Original Article Role of transient receptor potential channels in airway smooth muscle cell growth

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Abstract: The proliferation of airway smooth muscle cells (ASMCs) plays a vital role in airway remodeling. Besides, transient receptor potential channels (TRPC) play a key role in pathological cellular responses of ASMCs. In present studies, we investigated the effect of TRPC1 and TRPC3 on cell proliferation of ASMCs. Rats were sensitized with ovalbumin to construct asthmatic models. The pathological change of airway remodeling was detected by hematoxylin and eosin (HE) staining. Quantitative real-time PCR (qRT-PCR) and Western blot were used to detect the mRNA and protein expression of TRPC1, TRPC3 and proliferating cell nuclear antigen (PCNA) in asthmatic and non-asthmatic rats respectively. Furthermore, the cell proliferation was detected by MTT assay and ³H-TdR incorporation assay. The expression of TRPC1 and TRPC3 significantly increased at both transcriptional and translational levels in asthmatic rats. Moreover, SKF96365 could effectively inhibit cell over-proliferation caused by asthma. In addition, knockdown of TRPC1 and TRPC3 significantly decreased the absorbance of MTT and DNA synthesis, while overexpression of TRPC1 and TRPC3 had the opposite effect in asthmatic ASMCs. TRPC1 and TRPC3 were involved in the regulation of cell proliferation in asthmatic ASMCs.

Keywords: Asthma, airway smooth muscle cells (ASMC), transient receptor potential channels, proliferation

Introduction

Asthma is characterized by airway inflammation, airway remodeling and airflow obstruction [1]. The hyperplasia and hypertrophy of airway smooth muscle (ASM) cause the increasing of airway narrowing and airway wall thickening, which contribute to airway inflammation and obstruction [2]. Besides, the proliferation of ASM cells (ASMCs) plays a vital role in airway remodeling [3]. And airway remodeling which results from repeated airway wall damage and repair can play a key role in the pathophysiology of serious asthma [4]. Until now, some drugs have been applied to treat asthma airway remodeling, including Inhaled corticosteroid (ICS) [5], β-agonist bronchodilators [6] and anticholinergics [7]. However, the role of these drugs in retarding the process of airway remodeling is not efficient. Therefore, there is an urgent need to understand the molecular mechanisms of asthma progression in order to develop novel drug treatment for asthma.

Ca²⁺ is a well-known second messenger which can regulate a wide range of cell functions,

such as cell growth, membrane fusion, excitation-secretion coupling, excitation-contraction coupling, gene transcription and ion channel activation [8, 9]. Moreover, Ca²⁺ signaling plays a vital role in the regulation of airway smooth muscle functions, including proliferation, contraction and cytokine secretion [10]. Besides, Store-operated Ca²⁺ entry (SOCE) is the major Ca²⁺ influx pathway of ASMCs [11]. Storeoperated Ca²⁺ (SOC) channels will be activated when intracellular sarcoplasmic reticulum Ca2+ store is depleted [12]. And transient receptor potential (TRP) channels are characterized as one of the first molecular candidates to encode SOC channels, particularly the members of the transient receptor potential channels (TRPC) [13].

The transient receptor potential channels (TRPC) were originally identified in *Drosophila* [14]. And the first mammalian TRP gene was cloned from the human brain which was categorized into the TRPC family, and termed TRPC1 [15]. In addition, the TRPC family is found to constitute of seven members TRPC1-7 [16]. The

activity of TRPCs plays an important role in initiating and maintaining intracellular Ca²⁺ signaling, which is involved in different cellular responses, such as gene expression, proliferation, migration and contraction in airway SMCs [17]. Moreover, recent studies reveal that TRPC1 and TRPC3 mRNAs and proteins are expressed in primary mouse airway SMCs [18]. However, the role of TRPC1 and TRPC3 in cell proliferation of ASMCs remains unknown.

In this study, we investigated the expression of TRPC1 and TRPC3 in the ASM tissues of nonasthmatic and asthmatic rats and the effect of TRPC1 and TRPC3 on proliferation of ASMCs, in order to illustrate the molecular mechanisms of asthma and provide new drug treatment to asthmatic airway remodeling in clinics.

Materials and methods

Asthma model establishment

All experimental procedures were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by People's Hospital Affiliated to Zhengzhou University.

Twenty-four SPF grade Wistar rats (8-10 weeks old; 200~220 g) with a half of males and females were randomly divided into the control and experimental groups. The asthma-induced method was according to the methods described previously [19]. The experimental animals were intraperitoneal injected with 10 mg of ovalbumin (OVA) as well as 200 mg of aluminum hydroxide (dissolved in 1 ml of 0.9% NaCl solution). After sensitization for 3 times, experimental animals were exposed to aerosol consisting of 2% ovalbumin in normal saline for 30 min. Animals in the control group were exposed to saline. Exposure to aerosolized solutions was done for 7 consecutive days.

