and anti-apoptotic

effects by activating Erk-JNK and PI3K-Akt pathways respectively, providing potential therapeutic molecules to deal with smoking-associated human diseases.

Introduction

Cigarette smoking, which causes lung and other types of cancer, is also a risk factor of both established tumor metastasis and increases overall mortality in cancer patients (1,2). In addition, smokers have increased vulnerability to atherosclerosis and are predisposed to allergic airway diseases (3,4). These studies suggest that by altering cell viability, smoking enables tumor cells and vascular endothelial cells to evade appropriate immune responses. Nicotine, a major component of cigarette smoke, is widely accepted as a risk factor for carcinogenesis and atherosclerosis (1). However, to date, little is known about how and to what extent nicotine contributes to the adverse effects of chronic tobacco use, apart from its psychoactive actions and addictive properties. Although nicotine could promote lung cancer development, reduce the efficacy of chemotherapeutic agents (5) and activate hypoxia-inducible factor-1 α expression (6), the fast synaptic transmission in key regions controlling behavior mediated by nicotine via nicotinic acetylcholine receptors (nAChRs) was also reported (3), indicating that nicotine might be a survival agonist against various stresses inducing apoptosis (4). We previously studied the biological roles of nicotine and found that nicotine activates bone marrow-derived dendritic cells and nicotine-treated dendritic cells have potential antitumor effects (7-9). Hence, the exact effect of nicotine on cell survival has not been fully characterized and remains controversial.

nAChRs, which belong to a family of ionotropic receptors consisting of α -subunits or a combination of α and β -subunits, are widely distributed throughout the central and peripheral nervous system (10). In addition, non-neuronal cells such as monocytes, endothelial cells and epithelial cells were also found to express nAChRs (11). Despite documented data showing that $\alpha 1$, $\alpha 3$, $\alpha 5$ nAChRs play important roles in regulating cell proliferation, apoptosis and facilitating tumor formation (12-14), $\alpha 7$ nAChR, which is the main receptor of nACh in non-neuronal cells, has acquired more attention in recent years. For example, $\alpha 7$ nAChR was found to be

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involved in gastric and colon cancer migration (15,16) and to also upregulate PPAR β/δ expression in human lung carcinoma (17). The fact that inhibition of $\alpha 7$ -nicotinic receptor reduces tumorigenicity in A549 NSCLC xenografts (18) and facilitates lung cancer treatment (19) indicated that $\alpha 7$ nAChR plays important roles in tumor proliferation, angiogenesis and apoptosis (20-22). To date, however, the exact role of $\alpha 7$ nAChR in nicotine-mediated cell proliferation and anti-apoptotic effects has not been fully elucidated, and is important for potential tumor therapeutic target molecule discovery.

In the present study, to investigate the roles of $\alpha 7$ nAChR in nicotine-mediated cell proliferation and anti-apoptotic effects, Raw264.7 and E14 cells were treated with nicotine and cell proliferation was firstly determined by CCK-8 assay and western blot analysis, respectively. Then, the roles of $\alpha 7$ nAChR in nicotine-augmented cell proliferation and anti-apoptotic effects were further explored by pre-incubation of cells with $\alpha 7$ nAChR specific antagonist α -bungarotoxin and broad spectrum nicotinic antagonist tubocurarine chloride. The effects of nicotine on cisplatin-induced mitochondria translocation of Bax and the release of cytochrome *c* from mitochondria were investigated by mitochondria isolation and western blot analysis. Using kinase inhibitors, the roles of Erk-JNK-p38 and PI3K-Akt phosphorylation in nicotine-mediated cell proliferation and anti-apoptosis effects were further investigated. The results showed that, firstly, nicotine treatment clearly augmented cell viability, upregulated Mcl-1 and Bcl-2 expression and decreased caspase-3 activation in Raw264.7 and E14 cells. Secondly, the pretreatment of α -bungarotoxin and tubocurarine chloride efficiently attenuated nicotine-augmented cell proliferation, $\alpha 7$ nAChR upregulation and abolished the inhibitory effects of nicotine on cisplatin-induced caspase-3 activation. Further exploration demonstrated that nicotine efficiently abolished cisplatin-promoted mitochondria translocation of Bax and the release of cytochrome *c*. Notably, both Erk-JNK-p38 and PI3K-Akt signaling pathways could be activated by nicotine treatment in Raw264.7 and E14 cells. When Erk-JNK and PI3K-Akt activities were inhibited, nicotine-augmented cell proliferation ability and anti-apoptotic effects were reversed accordingly. The results presented here indicate that nicotine could achieve $\alpha 7$ nAChR-mediated proliferation and anti-apoptotic effects by activating the Erk-JNK and PI3K-Akt pathways respectively, providing potential molecules to deal with tobacco-associated human diseases.

Materials and methods

Reagents. Reagents were purchased from the following companies: nicotine, α -bungarotoxin and tubocurarine chloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cisplatin (DDP) was purchased from Calbiochem (San Diego, CA, USA). Annexin V/PI Apoptosis Detection kit was obtained from Promega (Madison, WI, USA). Cell Counting Kit-8 (CCK-8) was from Dojindo Laboratories (Kumamoto, Japan). p38 MAPK inhibitor SB203580, JNK MAPK inhibitor SP600125, Erk1/2 inhibitor U0126, PI3K inhibitor LY294002 and Akt inhibitor Wortmanin were from Cayman Chemical (Ann Arbor, MI, USA). Antibodies to β -actin, Cox IV, PCNA, $\alpha 7$ nAChR, Bcl-2, Mcl-1, Bax, cleaved caspase-3, cyto-

chrome *c*, phospho-p38, phospho-Erk1/2, phospho-Mek1/2, phospho-c-Raf, phospho-Msk, phospho-p90Rsk, phospho-JNK were from Cell Signaling Technology (Beverly, MA, USA). Mitochondria isolation kit for cultured cells was from Thermo Fisher Scientific (Rockford, IL, USA). RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone (Logan, UT, USA).

