Original Paper

Nicotine-Induced Airway Smooth Muscle
Cell Proliferation Involves TRPC6-
Dependent Calcium Influx Via $\alpha 7$ nAChR

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Key Words

chronic obstructive pulmonary disease • Store-operated calcium entry • Receptor-operated calcium entry • Canonical transient receptor potential protein 6 • $\alpha 7$ nAChR

Abstract

Background/Aims: The proliferation of human bronchial smooth muscle cells (HBSMCs) is a key pathophysiological component of airway remodeling in chronic obstructive pulmonary disease (COPD) for which pharmacotherapy is limited, and only slight improvements in survival have been achieved in recent decades. Cigarette smoke is a well-recognized risk factor for COPD; however, the pathogenesis of cigarette smoke-induced COPD remains incompletely understood. This study aimed to investigate the mechanisms by which nicotine affects HBSMC proliferation. **Methods:** Cell viability was assessed with a CCK-8 assay. Proliferation was measured by cell counting and EdU immunostaining. Fluorescence calcium imaging was performed to measure intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). **Results:** The results showed that nicotine promotes HBSMC proliferation, which is accompanied by elevated store-operated calcium entry (SOCE), receptor-operated calcium entry (ROCE) and basal $[\text{Ca}^{2+}]_i$ in HBSMCs. Moreover, we also confirmed that canonical transient receptor potential protein 6 (TRPC6) and $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) are involved in nicotine-induced upregulation of cell proliferation. Furthermore, we verified that activation of the PI3K/Akt signaling pathway plays a pivotal role in nicotine-enhanced proliferation and calcium influx in HBSMCs. Inhibition of $\alpha 7$ nAChR significantly decreased Akt phosphorylation levels, and LY294002 inhibited the protein expression levels of TRPC6. **Conclusion:** Herein, these data provide compelling evidence that calcium entry via the $\alpha 7$ nAChR-PI3K/Akt-TRPC6 signaling pathway plays an important role in the physiological regulation of airway smooth muscle cell proliferation, representing an important target for augmenting airway remodeling.

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Introduction

Airway remodeling has been described in a number of chronic airway diseases, including chronic obstructive pulmonary disease (COPD) and asthma, and it is characterized by the hypertrophy and/or hyperplasia of smooth muscle cells [1]. Recent studies have demonstrated a clear association between cigarette smoke and COPD [2-4]. Cigarette smoke has been reported to induce features of airway remodeling in COPD animal models, including airway wall thickening, increased airway smooth muscle (ASM) mass, goblet cell hyperplasia and collagen deposition [5]. The effects of nicotine, the major active and addictive component of tobacco [6], have been described in certain cell types, including bronchial smooth muscle cells [7]. We previously demonstrated that nicotine regulates cellular proliferation in rat airway smooth muscle cells (RASMCs) by activating the Akt signaling pathway [8]. However, the precise mechanism by which nicotine induces damage to airways remains largely unknown.

Previously, Feng's group developed a nicotine-dependent *C. elegans* model and determined that nicotinic acetylcholine receptors (nAChRs) were required for the behavioral responses to nicotine, suggesting the functional conservation of nAChRs in response to nicotine. The authors also observed that mutant worms lacking canonical transient receptor potential proteins (TRPCs) showed defective responses to nicotine that could be rescued by human TRPCs. These findings revealed an important role of TRPCs in regulating nicotine-dependent behavior [9]. However, literature regarding the link between TRPCs and nAChRs is limited.

TRPCs are encoded by genes that are most closely related to the *trp* gene, which was originally identified in *Drosophila*. Currently, the TRPC family is known to consist of seven members, designated TRPC1-7 [10], which are highly permeable to calcium. TRPCs have recently emerged as important players in controlling the function of smooth muscle cells [11, 12]. Recent studies have shown that TRPC genes, which encode store-operated Ca^{2+} channels (SOCC) and receptor-operated Ca^{2+} channels (ROCC), play important roles in the regulation of intracellular calcium homeostasis and cell proliferation [13-15]. Evidence suggests that SOCC mediates store-operated Ca^{2+} entry (SOCE) and ROCC mediates receptor-operated Ca^{2+} entry (ROCE). However, the influence of nicotine on TRPCs in HBSMCs has not been determined.

We previously showed that nicotine elevates intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in ASMCs through $\alpha 7$ nAChR-mediated signals pathways and highlighted the possibility that $\alpha 7$ nAChR could be considered a potential target for the treatment of airway remodeling [16]. However, information concerning the precise mechanism is lacking. nAChRs are non-specific cation channels that are widely expressed in the nervous system (neuronal nAChRs) and in skeletal muscle (muscle nAChRs). Among nAChRs, $\alpha 7$ nAChR are formed by the homomeric assembly of five subunits and are generally considered to be preferentially permeable to calcium [17, 18]. However, the expression and function of $\alpha 7$ nAChR in HBSMCs remain unclear. Given available information, targeting $\alpha 7$ nAChR has been considered a viable strategy for the treatment of a variety of diseases that involve the use of nicotine [19-21].

Dysfunction of the PI3K/Akt signaling pathway has been implicated in many human diseases, including COPD [22, 23]. In addition, various calcium-dependent signaling cascades are directly or indirectly influenced by the PI3K/Akt signaling pathway [22] via kinases and phosphatases. We previously showed that nicotine can induce activation of the Akt signaling pathway in RASMCs [8].