Airway smooth muscle cells isolation

A total of 24 rats were injected with 10% chloral hydrate to anesthetize them and then they were sacrificed by exsanguination according to the protocol approved by the Institutional Animal Care and Use Committees of People's Hospital Affiliated to Zhengzhou University. Primary rat ASMC cultures were isolated as previously described [20]. Briefly, large bronchi and airways without cartilages were used for the following experiment. Then pure airway smooth muscle bundles were cut free from surrounding tissues. After that, fresh culture medium (DMEM+FBS) was added and the cells were grown to confluency in an incubator with 5% CO_2 at 37°C. The cultured cells were pass aged using 0.25% trypsinization. After ASMCs pass aged 3-6 times, the cells were immunostained with anti- α -smooth muscle actin antibodies to confirm smooth muscle cells.

Immunocytochemistry

The cells were fixed with 4% paraformaldehyde and blocked with 2% bovine serum albumin. Then the cells were probed with primary antibodies specific to α -smooth muscle actin (1:100) at 4°C overnight and followed by incubation with secondary antibody (1:100) conjugated with a FITC fluorophore (green fluorescence) for 2 h at room temperature in the dark. Images were captured by using an Olympus LX-70 FluoView confocal laser-scanning microscope (Olympus, Japan).

Hematoxylin and eosin (HE) staining

Lung tissues were detached from the mice and fixed with 4% formalin solution overnight at 4°C. Then the tissues were embedded in paraffin and cut into 4-µm sections with a microtome (Leica, Germany). Hematoxylin and eosin staining (HE) staining was then performed as previously described [21]. In brief, hematoxylin treated for 8 minutes, 1% ethanol eosin for 3 minutes and mounted by neutral gum.

Cell transfection

Cells were cultured in six-well plates and transfected with control vector, si-TRPC1, TRPC1 overexpression vector, or si-TRPC3, TRPC3 overexpression vector using Lipofectamine 2000 (Sigma, USA) according to the manufacturer's instructions. Stable clones were acquired after antibiotic selection for 2 weeks. And control vector, si-TRPC1, TRPC1 overexpression vector, or si-TRPC3, TRPC3 overexpression vector were designed and synthesized by Genechem (Shanghai, China).

MTT assay

Rat ASMC were digested with 0.25% trypsin-EDTA and then seeded into 96-well culture plate. After 48 h, 20 μ L of the 12 mol/l MTT (Gibco, USA) was added to each well and incu-



Figure 1. Primary rat ASMCs. A. Typical "hill and valley" appearance in ASMCs visualized under phase-contrast microscopy. Magnification, $100 \times$. B. ASMCs were identified by immunocytochemistry following incubation with an anti- α -smooth muscle actin antibody. Magnification, $100 \times$. C and D. The airway of control and asthmatic groups observed by HE staining. In asthmatic groups, the mucosal fold membranes were broken and increased. Besides, the bronchial smooth muscle and the thickness of airway wall were increased significantly together with amounts of eosinophils were infiltrated into the airway submucosa. Magnification, $200 \times$. ASMCs, airway smooth muscle cells.

bated at 37°C for 4 h. Then medium was removed from the wells and added 150 μ l of DMSO (Sigma, USA) to dissolve the formazan crystals. Then the absorbance at 570 nm wavelength was read with an Elisa reader (Bio-tek ELX800, USA). Each group had six wells.

³H-TdR incorporation

³H-TdR incorporation method was performed to determine DNA synthesis. The cells were cultured in serum-free medium for 24 h. Then the cells were supplemented with 1 μ Ci ³H-TdR. The incorporation was stopped with cold PBS solution. Then 0.25% trypsin was added to digest cells. After that, cells were stabilized with 10% trichloroacetic acid, decolorized with absolute ethanol, dried at 80°C for 30 min, transferred

to scintillation fluid, and counted with a Tri-Carb 2810TR Low Activity Liquid Scintillation Analyzer (counts/min, cpm; PerkinElmer, USA).