Cell line. The Raw264.7 and E14, macrophage and T lymphoma cell lines respectively, were obtained from Shanghai Cell Bank (Shanghai, China). Cells were cultured in DMEM with 10% FBS at 37°C in 5% CO₂ and passed every 1-2 days to maintain logarithmic growth. Cells were synchronized by serum starvation for at least 12 h before the treatment of nicotine for indicated periods or concentrations. To investigate the role of $\alpha 7$ nAChR in nicotine-mediated proliferation and anti-apoptotic effect, the cells were pretreated with 1 μ g/ml $\alpha 7$ nAChR specific antagonist α -bungarotoxin or broad spectrum nicotinic antagonist tubocurarine chloride prior to the indicated nicotine treatment.

Cell apoptosis assay. Cell apoptosis assay was determined by flow cytometry according to the method previously described (23). Briefly, 5x10⁴ Raw264.7 or E14 cells seeded in 24-well plates were pretreated with 10 μ M U0126, SB203580 or SP600125 1 h prior to 24-h 10⁻⁷ M nicotine stimulation and were further treated with 2 μ g/ml cisplatin for 17 h. Then, the cells were removed by trypsinization, rinsed with PBS and re-suspended in binding buffer containing Annexin V-FITC and propidium iodide (PI) for 20 min at room temperature. The samples were analyzed on FACSCalibur and data were analyzed with CellQuest software.

Cell proliferation assay. Cell proliferation assay was performed as previously described (24). Briefly, 5x10⁴ E14 or Raw264.7 cells were inoculated in 96-well plate in 100 μ l/well medium in a humidified incubator (37°C, 5% CO₂). Then, the cells were treated with 10 μ M LY294002/Wortmanin or 1 μ g/ml tubocurarine chloride/ α -bungarotoxin 60 min prior to 10⁻⁷ mol/l nicotine stimulation for indicated periods. CCK-8 solutions (10 μ l) were added to each well of the plate and OD450 value was detected with the wavelength of 450 nm.

Mitochondria isolation. To explore the effect of nicotine on cisplatin-induced mitochondrial translocation of Bax and the release of cytochrome *c*, 2x10⁷ Raw264.7 cells were treated with 10⁻⁷ mol/l nicotine for 8 h prior to cisplatin (1 μ g/ml) treatment. The mitochondria isolation was performed according to the standard procedure. Briefly, cell pellet was harvested by 850 x g centrifuging and vortexed at medium speed in 800 μ l Mitochondria Isolation Reagent A. Then, 10 μ l Mitochondria Isolation Reagent B was added and incubated on ice for 5 min with further lysis by 800 μ l Mitochondria Isolation Reagent C. After 15 min 12,000 x g centrifugation, the pellet contained isolated mitochondria and the supernatant contained cytosol fraction. The Bax translocation and cytochrome *c* release were determined by western blot analysis, respectively. β -actin and Cox IV were used as internal control.