Therefore, the present study was designed to explore the function of nicotine in human bronchial smooth muscle cells (HBSMCs). We hypothesized that nicotine has a stimulatory effect on HBSMCs, promoting cell proliferation through an $\alpha 7$ nAChR-mediated mechanism that is dependent on intracellular calcium homeostasis and involves the PI3K/Akt signaling pathway.

Materials and Methods

Materials

Five HBSMC lines were obtained from ScienCell (USA, Cat. No. 3400), an EdU cell proliferation assay kit was obtained from RiboBio (Guangzhou, China), and 10× Cell Lysis Buffer was obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-TRPC1, 3, 4, 6 were obtained from Alomone Labs (Jerusalem, Israel). Anti- $\alpha 7$ nAChR were obtained from Santa Cruz (Cambridge, MA, USA). Fura 2-AM was obtained from Invitrogen (Carlsbad, CA, USA). An $\alpha 7$ nAChR-selective antagonist (methyllycaconitine, MLA), an $\alpha 7$ nAChR-specific antagonist (MG624) and a phosphoinositide 3-kinase (PI3K) inhibitor (LY294002) were obtained from Sigma (St. Louis, MO, USA).

Cell culture

Primary HBSMCs and smooth muscle cell medium (SMGM) were purchased from ScienCell (USA). HBSMCs were cultured in SMGM containing 2% fetal bovine serum (FBS) and 1% smooth muscle cell growth supplement. The medium was replaced every two days. Cells were incubated in a humidified atmosphere containing 5% CO_2 and 95% O_2 at 37°C. Cells at passages 3 and 8 were used for experiments.

Cell viability assay

To evaluate the cytotoxicity of nicotine on HBSMCs, a Cell Counting kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) was performed. In brief, HBSMCs were seeded at a density of 2×10^3 cells/well in 96-well plates and were allowed to attach overnight. Cells were treated with nicotine at different concentrations (0.01 μM , 0.1 μM , 1 μM , 10 μM , 100 μM and 1000 μM) and continuously cultured for 0, 24, 48 and 72 h. Six repeats were prepared for each group. CCK-8 solution (10 μL) was added to each well for 3 h, and then the absorbance at 450 nm was measured using a microplate spectrophotometer (Thermo Scientific, Rockford, IL, USA). The cytotoxic effect was indicated as a percentage of surviving cells (ratio of surviving cells after treatment to surviving cells without treatment) using the following formula: Cell viability = $[\text{A450 (treated group)} - \text{A450 (blank group)}] / [\text{A450 (negative group)} - \text{A450 (blank group)}] \times 100\%$.

Cell proliferation assays

Cell proliferation was analyzed by cell counting and EdU immunostaining. For cell counting, 35000 HBSMCs were plated in 6-well plates with 1 ml SMGM. After 24 h, the cells were washed and transferred to 0.2% FBS-containing DMEM-F12 for 24 h. After an additional 24 h, the cells were treated with 10 μM nicotine for 48 h. In each experiment, at least three wells were used for each condition. At the end of each experimental period, the cells were harvested with trypsin-EDTA and counted using a Zeiss Coulter Counter (Beckman Coulter, Miami, FL, USA). A Cell-Light™ EdU staining kit was used for the vitro labeling of the nuclei of dividing cells. HBSMCs were seeded in triplicate into 96-well plates at a density of 1×10^4 cells per well and incubated in SMGM overnight. Then, the cells were treated with 10^{-5} M nicotine. All EdU-incorporation experiments were performed according to the manufacturer's protocol [24, 25]. One hundred microliters of 50 μM EdU were added to each well for 2 h. The cells were fixed by using 4% paraformaldehyde and incubated with 2 mg/ml aminoacetic acid for 5 min with oscillation. The cells were incubated with 100 μL of penetrant per well for 10 min under oscillation followed by 100 μL of 1× EdU solution for 30 min. Hoechst 33342 was used to stain the cell nuclei. Images were acquired under a fluorescence microscope. For each EdU experiment, three random fields were imaged at 20× objective magnification, and the fraction of EdU-positive cells was expressed as the percentage of the total number of cells in each field.

RT-PCR and real-time PCR

Total RNA in HBSMCs was extracted using TRIzol (Invitrogen, Barcelona, Spain) following the manufacturer's instructions. Reverse transcription and PCR were performed using a Super Script cDNA synthesis kit (Takara Biotechnology, China) and KOD FX (ToYoBo, Japan), respectively. Real-time quantitative PCR was conducted by using Sso Fast™ Eva Green SuperMix (Bio-Rad, Hercules, CA, USA) with a Stratagene MX3000P real-time PCR detection system. Detection threshold cycle (CT) values were obtained using MX3000P software. The relative concentrations of each transcript were calculated by using the $\Delta\Delta\text{Ct}$ method. The mRNA copies were normalized and expressed as the percent change from control values. The

primer sequences were synthesized by TAKARA Biotechnology, and the details of the sequences are listed in Table 1.

Western blot analysis

HBSMCs were harvested in 10× Cell Lysis Buffer with PMSF, and the total protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). The proteins (30–50 µg) were separated by gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (pore size 0.45 µm, Bio-Rad). The membranes were blocked with 5% milk in TBS-blocking buffer and incubated overnight at 4°C with primary antibodies. The blots were washed and incubated with HRP-conjugated secondary antibody at RT for 1 h. Signals were then detected using an enhanced ECL chemiluminescence system (Bio-Rad, Hercules, CA, USA) and quantified by densitometry with image analysis software (ImageJ).

