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# Role of canonical transient receptor potential channel-3 in acetylcholine-induced mouse airway smooth muscle cell proliferation

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ABSTRACT

*Aims:* Canonical transient receptor potential channel-3 (TRPC3)-encoded $Ca^{2+}$-permeable nonselective cation channel (NSCC) has been proven to be an important native constitutively active channel in airway smooth muscle cell (ASMC), which plays significant roles in physiological and pathological conditions by controlling $Ca^{2+}$ homeostasis in ASMC. Acetylcholine (ACh) is generally accepted as a contractile parasympathetic neurotransmitter in the airway. Recently studies have revealed the pathological role of ACh in airway remodeling, however, the mechanisms remain unclear. Here, we investigated the role of TRPC3 in ACh-induced ASMC proliferation.

*Materials and methods:* Primary mouse ASMCs were cultured with or without ACh treatment, then cell viability, TRPC3 expression, NSCC currents and $[Ca^{2+}]_i$ changes were examined by MTT assay, cell counting, Western blotting, standard whole-cell patch clamp recording and calcium imaging, respectively. Small interfering RNA (siRNA) technology was used to confirm the contribution of TRPC3 to ACh-induced ASMC proliferation.

*Key findings:* TRPC3 blocker $Gd^{3+}$, antibody or siRNA largely inhibited ACh-induced up-regulation of TRPC3 protein, enhancement of NSCC currents, resting $[Ca^{2+}]_i$ and KCl-induced changes in $[Ca^{2+}]_i$, eventually inhibiting ACh-induced ASMC proliferation.

*Significance:* Our data suggested ACh could induce ASMC proliferation, and TRPC3 may be involved in ACh-induced ASMC proliferation that occurs with airway remodeling.

## 1. Introduction

Airway remodeling, defined by pathologically structural changes in the airway, has been described in many chronic respiratory diseases, such as asthma and chronic obstructive pulmonary disease (COPD) [1]. Increasing evidence demonstrates that airway remodeling is associated with the duration and severity of these diseases [2–4]. In asthma and COPD, airway remodeling represents a significant airway smooth muscle cell (ASMC) mass increase resulting from ASMC proliferation and/or hypertrophy [5–7]. ASMCs have been regarded as the target cells for protecting from the airway remodeling [2,5], while the mechanisms of ASMC proliferation remain unclear.

Acetylcholine (ACh) has been reported to be the primary parasympathetic neurotransmitter in the airway [8,9]. It is generally accepted that ACh is a contractile neurotransmitter by changing the intracellular free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$). Increasing evidence indicates that ACh regulates airway remodeling and inflammation in chronic respiratory diseases, except for its well-known functional role in regulating bronchoconstriction [9–12]. Previous studies have shown that muscarinic receptors stimulation with methacholine or carbachol could enhance ASMC proliferation induced by growth factors, including platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) [13–15]. Moreover, anticholinergics reduce airway remodeling in animal models of COPD and allergic asthma [16–20]. Although the mechanisms involved in the effects of ACh on airway remodeling are largely unknown, studies above suggest that $M_3$-R stimulation is presumably of major importance. A previous study has established that ACh could promote tracheal epithelial cells proliferation mediated by $M_1$ receptors [21]. However, evidence for the direct effect of ACh on ASMC proliferation is still insufficient.

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Canonical transient receptor potential channels (TRPCs), including TRPC1-7, have been indicated as the molecular identities of $Ca^{2+}$-permeable nonselective cation channels (NSCCs) [22] and play important roles in physiological and pathological cellular responses in many types of cells [23,24]. TRPC3 mRNA and protein have been consistently proven to be expressed in cultured and passaged ASMCs [25,26]. In ASMC from asthmatic mice, TRPC3 protein was significantly overexpressed. TRPC3 could form native constitutively active NSCCs and play significant roles in ASMC contraction under physiological and asthmatic conditions through controlling resting $[Ca^{2+}]_i$ and $Ca^{2+}$ response to methacholine [26]. In human ASMC treated with TNF-α or mixed allergens, TRPC3 protein was up-regulated and its up-regulation was involved in TNF-α-induced changes in $[Ca^{2+}]_i$ [25,27]. $Ca^{2+}$ signaling plays key roles in smooth muscle cell contractility, migration and proliferation [28]. Abnormal $Ca^{2+}$ homeostasis and changes in expression of $Ca^{2+}$-permeable channels have been reported to be associated with airway remodeling [29,30]. Still, the role of TRPC3-mediated $Ca^{2+}$ entry in airway remodeling remains unclear.

In this study, we sought the evidence of the direct effect of ACh on ASMC proliferation and meanwhile explored whether TRPC3 may be involved in the underlying mechanisms of ACh-induced ASMC proliferation, anticipating to find the intervention target of airway remodeling.

## 2. Materials and methods

### 2.1. ASMCs dispersion and culture

20–25 g Kunming mice of both sexes were purchased from the Department of Experimental Animal of Tongji Medical College of HuaZhong University of Science and Technology (HUST). This investigation was carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health. All animal experiments were approved by the Ethics Committee of Laboratory Animals of Tongji Medical College of HUST. Primary ASMCs were prepared as previously described [31,32]. 5–6 mouse tracheas were isolated and placed in phosphate-buffered saline (PBS, composition [mM]:137 NaCl, 2.7 KCl, 10 $Na_2HPO_4$, 1.47 $KH_2PO_4$; pH 7.2–7.4). After removal of the connective tissue and mucosa, the epithelial layer was stripped off by gently rubbing the intimal surface. The remaining smooth muscle layer was cut longitudinally through the cartilage, minced into tissue particles, transferred to Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (FBS, 2%), penicillin (100 U/ml), streptomycin (100 U/ml) (Gibco life Technologies, USA) and collagenase II (2 mg/ml, Worthington Biochemical Corporation, USA), and digested for 3 h at 37 °C.