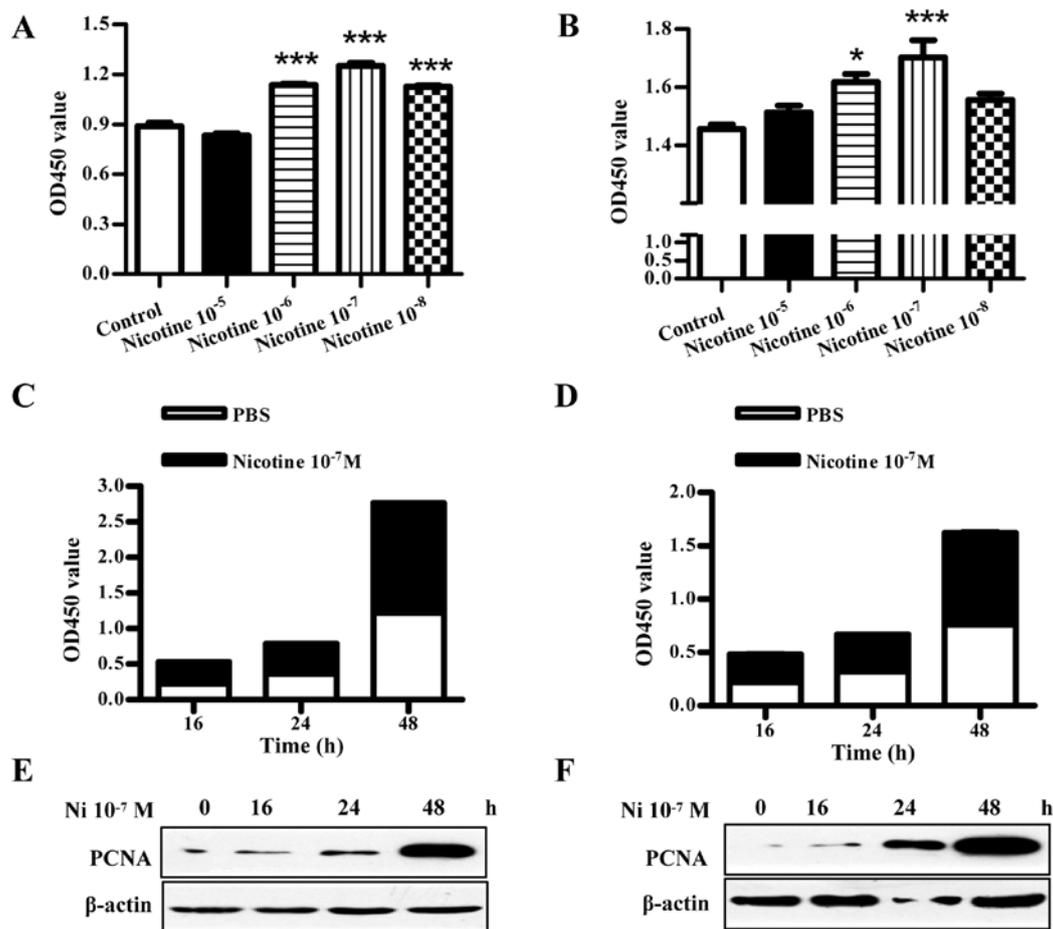


Figure 1. Nicotine treatment augments cell proliferation in Raw264.7 and E14 cells. (A, C and E) 3×10^4 Raw264.7 cells or (B, D and F) E14 cells were treated with the indicated concentrations of nicotine for 24 h (A and B) or 10^{-7} mol/l nicotine for the indicated periods (C-F). Then, the cell viabilities of Raw264.7 (A and C) and E14 (B and D) were determined by CCK-8 assay. Data are given as mean \pm SEM, $n=4$. *** $P < 0.001$, * $P < 0.05$, one way ANOVA with post Newman-Keuls test. Meanwhile, the whole cellular protein of (E) Raw264.7 or (F) E14 stimulated with 10^{-7} mol/l nicotine for 24 h was extracted and the effects of nicotine on PCNA expression were determined by western blot analysis. β -actin was used as internal control. A representative of three independent experiments is shown ($n=3$). Ni, nicotine.

Western blot analysis. Proteins were obtained in lysis buffer as previously described (7). For analysis of PI3K-Akt, MAPK kinases phosphorylation and upregulation of Bcl-2, Mcl-1, PCNA, $\alpha 7$ nAChR induced by nicotine stimulation, 2×10^7 Raw264.7 or E14 cells were treated with 10^{-7} mol/l nicotine stimulation for indicated periods. To explore the role of $\alpha 7$ nAChR in nicotine-augmented cell proliferation and anti-apoptotic effect, cells were treated with 1 μ g/ml tubocurarine chloride or α -bungarotoxin 60 min prior to 24 h 10^{-7} mol/l nicotine stimulation. Proteins were loaded onto SDS-PAGE gels for electrophoresis and then transferred onto PVDF membranes. After blocking in 5% fat-free milk in TBST for 1.5 h, the membranes were incubated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with corresponding HRP-conjugated secondary antibodies at room temperature for 1.5 h. After washing six times with TBST (for 10 min each), bound antibodies were visualized using enhanced chemiluminescence ECL. β -actin was used as loading control.

Statistical analysis. Each experiment was repeated at least 3 times and confirmed that similar data were obtained. All data are expressed as mean and standard errors. Statistical

significance was tested using one-way ANOVA with post Newman-Keuls test or two-way ANOVA with post Bonferroni test. Statistical differences were considered to be significant if $P < 0.05$.

Results

Nicotine treatment augments cell proliferation abilities in Raw264.7 and E14 cells. Our previous studies showed that nicotine could activate bone marrow-derived dendritic cells and nicotine-treated dendritic cells have potential antitumor effects (7-9). To explore the effect of nicotine on cell proliferation, both Raw264.7 and E14 cells were treated with 10^{-5} - 10^{-8} mol/l nicotine for 12 h or 10^{-7} mol/l nicotine for indicated periods and cell viabilities, PCNA expression were determined by CCK-8 assay and western blot analysis, respectively. The results showed that compared to PBS-treated control, 10^{-6} - 10^{-8} mol/l nicotine treatment clearly augmented Raw264.7 cell viability (** $P < 0.001$, one-way ANOVA with post Newman-Keuls test) (Fig. 1A). Meanwhile, 16, 24 and 48 h 10^{-7} mol/l nicotine treatment augmented Raw264.7 cell proliferation to 137.5, 118.7 and 126.7% respectively (Fig. 1C). PCNA protein determination also showed that nicotine treatment upregulated