Measurement of intracellular Ca^{2+}

The $[\text{Ca}^{2+}]_i$ was measured in HBSMCs by using fura-2 dye, which is a Ca^{2+} -sensitive fluorescent dye. The cells were incubated with 5 µM fura-2 (Molecular Probes) for 40 min at 37°C, washed three times, and then maintained at room temperature for an additional 30 min to allow for complete de-esterification of the intracellular AM esters. Coverslips with HBSMCs were then mounted onto a closed polycarbonate chamber clamped in a heated aluminum platform (PH-2; Warner Instrument, Hamden, CT, USA) on the stage of a Leica inverted microscope (Melville, NY, USA). The chamber was perfused at 0.5 ml/min with Krebs-Ringer bicarbonate solution (KRBS) containing (in mM) 118 NaCl, 4.7 KCl, 0.57 MgSO_4 , 2.5 CaCl_2 , 1.18 KH_2PO_4 , 25 NaHCO_3 and 10 glucose. The KRBS was equilibrated with 16% O_2 –5% CO_2 at 37°C in heated reservoirs and added to the chamber through stainless steel tubing. The chamber temperature was maintained at 37°C with an in-line heat exchanger and a dual channel heater controller (SF-28 and TC-344B, Warner Instruments). Fluorescent emissions were acquired at 510 nm from regions of interest corresponding to a single cell after excitation at 340 and 380 nm (shift in excitation wavelength upon binding to Ca^{2+}) in 15–30 cells using a xenon arc lamp. The 340/380 nm emission fluorescence ratio, which is known to be proportional to $[\text{Ca}^{2+}]_i$, is shown for most of the data.

We first measured $[\text{Ca}^{2+}]_i$ at 12-s intervals before and after the restoration of extracellular $[\text{Ca}^{2+}]_o$ to 2.5 mM. SOCE was evaluated based on the increase in $[\text{Ca}^{2+}]_i$ caused by the restoration of extracellular $[\text{Ca}^{2+}]_o$ in the continuous presence of nifedipine and cyclopiazonic acid (CPA). ROCE was evaluated based on the increase in $[\text{Ca}^{2+}]_i$ caused by the restoration of extracellular $[\text{Ca}^{2+}]_o$ in the continuous presence of nifedipine and 1-oleoyl-2-acetyl-sn-glycerol (OAG).

SOCE and ROCE were further quantified by quenching fura-2 fluorescence with MnCl_2 (200 µmol/L). Fura-2 was excited at 360 nm in 30-s intervals. After a stable baseline of fluorescence was attained, Mn^{2+} was applied to determine the maximum rate of the quenching of fura-2 fluorescence.

siRNA knockdown

HBSMCs were seeded and grown to 60% confluence in six-well plates. A specific siRNA against TRPC6 (siTRPC6) and non-targeted siRNA (siNT) were synthesized (Life Technologies, Gaithersburg, MD, USA) and used to transfect HBSMCs with serum-free Opti-MEM at a final concentration of 50 nM, according to the manufacturer's instructions. siRNA transfection was monitored under a fluorescence microscope after 24 h, and the efficiency of the siRNA-mediated gene silencing was evaluated by western blot analysis. The medium was removed to measure TRPC6 protein expression at 48 h after transfection. All the cell groups in the experiments consisted of three replicates.

Statistical analyses

The data were analyzed using SPSS16.0 statistical software. The data are presented as the mean ± SD. The results are representative of at least n = 3 unless otherwise indicated. Differences between groups were

Table 1. Primer sequences used for qRT-PCR

Gene	Primer	Products (bp)
TRPC1	5'-agcctcttgacaaacgggga-3' 5'-accgacatctgtccaaacc-3'	146
TRPC3	5'-gcgcactgccagaaatacga-3' 5'-agtggctgaaggagtcgtg-3'	130
TRPC4	5'-gaggaacctgggtgaagcgat-3' 5'-caggacttcaaagcggaaactag-3'	124
TRPC6	5'-gacagcagacaatggcggc-3' 5'-tgctgatggagcaaacagat-3'	96
GAPDH	5'-gcaccgtcaaggctgagaac-3' 5'-tggtgaagacgcagctgga-3'	138

compared by one-way ANOVA and Bonferroni's post hoc test. P values less than 0.05 were considered to be significant.

Results

Nicotine-induced enhancement of HBSMC proliferation depends on $\alpha 7$ nAChR

We first assayed the cytotoxicity of nicotine on HBSMCs by CCK-8 assays. The viability of the HBSMCs did not change when the cells were treated with nicotine below $0.1 \mu\text{M}$ at different time points. However, the cell viability of HBSMCs was significantly increased by nicotine at the concentration of 1 – $10 \mu\text{M}$. Moreover, the cell viability of HBSMCs treated with $10 \mu\text{M}$ nicotine reached a peak at 48 h. However, a time- and dose-dependent decrease in cell viability was observed at higher doses of nicotine (more than $10 \mu\text{M}$) (Fig. 1A). This result