After washed three times with DMEM containing 10% FBS, the digested tissues were gently triturated with a glass pipette to disperse single cells. The cell suspension was seeded into culture flasks. All cultures were maintained at 37 °C in a 5% $CO_2$-95% atmospheric incubator. Upon reaching 80–90% confluence, cells were split with 0.25% trypsin (Worthington Biochemical Corporation, USA). Cells from passages 3–6 were used for all experiments.

### 2.2. MTT assay and cell counting

Cell viability was quantified by methyl thiazolyl tetrazolium (MTT) assay as described previously [33,34]. In brief, 5000 ASMCs per well were seeded in 96-well plates and cultured to attach in 10% FBS-containing DMEM at 37 °C in 5% $CO_2$ humidified incubator for 24 h. Cells were stimulated with different concentrations of ACh (1 nM–1 mM; Sigma-Aldrich, St. Louis, MO, USA) in 2% FBS-containing DMEM. When necessary, a pan muscarinic receptor antagonist Atropin (0.1 μM–100 μM, Sigma-Aldrich, St. Louis, MO, USA), a nicotinic receptor antagonist Mecamylamine (0.1 μM–100 μM, Sigma-Aldrich, St. Louis, MO, USA), TRPC3 channel blockers Gadolinium chloride ($Gd^{3+}$, 3 μM, Sigma-Aldrich, St. Louis, MO, USA) or TRPC3 antibody (dilution 1:200; Alomone Laboratories, Israel) were added into the medium. After drugs treatment for different time, the cells were cultured with MTT (0.5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) for another 4 h. The supernatant was removed and dimethyl sulfoxide was added to dissolve the formazan salt crystals. After incubated for 10 min on a swing bed at room temperature, the solubilized products were quantified at 490 nm wavelength using a microplate reader (Tecan sunrise, Switzerland).

20,000 cells per well were seeded in 24-well plates. Cell numbers were manually counted after drugs treatment.

### 2.3. Western blotting

Protein extraction and Western blotting were performed as described previously [35,36]. Briefly, adherent ASMCs were rinsed with PBS for twice, homogenized in ice-cold RIPA buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF) for 40 min. The homogenates were centrifuged at 12,000 × g for 15 min to remove cellular debris to obtain total cell lysate. The protein concentration was assayed using BCA (Roche, Switzerland). 30 μg total proteins from each sample were separated on an 8% acrylamide gel by SDS-PAGE at 80 V for 30 min, and at 100 V for 2 h, transferred onto a polyvinylidene fluoride (PVDF) membrane at 200 mA for 2 h at 4 °C. The blocked membrane with 5% nonfat milk was incubated with specific antibody against TRPC3 (dilution 1:1000; Alomone Laboratories, Israel) and β-actin (dilution 1:1000; Santa Cruz Biotechnology) overnight at 4 °C. After incubation with horseradish peroxidase-conjugatedanti-rabbit IgG or anti-mouse secondary antibody (dilution 1:10,000; NovoGene Biotech, Co. Ltd., China) for 1 h at room temperature, the immunoblotting bands were visualized by electrochemiluminescence (ECL, EngreenBiosytem, Co. Ltd., China) reagent and detected by DNR Bio Imaging systems (DNR Bio Imaging systems, Co. Ltd., Israel). The results were assayed by densitometry using Image J software. The value of the relative density of the TRPC3 band was normalized to the density of the β-actin band to represent the level of TRPC3 protein. The ratio of control group was set at 100%.

### 2.4. Whole-cell patch clamp

The NSCC currents were measured by a standard whole-cell patch clamp technique with an EPC-10 amplifier (HEKA Electronic, Lambrecht, Germany), as previously published [37–40]. All experiments were performed at room temperature (20–25 °C). Pipettes with tip resistance of 3–5 MΩ were pulled from borosilicate glass capillaries (1.5 mm in diameter) with a two-stage microelectrode puller (PC-10, Narishige, Japan). Only cells with tight seals (> 1 GΩ) were selected to break in. Whole cell currents were low-pass filtered at 3 kHz, sampled at a rate of 10 kHz. The data were analyzed offline using Clampfit-9 software (Axon Instruments, CA, USA). Currents were normalized to cell membrane capacitance and presented as current densities (pA/pF).

The bath solution contained (mM): 126 NaCl, 1.5 $CaCl_2$, 10 HEPES, and 11 D-glucose, as well as 100 μM DIDS, 5 μM nicardipine, and 100 μM niflumic acid; pH 7.2–7.4 adjusted with NaOH. Under these conditions, voltage-gated $Ca^{2+}$, $K^+$, as well as swell-activated and $Ca^{2+}$-activated $Cl^-$ channels were inhibited in order to further purify the NSCC currents [41]. The patch pipette solution contained (mM):18 CsCl, 108 Cs-aspartate, 1.2 $MgCl_2$, 10 HEPES, 11 D-glucose, 10 BATPA, 1 $CaCl_2$, 0.2 NaGTP, 1 $Na_2ATP$; pH 7.2–7.4 adjusted with CsOH. All drugs were purchased from Sigma-Aldrich, St. Louis, MO, USA. In some experiments, TRPC3 antibody (1:200) were added to the pipette solution.

### 2.5. Calcium imaging

$[Ca^{2+}]_i$ of single cell was measured by a ratio vision digital

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