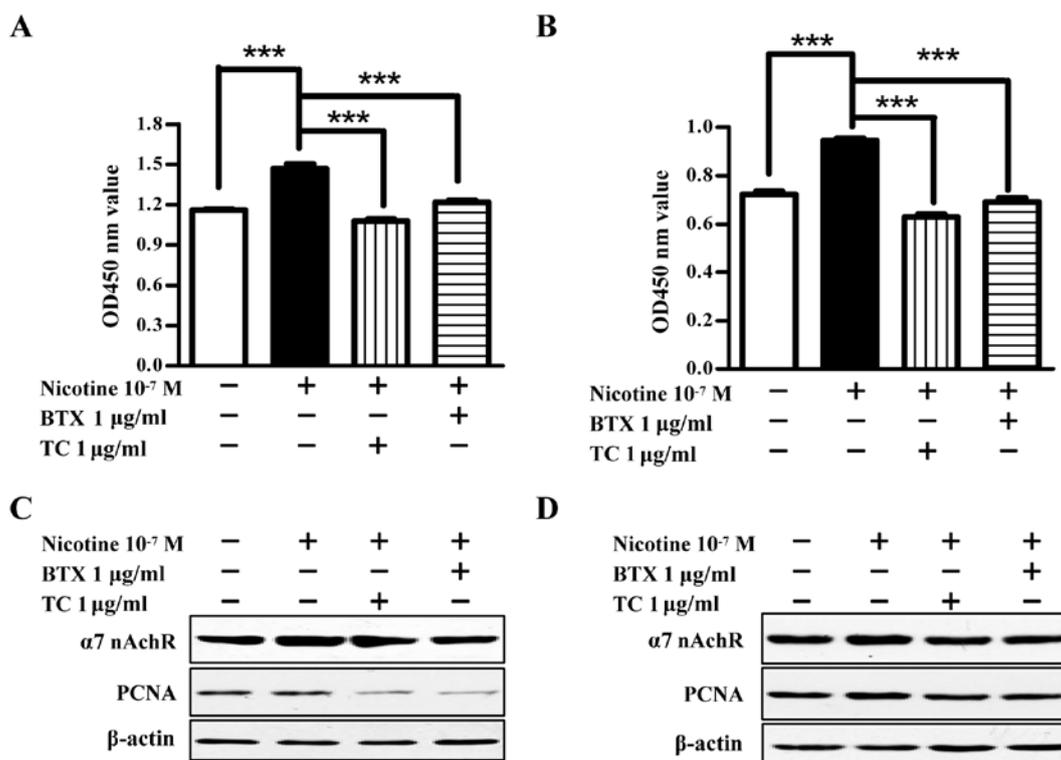


Figure 2. $\alpha 7$ nAChR is involved in nicotine-augmented cell proliferation in Raw264.7 and E14 cells. To explore the role of $\alpha 7$ nAChR in nicotine-augmented cell proliferation, Raw264.7 and E14 cells were pretreated with 1 μ g/ml tubocurarine chloride or α -bungarotoxin prior to 10⁻⁷ mol/l nicotine stimulation. Cell viabilities of (A) Raw264.7 and (B) E14 cells were determined by CCK-8 assay. Data are given as mean \pm SEM, n=4. ***P<0.001, one way ANOVA with post Newman-Keuls test. PCNA and $\alpha 7$ nAChR expression of (C) Raw264.7 and (D) E14 cells was determined by western blot analysis. β -actin was used as internal control. A representative of three independent experiments is shown (n=3). BTX, α -bungarotoxin; TC, tubocurarine chloride.

PCNA expression in Raw264.7 cells (Fig. 1E). Cell viability and PCNA expression investigations in E14 cells presented similar results (Fig. 1B, D and F). These results illustrate that nicotine augments cell proliferation in both a concentration and period manner.

$\alpha 7$ nAChR is involved in nicotine-augmented cell proliferation in both Raw264.7 and E14 cells. $\alpha 7$ nAChR, expressed in non-neuronal cells such as monocytes and endothelial cells, is one of the most abundant nicotinic acetylcholine receptors (11). To investigate the role of $\alpha 7$ nAChR in nicotine-increased cell proliferation, Raw264.7 and E14 cells were pretreated with $\alpha 7$ nAChR specific antagonist α -bungarotoxin or non-specific antagonist tubocurarine chloride prior to nicotine treatment. Then, cell viability, $\alpha 7$ nAChR and PCNA expression were determined by CCK-8 assay and western blot analysis, respectively. The results showed that compared with vehicle-treated cells, nicotine treatment significantly enhanced cell viability in both Raw264.7 and E14 cells (Fig. 2A and B). The pretreatment of α -bungarotoxin or tubocurarine chloride clearly abolished the effect of nicotine on cell viability. For example, the pretreatment of α -bungarotoxin or tubocurarine chloride achieved ~83 and 73% inhibitory rate, respectively, in Raw264.7 cells (***P<0.001, one-way ANOVA with post Newman-Keuls test). As nicotine significantly upregulated $\alpha 7$ nAChR and PCNA expression in both Raw264.7 and E14 cells, the preincubation of α -bungarotoxin or tubocurarine chloride abolished the effect of nicotine on $\alpha 7$ nAChR and PCNA expression (Fig. 2C and D). These data indicate that

the upregulation of $\alpha 7$ nAChR induced by nicotine treatment contributes to nicotine-enhanced cell proliferation abilities.

Nicotine treatment upregulates anti-apoptotic protein Mcl-1 and Bcl-2 expression in Raw264.7 and E14 cells. To explore the effect of nicotine on anti-apoptotic protein Mcl-1 and Bcl-2 expression, Raw264.7 and E14 cells were treated with 10⁻⁷M nicotine for indicated periods, the expression of Mcl-1 and Bcl-2 was determined by western blot analysis. The results showed that the expression of Mcl-1 and Bcl-2 was continuously augmented by nicotine treatment in both Raw264.7 (Fig. 3A) and E14 (Fig. 3B) cells, indicating that nicotine treatment might achieve anti-apoptotic effects on these cells.

$\alpha 7$ nAChR is involved in nicotine-inhibited cisplatin-induced caspase-3 activation in both Raw264.7 and E14 cells. To investigate the role of $\alpha 7$ nAChR in nicotine-mediated anti-apoptotic effects, Raw264.7 and E14 cells were treated with α -bungarotoxin or tubocurarine chloride prior to nicotine stimulation, the cleaved caspase-3 was determined by western blot analysis. The results showed that compared with cisplatin-treated cells, nicotine treatment significantly inhibited cisplatin-induced cleaved caspase-3 in both Raw264.7 (Fig. 4A) and E14 (Fig. 4B) cells. Notably, the effect of nicotine on cisplatin-induced caspase-3 activation was efficiently abolished by the pretreatment of α -bungarotoxin or tubocurarine chloride (Fig. 4), indicating that the upregulation of $\alpha 7$ nAChR induced by nicotine contributed to nicotine-mediated anti-apoptotic effects.

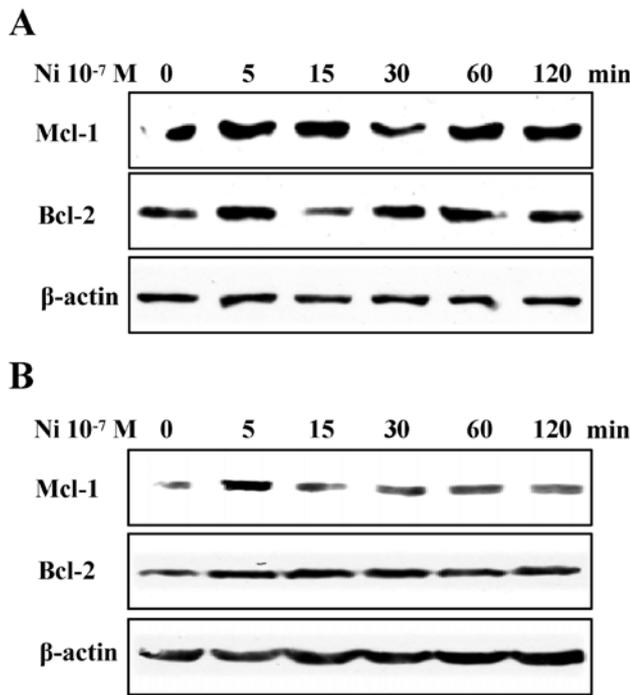


Figure 3. Nicotine treatment upregulates Mcl-1 and Bcl-2 expression in Raw264.7 and E14 cells. To explore the effects of nicotine on anti-apoptotic protein Mcl-1 and Bcl-2 expression, 2x10⁷ (A) Raw264.7 and (B) E14 cells treated with 10⁻⁷ mol/l nicotine for the indicated periods and whole cellular protein was extracted. The effect of nicotine on Mcl-1 or Bcl-2 expression in (A) Raw264.7 and (B) E14 cells was determined by western blot analysis. β-actin was used as internal control. A representative of three independent experiments is shown (n=3). Ni, nicotine.