Fig. 1. Nicotine-induced enhancement of HBSMCs proliferation depends on $\alpha 7$ nAChR. (A) The effects of nicotine treatment on the cell viability of HBSMCs. (B) The effects of nicotine treatment on the cell number of HBSMCs. (C) Nicotine ($10 \mu\text{M}$ for 48 h) promoted HBSMCs proliferation. Nicotine promoted DNA replication in the S-phase (left). Proliferation was determined by EdU incorporation (red), with EdU-positive cells expressed as the percentage of the number of total cells in each field. Total cell numbers were determined by Hoechst 33342 staining of the nuclei (blue). Average proliferation rates of HBSMCs in control and nicotine treatment group (right). (D) nAChR antagonists, MLA ($100 \mu\text{M}$) and MG624 ($1 \mu\text{M}$), attenuated nicotine-induced enhancement of HBSMCs proliferation. Representative DNA replication in the S-phase in each group (left). Average proliferation rates of HBSMCs in each group (right). (E) MLA ($100 \mu\text{M}$) and MG624 ($1 \mu\text{M}$) attenuated nicotine-induced increases of cell numbers of HBSMCs. (F) $\alpha 7$ nAChR-specific siRNA (si $\alpha 7$ nAChR) suppressed nicotine-induced enhancement of HBSMCs proliferation. Representative DNA replication in the S-phase in each group (left). Average proliferation rates of HBSMCs in each group (right). (G) si $\alpha 7$ nAChR suppressed nicotine-induced increases of cell numbers of HBSMCs. The data represent the mean $\pm$ SD of three independent experiments. * $P < 0.05$, compared with the control group; # $P < 0.05$, compared with nicotine treatment group.

implied that a wide concentration range of nicotine (1–10 μM) can promote proliferation in HBSMCs. According to this result, the nicotine concentration of 10 μM and the drug treatment time of 48 h were adopted in the following in-vitro experiments. Next, we demonstrated that the cell viability increase resulted from the increase in cell numbers (Fig. 1B) due to exposure to 10 μM nicotine. To verify the upregulation of cell proliferation related to cell cycle progression, we evaluated the S-phase cell ratio of HBSMCs by fluorescent EdU. We found that the S-phase cell ratio of the HBSMCs was remarkably increased by 48 h treatment of 10 μM nicotine (Fig. 1C). These results indicated that 10 μM nicotine provokes proliferation of HBSMCs.

It is well accepted that nicotine exerts its effects by activating nAChRs [8, 16]. To determine whether nAChRs play a role in the nicotine-induced proliferation of HBSMCs, we treated HBSMCs with the non-specific pharmacological nAChR antagonist MLA or the $\alpha 7$ nAChR-specific antagonist MG624 and then measured cell proliferation. Data showed that pretreatment with 100 μM MLA or 1 μM MG624 attenuated nicotine-induced increase of HBSMCs proliferation (Fig. 1D). Direct cell counting revealed that MLA or MG624 inhibited nicotine-induced upregulation of the number of HBSMCs (Fig. 1E). These results implied that $\alpha 7$ nAChR plays a pivotal role in nicotine-mediated cell proliferation of HBSMCs. To further support this finding, $\alpha 7$ nAChR-specific siRNA was used in the following experiments. As shown in Fig. 1F–G, nicotine-mediated enhancement of HBSMC proliferation could be suppressed by $\alpha 7$ nAChR-specific siRNA. Thus, we concluded that the pro-proliferation effect of nicotine on HBSMCs was nAChR-dependent and that among nAChRs, $\alpha 7$ nAChR may play a major role.

Nicotine exposure enhanced SOCE and ROCE in HBSMCs

An important mechanism of smooth muscle cell proliferation is the maintenance of intracellular calcium homeostasis. Increased $[\text{Ca}^{2+}]_i$ has been proven to promote smooth muscle cell proliferation. As a type of calcium channel, TRPCs can facilitate calcium influx, which may further evoke SOCE and ROCE, finally leading to elevation of cytoplasmic calcium levels. SOCE can be activated by depletion of Ca^{2+} stores using CPA or thapsigargin, whereas ROCE can be activated directly by diacylglycerol (DAG) via a protein kinase C-independent mechanism. As a membrane-permeable analogue of DAG, OAG is commonly used as a ROCE activator. To determine whether nicotine-induced HBSMCs proliferation is associated with SOCE and/or ROCE, $[\text{Ca}^{2+}]_i$ were monitored by fura-2 fluorescence imaging (340/380 nm ratio, F340/F380). As shown in Fig. 2A, SOCE activated by depletion of Ca^{2+} stores with CPA significantly increased in HBSMCs treated by 10 μM nicotine for 48 h (0.66 ± 0.10 for nicotine treatment cells, and 0.38 ± 0.04 for control cells; $P < 0.05$). Consistent with SOCE, ROCE activated directly by OAG was also significantly elevated in HBSMCs treated by 10 μM nicotine for 48 h (0.38 ± 0.01 for nicotine treatment cells, and 0.18 ± 0.01 for control cells; $P < 0.05$; Fig. 2B).

With the 48 h treatment of 10 μM nicotine, the increases in both SOCE and ROCE resulted in a significant rise in basal $[\text{Ca}^{2+}]_i$ in HBSMCs (1.01 ± 0.05 for nicotine treatment cells, and 0.83 ± 0.04 for control cells; $P < 0.05$; Fig. 2C). These results suggested that the expression of putative SOCC and ROCC and their functional activities were augmented in HBSMCs proportional to nicotine treatment. SOCE and ROCE were further evaluated by the rate of Mn^{2+} -quenched fura-2 fluorescence. As shown in Fig. 2D, the rate of CPA-induced Mn^{2+} quenching was increased from $33.45 \pm 2.17\%$ in control HBSMCs to $52.79 \pm 2.33\%$ in nicotine-treated HBSMCs, whereas the rate of OAG-induced Mn^{2+} quenching was accelerated from $26.09 \pm 1.54\%$ in control cells to $44.19 \pm 4.96\%$ in nicotine-treated cells (Fig. 2E). Together, these results clearly indicated that both SOCE and ROCE are involved in nicotine-mediated enhancement of proliferation of HBSMCs.