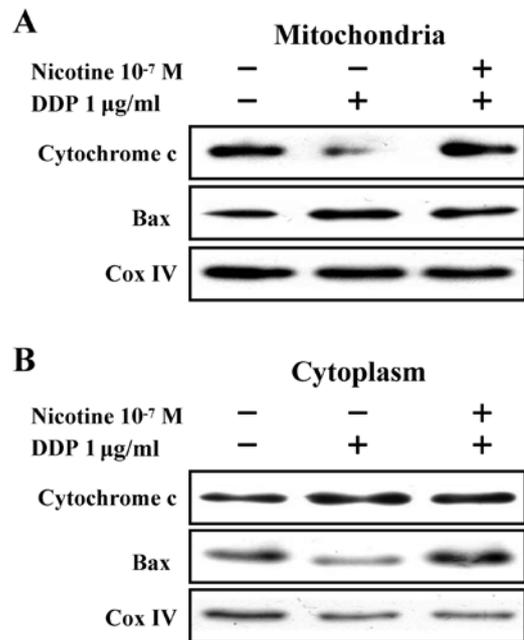


Figure 5. Nicotine attenuates cisplatin-induced mitochondrial translocation of Bax and cytochrome c release in Raw264.7 cells. To investigate the effect of nicotine on cisplatin-induced mitochondrial translocation and cytochrome c release, 2x10⁷ Raw264.7 cells were treated with 10⁻⁷ mol/l nicotine prior to cisplatin (1 μg/ml) treatment. Then, (A) mitochondria and (B) cytosol fraction were extracted and the levels of Bax and cytochrome c in both mitochondria and cytoplasm were determined by western blot analysis, respectively. A representative of three independent experiments is shown (n=3). β-actin and Cox IV were used as cytoplasm and mitochondria internal control respectively. DDP, cisplatin.

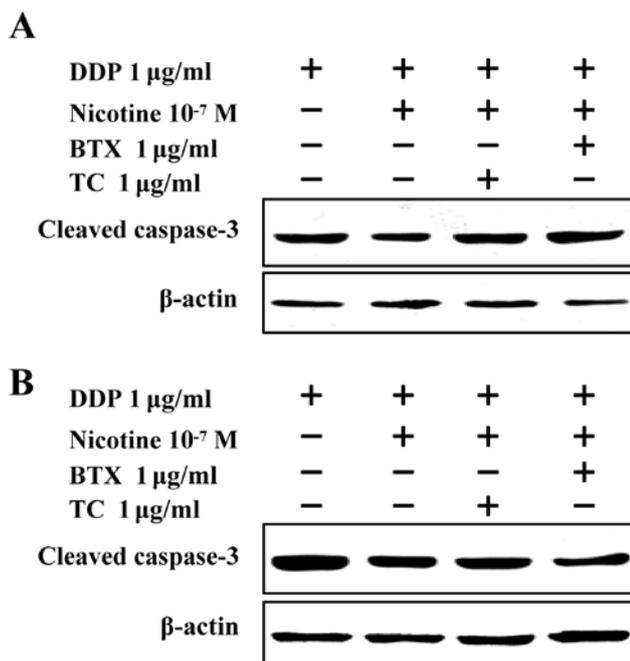


Figure 4. α7 nAChR is involved in nicotine-decreased cisplatin-induced caspase-3 activation in Raw264.7 and E14 cells. To explore the role of α7 nAChR in nicotine-mediated anti-apoptotic effect, 2x10⁷ Raw264.7 or E14 cells were pretreated with tubocurarine chloride or α-bungarotoxin prior to 10⁻⁷ mol/l nicotine stimulation which was further treated with 1 μg/ml cisplatin. The cleaved caspase-3 of (A) Raw264.7 and (B) E14 cells was assessed by western blot analysis. A representative of three independent experiments is shown (n=3). β-actin was used as internal control. BTX, α-bungarotoxin; TC, tubocurarine chloride; DDP, cisplatin.

The mitochondrial translocation of Bax is involved in nicotine-inhibited cisplatin-induced cytochrome c release in Raw264.7 cells. To investigate the role of Bax translocation in nicotine-mediated anti-apoptotic effects, Raw264.7 cells were stimulated with nicotine prior to cisplatin treatment and mitochondria/cytosol fraction was extracted. The mitochondrial translocation of Bax and the release of cytochrome c were determined by western blot analysis. The results showed that the decreased cytochrome c level and increased Bax existence in mitochondria was derived from cisplatin treatment (Fig. 5A). On the other hand, cisplatin stimulation was found to augment cytochrome c level and decrease Bax expression in cytoplasm (Fig. 5B), indicating that mitochondrial translocation of Bax was involved in cisplatin-induced cytochrome c release in Raw264.7 cells. The pretreatment of nicotine abolished cisplatin's effects on Bax translocation and cytochrome c release (Fig. 5). Therefore, nicotine achieved anti-apoptotic effects by attenuating Bax translocation from cytoplasm to mitochondria.