Nicotine exposure increased TRPC6 expression in HBSMCs

It is well accepted that TRPCs encode SOCC and ROCC based on their participation in calcium entry routes that were initially shown to be activated downstream of PLC-coupled

receptors [26-28]. Researchers have demonstrated that upregulation of these proteins is associated with contraction and proliferation of smooth muscle cells [10, 29]. In this study, we evaluated the mRNA expression of the family of TRPCs to determine their changes in response to nicotine exposure in HBSMCs. We confirmed that TRPC1, TRPC3, TRPC4 and TRPC6 mRNA were expressed in HBSMCs, while TRPC2, TRPC5 and TRPC7 mRNA was not detected in HBSMCs (Fig. 3A). In addition, nicotine selectively increased the mRNA and

Fig. 2. Nicotine enhanced store-operated Ca^{2+} entry (SOCE), receptor-operated Ca^{2+} entry (ROCE) and intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in HBSMCs. (A) Nicotine (10 μM for 48 h) enhanced SOCE in HBSMCs. Time-course of $\Delta[\text{Ca}^{2+}]_i$ ($\Delta\text{F340}/\text{F380}$) before and after restoration of extracellular Ca^{2+} to 2.5 mM in control HBSMCs (left) and nicotine treatment (10 μM for 48 h) HBSMCs (middle) perfused with Ca^{2+} -free Krebs-Ringer bicarbonate solution (KRBS) containing 10 μM cyclopiazonic acid (CPA) and 5 μM nifedipine. Ca^{2+} -free KRBS also contained 1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) to chelate any residual Ca^{2+} . Average peak change in $\Delta[\text{Ca}^{2+}]_i$ ($\Delta\text{F340}/\text{F380}$) after restoration of extracellular Ca^{2+} (SOCE) in control and nicotine treatment HBSMCs (right). (B) Nicotine enhanced ROCE in HBSMCs. Time-course of $\Delta[\text{Ca}^{2+}]_i$ before and after restoration of extracellular Ca^{2+} to 2.5 mM in control (left) and nicotine treatment HBSMCs (middle) perfused with Ca^{2+} -free KRBS containing 100 μM 1-oleoyl-2-acetyl-sn-glycerol (OAG) and 5 μM nifedipine. Average peak change in $\Delta[\text{Ca}^{2+}]_i$ after restoration of extracellular Ca^{2+} (ROCE) in control and nicotine treatment HBSMCs (right). (C) Nicotine increased basal $[\text{Ca}^{2+}]_i$ in HBSMCs. (D) Time-course of fura-2 fluorescence at 360 nm normalized to values at time 0 before and after administration of MnCl_2 (200 μM) to control and nicotine treatment HBSMCs perfused with Ca^{2+} -free KRBS containing 10 μM CPA and 5 μM nifedipine (left). Average change in fura-2 fluorescence at 360 nm in control and nicotine treatment HBSMCs (right). (E) Time-course of fura-2 fluorescence at 360 nm normalized to values at time 0 before and after administration of MnCl_2 (200 μM) to control and nicotine treatment HBSMCs perfused with Ca^{2+} -free KRBS containing 100 μM OAG and 5 μM nifedipine (left). Average change in fura-2 fluorescence at 360 nm in control and nicotine treatment HBSMCs (right). * $P < 0.05$ vs. control.

Fig. 3. Nicotine increased TRPC6 expression in HBSMCs. (A) Evaluation of TRPC mRNA expression in HBSMCs by RT-PCR. (B-C) Effect of nicotine ($10 \mu\text{M}$ for 48 h) on TRPC expression in HBSMCs. (B) Bar graph showing real-time quantitative PCR results of TRPC mRNA expression in HBSMCs with GAPDH as an internal standard. (C) Western blot analysis of TRPC proteins (Left). Bar graph showing the normalized amount of TRPC proteins with GAPDH as an internal standard (Right). TRPC6 siRNA (siTRPC6) attenuated the increases of basal $[\text{Ca}^{2+}]_i$ (D), SOCE (E), ROCE (F), CPA-induced Mn^{2+} -quenching (G) and OAG-induced Mn^{2+} -quenching (H) in HBSMCs induced by 48 h treatment of $10 \mu\text{M}$ nicotine. siTRPC6 attenuated the enhancement of proliferation of HBSMCs (I, determined by EdU immunostaining; J, determined by cell counting) induced by nicotine. * $P < 0.05$ vs. control, # $P < 0.05$ vs. nicotine treatment HBSMCs.

protein expression of TRPC6 in HBSMCs ($P < 0.05$), whereas the expression of TRPC1, TRPC3 and TRPC4 was unaltered after nicotine treatment (Fig. 3B-C).