Nicotine activates MAPK and PI3K-Akt pathways in both Raw264.7 and E14 cells. Erk1/2-p38-JNK and PI3K-Akt pathways are reported to be involved in regulating anti-apoptosis protein expression (25-27) and promoting lung and colon cancer cell proliferation (27,28), respectively. To investigate the roles of Erk1/2-p38-JNK and PI3K-Akt pathways in nicotine-mediated anti-apoptotic effects and promoting cell proliferation, Raw264.7 and E14 cells were treated with nicotine and the effects of nicotine on Erk1/2-p38-JNK and

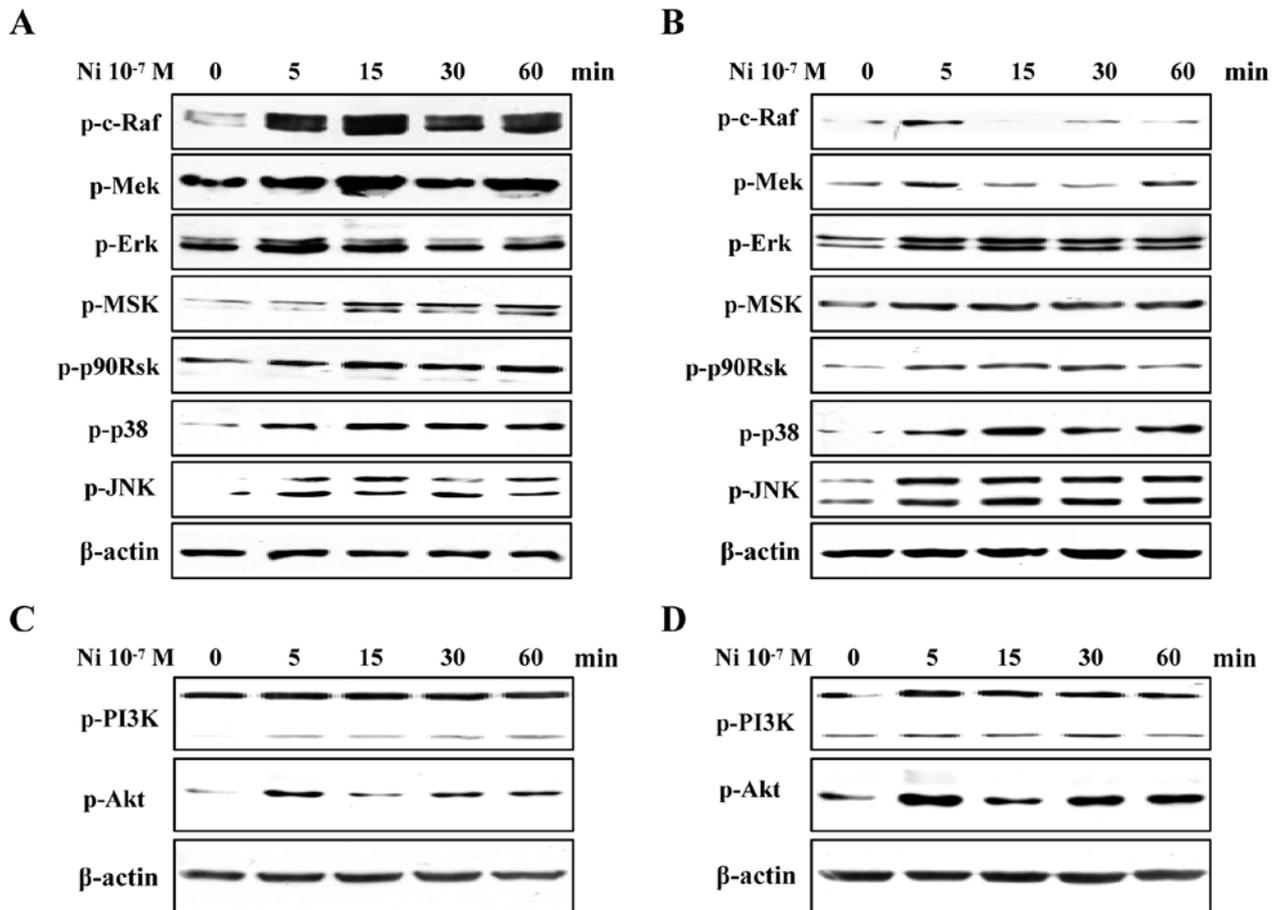


Figure 6. Nicotine efficiently activates Erk1/2-p38-JNK and PI3K-Akt pathways in both Raw264.7 and E14 cells. To determine effect of nicotine on MAPK and PI3K-Akt pathway activation, 2×10^7 Raw264.7 (A and C) or E14 (B and D) cells were treated with 10^{-7} mol/l nicotine for the indicated periods and the phosphorylation of Erk1/2, MSK, p90rsk, Mek, c-raf, p38, SAPK/JNK (A and B) and PI3K, Akt (C and D) were determined by western blot analysis. β -actin was used as internal control. A representative of three independent experiments is shown ($n=3$). Ni, nicotine.

PI3K-Akt pathway activation was determined by western blot analysis. The results showed that Erk1/2-p38-JNK kinases and other components of these pathways, such as c-Raf, Mek, MSK and p90Rsk, were clearly activated by nicotine (10^{-7} M) treatment in both Raw264.7 (Fig. 6A) and E14 (Fig. 6B) cells. Meanwhile, the augmented phosphorylation levels of PI3K p55 (Tyr199), PI3K p85 (Tyr458) and Akt were visible within 5 min and continued to 60 min in both Raw264.7 (Fig. 6C) and E14 cells (Fig. 6D). These results indicate that nicotine treatment activated the MAPK and PI3K-Akt pathways.

The PI3K-Akt pathway is involved in nicotine-augmented cell proliferation. To investigate the role of the PI3K-Akt pathway in nicotine-augmented cell proliferation, Raw264.7 and E14 cells were pretreated with PI3K-Akt kinase inhibitor prior to nicotine stimulation and cell viabilities were determined by CCK-8 assay. The results showed that while nicotine obviously augmented both Raw264.7 and E14 cell proliferation, the inhibition of PI3K kinase activities which was derived from LY294002 pretreatment achieved 36.6 and 44.9% inhibitory rates in Raw264.7 (Fig. 7A) and E14 (Fig. 7B) cells, respectively. Meanwhile, the usage of Wortmanin which is an inhibitor of Akt kinase also abolished the effects of nicotine on cell proliferation, which revealed 42.1 and 79.5% inhibitory

rates in Raw264.7 (Fig. 7A) and E14 (Fig. 7B) cells, respectively ($^{***}P < 0.001$, one-way ANOVA with post Newman-Keuls test) (Fig. 7). Hence, nicotine-induced PI3K-Akt activation is involved in the nicotine effects on cell proliferation.