To validate whether nicotine-induced increase in TRPC6 expression is responsible for the enhanced calcium entry into HBSMCs, HBSMCs were transfected with specific siRNA for TRPC6, and the changes in SOCE and ROCE were detected. Transfection with TRPC6 siRNA significantly attenuated the increases of basal $[\text{Ca}^{2+}]_i$ (Fig. 3D), SOCE (Fig. 3E) and ROCE (Fig. 3F) in HBSMCs induced by 48 h treatment of $10 \mu\text{M}$ nicotine. Similar results were also obtained for the maximum rate of CPA/OAG-induced Mn^{2+} -quenching (Fig. 3G-H). Furthermore, a

Fig. 4. Nicotine activated the PI3K/Akt pathway in HBSMCs. (A) Effects of nicotine (10 μM for 5, 15, 30 and 60 min) on p-Akt expression in HBSMCs. (B) An inhibitor of PI3K, LY294002 (1–10 μM), attenuated the upregulation of p-Akt expression in HBSMCs induced by nicotine (10 μM for 15 min). Pretreatment with LY294002 inhibited the increases in TRPC6 protein expression (C), basal $[\text{Ca}^{2+}]_i$ (D), SOCE (E), ROCE (F), CPA-induced Mn^{2+} -quenching (G) and OAG-induced Mn^{2+} -quenching (H) in HBSMCs induced by 48 h treatment of 10 μM nicotine. LY294002 attenuated the enhancement of proliferation of HBSMCs (I, determined by EdU immunostaining; J, determined by cell counting) induced by nicotine (10 μM for 48 h). * $P < 0.05$ vs. control, # $P < 0.05$ vs. nicotine treatment HBSMCs.

significant attenuation of the ratio of EdU-positive HBSMCs was detected after transfection with TRPC6 siRNA in both the presence and absence of nicotine (Fig. 3I). Similar changes in cell numbers were observed according to the cell counting results (Fig. 3J). Taken together, these results strongly suggested that the nicotine-induced upregulation of calcium entry was dependent on TRPC6 in HBSMCs. TRPC6 is therefore a major determinant of both SOCE and ROCE in HBSMCs. Nicotine exposure elevated $[\text{Ca}^{2+}]_i$ levels in HBSMCs, which is believed to promote cell proliferation.

Nicotine activated the PI3K/Akt pathway in HBSMCs

The PI3K/Akt signaling pathway has been reported to be an important regulator of nicotine-mediated RSMC proliferation [8]. Therefore, we supposed that increased TRPC6

Fig. 5. Nicotine induced the activation of the PI3K/Akt pathway via $\alpha 7$ nAChR. $\alpha 7$ nAChR antagonists, MLA (10 - 100 μM) (A) and MG624 (0.1 - 1 μM) (B), and $\alpha 7$ nAChR-specific siRNA (si $\alpha 7$ nAChR) (C) attenuated nicotine-induced upregulation of p-Akt expression in HBSMCs (upper: representative blots; bottom: mean protein expression of p-Akt relative to Akt normalized to values of control.). * $P < 0.05$ vs. control, # $P < 0.05$ vs. nicotine treatment HBSMCs.

expression may be a downstream effect of the PI3K/Akt pathway in HBSMCs. We first checked if a component of this pathway, Akt, was activated after nicotine treatment. We found that nicotine increased the phosphorylation of Akt at Ser473 in a time-dependent manner (Fig. 4A). The phosphorylation of Akt at Ser473 in HBSMCs was remarkably increased after 15 to 30 min of 10 μM nicotine exposure, whereas the total levels of Akt remained unchanged. However, pretreatment with a pharmacological inhibitor of PI3K (LY294002) effectively prevented the nicotine-induced phosphorylation of Akt (Fig. 4B). Our results demonstrated that nicotine-mediated activation of Akt depends on PI3K activity.

We next studied the potential role of the PI3K/Akt pathway in the nicotine-induced elevation of calcium entry into HBSMCs, which is also TRPC6 dependent. As shown in Fig. 4C-F, pretreatment with LY294002 significantly inhibited the nicotine-induced increases in TRPC6 protein expression, basal $[\text{Ca}^{2+}]_i$, SOCE and ROCE in HBSMCs. Similar results were also obtained for the maximum rate of CPA/OAG-induced Mn^{2+} -quenching (Fig. 4G-H). Consistent with SOCE and ROCE, the nicotine-mediated promotion of the EdU-positive HBSMC ratio (Fig. 4I) and cell numbers that were observed based on cell counting (Fig. 4J) could be significantly attenuated by LY294002. These results provide further evidence that nicotine-upregulated calcium entry in HBSMCs involves activation of the PI3K/Akt signaling pathway.

Nicotine induced the activation of the PI3K/Akt pathway via $\alpha 7$ nAChR

To determine whether $\alpha 7$ nAChR might participate in the nicotine-induced activation of the PI3K/Akt signaling pathway, we evaluated the activation of Akt after treatment with nicotine combined with inhibition of $\alpha 7$ nAChR via its antagonists (MLA or MG624) or specific siRNA. The results revealed that pretreatment with $\alpha 7$ nAChR antagonists or siRNA targeting $\alpha 7$ nAChR effectively prevented nicotine-induced phosphorylation of Akt (Fig. 5A-C). These results imply that nicotine-induced activation of the PI3K/Akt signaling pathway mediates $\alpha 7$ nAChR. We deduced that PI3K/Akt pathway activation is the link between $\alpha 7$ nAChR and TRPC6.

Discussion

COPD is characterized by irreversible remodeling of the airway walls, including thickening of the airway smooth muscle layer. The mechanisms underlying such remodeling of smooth muscle remain largely unknown, and the role of nicotine in this process has not been fully explored. The present study provides evidence strongly indicating that nicotine promotes cell proliferation by increasing basal calcium levels and calcium influx in HBSMCs, which result from the $\alpha 7$ nAChR-dependent activation of the PI3K/Akt pathway and upregulation of TRPC6 protein expression. These findings have important implications for our understanding of nicotine-induced cell proliferation and offer a mechanism by which TRPC6-dependent calcium entry occurs.

The proliferation of airway smooth muscle cells is a typical pathological change in COPD patients, which is primarily related to cigarette smoke [8, 30]. Traditionally, nicotine has been studied as the addictive component in cigarettes, but increasing evidence has indicated that nicotine is also a key factor in the initiation and progression of COPD. Nicotine hinders the hydration of mucus, promotes cell proliferation by causing calcium influx in ASMCs, induces pro-inflammatory dendritic cell responses, and blocks neutrophil apoptosis [31]. Despite the absence of a clear understanding of nicotine-induced health risk, some manufacturers have suggested that using nicotine can help someone who extensively smoke quit their smoking habit, which is widely known to be hazardous to health. In this study, we demonstrated that the proliferation of HBSMCs was markedly enhanced after exposure to the concentrations used in this study. The viability of HBSMCs was also significantly increased after 48-h treatment of nicotine (10 μM), which is similar to the concentration of nicotine in the lungs of smokers [32, 33]. The proliferation of ASMCs has been the focus of consideration and extensive research, as it is an important airway wall remodeling response in cigarette smokers with COPD [34]. Although many groups have identified mediators that have the potential to increase the proliferation of HBSMCs, no groups have clearly reported the effects of nicotine on the proliferation of HBSMCs. In the present study, the EdU incorporation and direct cell counting experiments clearly showed that nicotine promoted HBSMC proliferation. This is consistent with previous findings that the upregulation of cell proliferation is associated with nicotine in some nonneuronal cells [8, 35-37]. Environmental factors such as cigarette smoke also contribute to airway diseases such as COPD, in which altered cell proliferation in airway smooth muscle is the key feature of airway remodeling. Thus, understanding the precise mechanisms by which nicotine affects airway structural and functional changes is vital. To better understand how nicotine enhances HBSMC proliferation, we focused our research on the alterations of intracellular calcium homeostasis. Changes in $[\text{Ca}^{2+}]_i$ regulate a wide variety of cell functions, including gene expression, cell proliferation and cell death [38-40]. Several studies have recently suggested that calcium homeostasis is dysregulated in ASMCs from COPD patients. Calcium regulation is central to ASMCs and as an early signal in cell proliferation. Many mechanisms are involved in calcium regulation in ASMCs, among which calcium channels play a major role. The role of calcium entry channel proteins belonging to TRPC family has been recently identified in some cells such as vascular smooth muscle cells, mast cells, pancreatic β -cells, podocytes and B-lymphocytes [41-45]. Z Feng et al [9]. showed that the multiple effects of nicotine in *C. elegans* is dependent on nAChRs and downstream target TRPC protein, a major ion channel component for calcium influx. Within the TRPC family, TRPC1/4/5 can be activated by calcium mobilization from intracellular stores mediated by inositol 1, 4,5-trisphosphate (IP3) or thapsigargin; this process is referred as SOCE. In addition, TRPC3/6/7 can be directly activated by DAG through a process known as ROCE [46, 47]. Our results showed that nicotine enhanced both SOCE and ROCE and finally led to an elevation of basal $[\text{Ca}^{2+}]_i$ in HBSMCs. SOCE and ROCE are enhanced during PASMC proliferation to increase calcium influx, which provides sufficient calcium for activation of the intracellular mechanisms responsible for cell proliferation and growth [48].

We thus expected that more than one TRPC family member should be increased in HBSMCs, at least one for SOCE and another for ROCE. However, our data showed that

only TRPC6 was selectively increased in response to nicotine, while the other subtypes TRPC1/3/4 did not change in HBSMCs. Interestingly, this finding differs from a previous study reporting that TRPC6 alone induced OAG-activated calcium influx in human epithelial breast cancer cells [49]. It also differs from our previous study reporting that the enhanced ROCE due to upregulated TRPC6 is a novel pathogenic mechanism contributing to the increased basal $[\text{Ca}^{2+}]_i$ in pulmonary venous smooth muscle cells (PVSMC) and excessive PVSMC proliferation during the development of hypoxic pulmonary hypertension [50]. However, more recent studies have indicated that TRPC6 is also an important component of SOCC and contributes to the SOCE process [12, 51, 52]. Evidences have also shown that upregulation of TRPC6 expression increases SOCC activity, which is related to elevated SOCE, and finally promotes the proliferation of PSMCs [53, 54]. However, the reasons behind these discrepancies remain unclear. TRPCs have been shown to assemble as both homomeric and heteromeric complexes. Thus, it is difficult to unambiguously assign specific functions to specific TRPCs, and in many cases, the functions of various TRPCs may overlap. In PSMCs, the detection of the putative receptor-operated channel TRPC6, in addition to TRPC1, suggested the presence of ROCE pathway [55]. However, it is noteworthy that TRPC6 expression closely relates to CPA-induced calcium entry, which contributes to the proliferation of PSMCs [56]. It remains unclear whether TRPC6 exhibits a different phenotype in PSMCs proliferating by heterodimerizing with proteins such as TRPC3, which has been suggested to be a receptor- and store-operated calcium channel [46], or whether CPA activates TRPC6 through mechanisms other than SR calcium depletion. Recently, numerous evidences have reported that TRPC6 is associated with ROCE [57-60]. It is unclear if TRPC6 displays a different phenotype among different species, eg, by heteromerization with other TRPC proteins. These interesting possibilities require future investigations. Our data showed that nicotine induces elevation of SOCE and ROCE in HBSMCs, leading to enhanced basal $[\text{Ca}^{2+}]_i$. This enhancement is potentially correlated with increased expression of TRPC6 and finally contributes to promoting the proliferation of HBSMCs. To the best of our knowledge, this is the first study regarding the importance of TRPC6 in HBSMC proliferation. Recent studies also have suggested a key role of TRPC6 calcium entry in the mediation of $[\text{Ca}^{2+}]_i$ elevation and cell proliferation [61, 62]. Based on the observations from this study, the attenuation of SOCE and ROCE may serve as a potential therapeutic approach for COPD.