The Erk-JNK pathway is involved in nicotine-mediated anti-apoptotic effects. To explore the role of Erk-p38-JNK MAPK pathway in nicotine-augmented anti-apoptotic effects, Raw264.7 and E14 cells were pretreated with kinase inhibitors prior to nicotine stimulation and cell apoptosis was determined by flow cytometry. The results showed that while nicotine pretreatment decreased cisplatin-induced apoptosis from 51.2 to 33.8% and 24.5 to 12.6% in Raw264.7 (Fig. 8A) and E14 (Fig. 8B) cells, respectively, the usages of Erk kinase U0126 and JNK kinase SP600125 clearly abolished the effects of nicotine on cell anti-apoptosis in both Raw264.7 (Fig. 8A) and E14 (Fig. 8B) cells, respectively ($^{***}P < 0.001$, one-way ANOVA with post Newman-Keuls test) (Fig. 8). The inhibition of p38 kinase activities by the usage of SB203580 did not reverse nicotine's effects on cell apoptosis, indicating that p38 phosphorylation is not associated with nicotine's anti-apoptotic function. These results indicate that the Erk-JNK MAPK pathway is involved in nicotine-augmented anti-apoptotic effects.

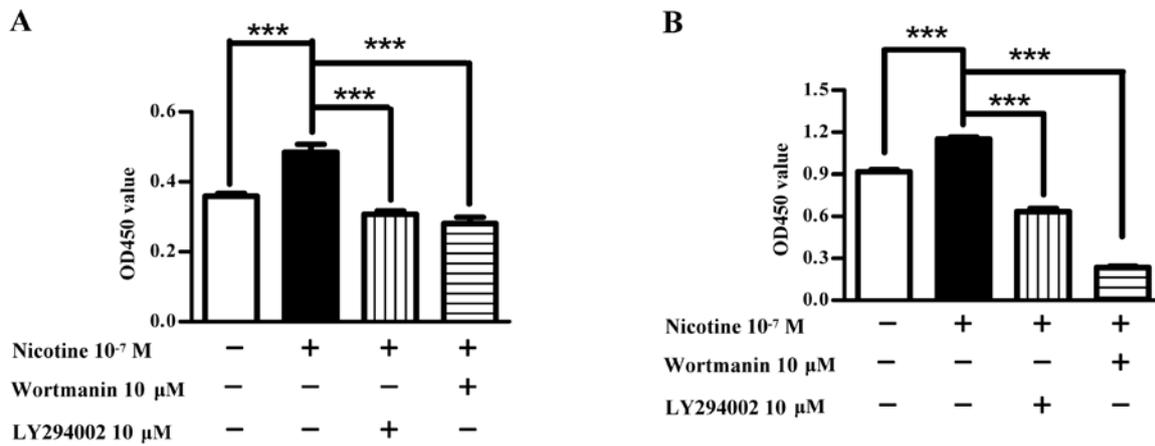


Figure 7. PI3K-Akt pathway activation is involved in nicotine-augmented cell proliferation in Raw264.7 and E14 cells. To explore the role of PI3K-Akt pathway in nicotine-augmented cell proliferation, 5×10^4 Raw264.7 or E14 cells were pretreated with 10 μ M LY294002 or Wortmanin prior to 10^{-7} mol/l nicotine stimulation. Then, cell viabilities of (A) Raw264.7 or (B) E14 cells were determined by CCK-8 assay. Data are given as mean \pm SEM, n=4. ***P<0.001, one way ANOVA with post Newman-Keuls test. A representative of three independent experiments is shown (n=3).

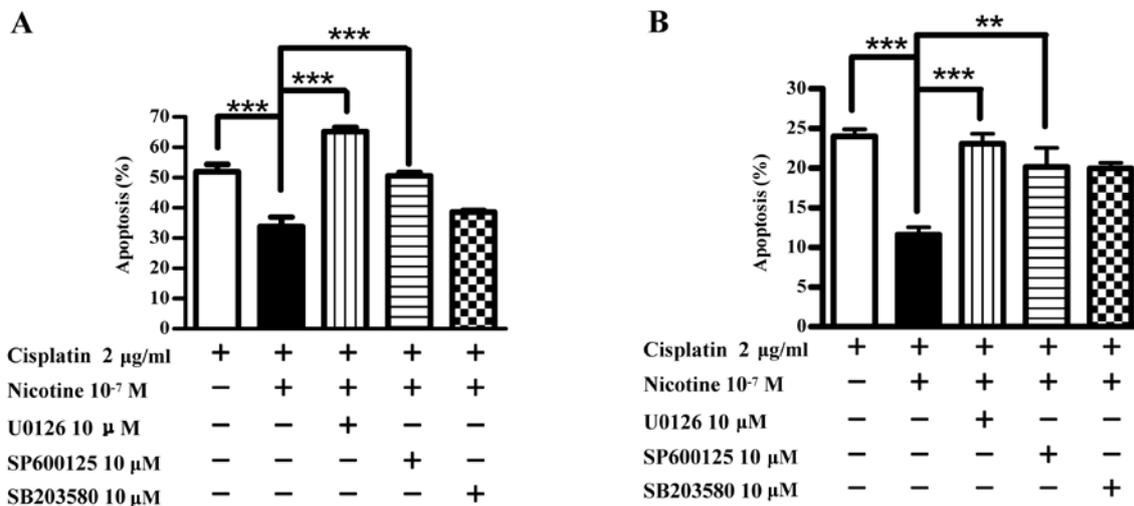


Figure 8. The phosphorylation of Erk1/2-JNK is responsible for nicotine-mediated anti-apoptotic effects in Raw264.7 and E14 cells. To explore the role of Erk1/2, JNK and p38 in nicotine-mediated anti-apoptotic effects, 5×10^4 Raw264.7 (A) or E14 (B) cells were pre-incubated with 10 μ M U0126, SB203580 or SP600125 prior to 10^{-7} mol/l nicotine stimulation which were further treated with 2 μ g/ml cisplatin. Then, cell apoptosis was assessed by flow cytometry with Annexin V/PI staining. Data are given as mean \pm SEM, n=4. ***P<0.001; **P<0.01, one way ANOVA with post Newman-Keuls test. A representative of three independent experiments is shown (n=3).

Discussion

In recent years, our studies have focused on the effect of neurotransmitter on immune cells and we have found that nicotine, an acetylcholine agonist, could activate bone marrow-derived dendritic cells and have potential antitumor effects (7-9). Further exploration showed that the biological effect of nicotine on lymphocyte is dependent on nicotine dose, duration of exposure and lipopolysaccharide existing in experiment system (23). However, other studies indicated that inhibition of $\alpha 7$ nACh mediated signaling could reduce tumorigenicity (18) and facilitate lung cancer treatment (19). Hence, the exact roles of $\alpha 7$ nACh in acetylcholine-mediated biological characterizations remain controversial and require further investigation. In the present study, Erk-JNK and PI3K-Akt signaling pathways

were found to be respectively responsible for $\alpha 7$ nACh-mediated cell proliferation and anti-apoptotic effects which were supported by the following data; firstly, nicotine-augmented cell viabilities, upregulated $\alpha 7$ nACh and PCNA expression were obviously abrogated by the pretreatment of $\alpha 7$ nAChR antagonist α -bungarotoxin and tubocurarine chloride; secondly, nicotine attenuated cisplatin-induced caspase-3 activation and cytochrome *c* release from mitochondria were reversed by the treatment of α -bungarotoxin or tubocurarine chloride. Further studies showed that nicotine could efficiently activate the Erk-JNK-p38 and PI3K-Akt signaling pathway. The inhibition of Erk-JNK MAPK activities significantly decreased nicotine-mediated anti-apoptotic effects. Of note, the downregulation of nicotine-augmented proliferation abilities was achieved by the usage of PI3K-Akt kinase inhibitor.

AChRs, which is an integral membrane protein that responds to the binding of acetylcholine, can be classified as nAChR and mAChR according to their relative affinities and sensitivities to nicotine or muscarine. As nAChRs are widely expressed by nervous system (10) and non-neuronal cells (11), $\alpha 1$, $\alpha 3$, $\alpha 5$ nAChRs has been found to regulate cell proliferation and apoptosis (12-14). In the present study, the pretreatment of $\alpha 7$ nAChR specific antagonist α -bungarotoxin or non-specific antagonist tubocurarine chloride not only abrogated nicotine-augmented $\alpha 7$ nAChR upregulation, cell proliferation (Fig. 2) but also reversed nicotine-mediated anti-apoptotic effects (Fig. 4), indicating that $\alpha 7$ nAChR might be mainly acetylcholine receptor in Raw264.7 and E14 cells. However, it should be noted that $\alpha 7$ nAChR, which was found to be involved in tumor migration (15,16) and PPAR β/δ expression (17), has also been documented to facilitate dendritic cell-mediated antitumor immune response (7-9). Hence, the exact roles of $\alpha 7$ nAChR and other AChRs in nicotine-mediated proliferation and anti-apoptotic effects require further investigation.

Bcl-2 and Mcl-1, which belong to the Bcl-2 family, are located in the outer mitochondrial membrane and protect cells against a variety of apoptotic stimuli (29). In the present study, both Bcl-2 and Mcl-1 expression was upregulated by nicotine treatment in both Raw264.7 and E14 cells (Fig. 3). Hence, nicotine clearly inhibited cisplatin-induced mitochondrial dependent caspase-3 activation (Fig. 4) and cytochrome *c* release (Fig. 5). The decreased Bax level in mitochondria and increased Bax level in cytoplasm induced by nicotine stimulation (Fig. 5) indicated that Bax translocation from cytoplasm to mitochondria might be a key step involved in nicotine-mediated anti-apoptotic effects. Upon apoptotic signalling, Bak/Bax translocated to the mitochondrial outer membrane, inserted its transmembrane domain, oligomerized and released cytochrome *c* (30). Cytochrome *c* then binds to and penetrates lipid structures which contain the inner mitochondrial membrane lipid cardiolipin, leading to protein conformational changes and increased peroxidase activity (31). Hence, the exact mechanisms of Bax in nicotine decreased cytochrome *c* release remain to be further examined.

Erk-JNK, one of the vital signal transduction pathways transmitting and converting stress signaling into apoptosis signaling (32), has been reported to upregulate Fas ligand expression (33) and is necessary for mitochondrial mediated apoptosis (34). Meanwhile, JNK activation playing an anti-apoptotic function was also documented (35). In the present study, the inhibition of Erk-JNK kinase abrogated nicotine-mediated anti-apoptotic effects in both Raw264.7 and E14 cells (Fig. 8), indicating that Erk-JNK phosphorylation facilitated cell survival in Raw264.7 and E14 cells. Although p38 could be efficiently activated (Fig. 6), the preincubation of SB203580 could not reverse nicotine-mediated anti-apoptotic effects, indicating that p38 phosphorylation is not associated with the anti-apoptotic function of nicotine.

Collectively, our study revealed that nicotine could achieve $\alpha 7$ nAChR-mediated proliferation effects by activating the PI3K-Akt pathway. Nicotine's anti-apoptotic effect might result from Bcl-2 family protein upregulation and Erk-JNK MAPK kinase phosphorylation, which provides potential molecules to deal with nicotine-associated human diseases.

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