Our findings suggest that $\alpha 7$ nAChR is the major nicotinic receptor subtype responsible for upregulation of TRPC6 expression and proliferation in HBSMCs. Thus far, 12 different neuronal nicotinic subunits have been identified: nine α ($\alpha 2$ - $\alpha 10$) and three β ($\beta 2$ - $\beta 4$) subunits [63]. Multiple functionally distinct nAChR subunits can be assembled either as homomeric functional pentamers, such as $\alpha 7$ nAChR, or as heteropentamers with at least two different subunits, such as $\alpha 4\beta 2$ nAChRs [64]. We recently became interested in $\alpha 7$ nAChR because of their physiological roles in non-neuronal cells such as macrophages, vascular endothelial cells and aortic smooth muscle cells [65]. In addition, there is growing evidence that $\alpha 7$ nAChR plays an important role in nicotine-mediated signaling transduction pathways in cell proliferation [8]. Some studies have demonstrated that PGE2 promotes cell growth of nonsmall-cell lung cancer (NSCLC) by increasing the expression of $\alpha 7$ nAChR [66]. Our results showed that the $\alpha 7$ nAChR-specific antagonist MG624 abolished the nicotine-induced elevation of TRPC6 expression, SOCE, ROCE and cell proliferation in HBSMCs, which led us to conclude that nicotine specifically enhances HBSMC proliferation through an $\alpha 7$ nAChR-TRPC6- Ca^{2+} signaling pathway. It was confirmed in our previous study and other investigations that nicotine can bind to $\alpha 7$ nAChR to regulate cell proliferation [8, 60-64]. Although nicotine acts via $\alpha 7$ nAChR to promote cell proliferation [67-71], it remains to be determined which signaling pathway activation contributed to the pathogenic changes that occurred. Indeed, identifying the signaling mechanisms responsible for these nicotine-mediated effects remains a key goal.

Between $\alpha 7$ nAChR and TRPC6, phosphoinositide 3-kinase (PI3K) was necessary for the effects induced by nicotine. In this study, we observed that nicotine magnified the phosphorylation of Akt in a time-dependent manner (0-60 min). In addition, PI3K inhibitor

(LY294002) abrogated the nicotine-induced phosphorylation of Akt. Additionally, the related nicotine-induced changes, such as elevated TRPC6 expression, SOCE, ROCE and proliferation of HBSCMs were abolished by LY294002. Thus, we inferred that nicotine-induced HBSCM proliferation is mediated, at least partly, through activation of the Akt pathway. In line with previous observations made in our laboratory that nicotine could induce activation of the Akt pathway in RASMCs, we demonstrated in this study that activation of the Akt pathway, which controls cell proliferation, is a further adaptive response to nicotine. Akt is known as a major downstream target of phosphoinositide 3-kinase (PI3K), which plays a crucial role in a broad range of cellular functions, such as cell survival, migration, division, differentiation, proliferation, etc., in response to extracellular signals [72]. It was recently identified that PI3K-mediated signaling pathways are novel regulators of native TRPCs in rabbit coronary artery vascular smooth muscle cells (VSMCs) [73]. It was also reported that the PI3K pathway is involved in the externalization of TRPC6 and TRPC6-dependent calcium entry [74]. Furthermore, it has been well accepted that TRPC6 mediates glucagon-induced increase of $[\text{Ca}^{2+}]_i$ in glomerular mesangial cells through the cAMP-PI3K-MEK-ERK1/2 axis, which leads to the proliferation of renal mesangial cells [75].

In summary, calcium appears to be a critical second messenger in the signal transduction underlying the regulation of nicotine-induced cell proliferation. PI3K is activated by G-protein-coupled receptors (GPCR) [76] and is possibly involved in calcium entry [77]. As PI3K is a key protein involved in calcium regulation, we hypothesized that it might influence the activation of TRPC6. Activation of the PI3K/Akt pathway may increase calcium influx by stimulating TRPC6. Thus, TRPC6 appears to be a downstream target of the PI3K/Akt signaling pathway in nicotine-treated HBSCMs. The PI3K/Akt signaling pathway is a major mechanism through which $\alpha 7$ nAChR mediates cell proliferation. Recent studies have shown that nicotine can induce $\alpha 7$ nAChR-mediated proliferation effects by activating the PI3K/Akt pathway, shedding light on potential therapeutic molecules that could be targeted to combat smoking-associated human diseases [68]. Our study shows that $\alpha 7$ nAChR antagonists or siRNA can also suppress Akt phosphorylation.

Conclusion

The results presented here provide evidence that nicotine-induced activation of $\alpha 7$ nAChR enhances TRPC6 expression by phosphorylating Akt at Ser473, leading to increased calcium influx and ultimately promotion of HBSCMs proliferation. Thus, understanding the underlying regulatory mechanisms is of important potential clinical value and may provide further insight regarding new treatment strategies for airway remodeling diseases such as COPD and asthma.

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Disclosure Statement

The authors declare no competing financial interests

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