Quantitative real-time PCR

Total RNA was extracted by using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocols. And cDNA was reverse transcribed from mRNA with an omniscript reverse transcriptase (Qiagen, CA). Then mRNA levels were quantified by RT-PCR with a Takara RNA PCR Kit (Takara, Dalian, China) on an Applied Biosystems 7900HT real-time PCR system. The relative expression levels were subsequently calculated using the $2^{-\Delta\Delta Ct}$ method [22]. GAPDH was used as an internal control. The primers used for real-time PCR were listed as follows: A novel therapeutic strategy for asthma



Figure 2. The expression of TRPC1 and TRPC3 in non-asthmatic and asthmatic rats. A and C. The mRNA level of TRPC1 and TRPC3 in non-asthmatic and asthmatic rats detected by qRT-PCR. B and D. The protein level of TRPC1 and TRPC3 in non-asthmatic and asthmatic rats measured by Western blot. Data are expressed as means \pm SD for each group. *P<0.05 vs. non-asthma group.

TRPC1-Forward: 5'-ATTCTCGCAGCATTTCCAGTT-AAG-3'; TRPC1-Reverse: 5'-TTACAGACCAAGG-GTTACCTGCAC-3'; TRPC3-Forward: 5'-CATTC-TCAATCAGCCAACACGTTAT-3'; TRPC3-Reverse: 5'-CTCAGTTGCTTGGCTCTTGTCTTC-3'; GAPDH-Forward: 5'-ACCACAGTCCATGCCATCAC-3'; GAP-DH-Reverse: 5'-TCCACCACCCTGTTGCTGTA-3'.

Western blot analysis

Cellular proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad, USA) and transferred to nitrocellulose membranes (Millipore, USA). Each membrane was incubated with a primary antibody against TRPC1, TRPC3, PCNA or GAPDH overnight at 4°C and then incubated with secondary antibodies for 2 h at room temperature. The bands were visualized by ECL Detection Reagents (Amersham Bioscience). And the images were analyzed by the NIH Image J software.

Statistical analysis

The data are expressed as the means \pm SD. Comparisons between two groups were evaluated by Student's *t*-test. All experiments were performed at least three times. Statistical analysis was performed using the software of SPSS 16.0 (SPSS Inc, USA). P<0.05 was considered as statistically significant. GraphPad Prism 6.0 software (GraphPad, USA) was used to make the figures.

Results

Characteristics and identification of rat ASMCs in primary culture

After 8-10 d cultivation, cells began to grow confluence and displayed the typical "hill and valley" appearance (**Figure 1A**). Moreover, α -actin was observed by immunocytochemistry and showed positive immunostaining of green parallel fiber in cytoplasm (**Figure 1B**). These results demonstrated ASMCs were isolated from airways successfully.

Pathological changes of airway remodeling

As shown in **Figure 1C** and **1D**, the mucosal fold membranes of bronchiole were broken and increased in asthmatic rats. Besides, the bronchial smooth muscle and thickness of airway wall were dramatically increased. Furthermore, amounts of eosinophils were infiltrated into the bronchial submucosa. These results meant airway remodeling was formed in the asthmatic model.

Expression of TRPC1 and TRPC3 in non-asthmatic and asthmatic rats

The mRNA expression of TRPC1 and TRPC3 in asthmatic rats remarkably increased compared with that in non-asthmatic group (Figure 2A and 2C). And as shown in Figure 2B and 2D, the



Figure 3. SKF96365 reduced the increased expression of PCNA and absorbance of MTT assay in asthmatic rats. A. The protein expression of PCNA in non-asthmatic and asthmatic rats measured by Western blot. B. The cell proliferation detected by MTT assay in non-asthmatic and asthmatic rats. C. The protein expression of PCNA in asthmatic rats with or without application of SKF96365, which is a TRP channel inhibitor. D. The cell proliferation detected by MTT assay in asthmatic rats with or without application of SKF96365, which is a TRP channel inhibitor. D. The cell proliferation detected by MTT assay in asthmatic rats with or without application of SKF96365. *P<0.05, **P<0.01 vs. non-asthma group or control group.

protein expression of TRPC1 and TRPC3 in asthmatic rat was significantly upregulated than that in non-asthmatic group. The results showed that the expression of TRPC1 and TRPC3 significantly increased at both transcriptional and translational levels in asthmatic rats.

SKF96365 reduced the upregulation of proliferating cell nuclear antigen (PCNA) expression and absorbance of MTT assay in asthmatic rats

It has been demonstrated that PCNA is chosen as a marker for cell proliferation [23]. As shown in **Figure 3A**, the protein expression of PCNA in asthmatic rats was notably increased compared with that in non-asthmatic group. In addition, the value A570 of asthma group was also significantly increased than that of non-asthmatic group (**Figure 3B**). However, SKF96365 could suppress the upregulation of PCNA expression induced by asthma (**Figure 3C**), Moreover, after SKF96365 treatment, the value A570 was decreased significantly in asthmatic rats (**Figure 3D**). These meant that SKF96365 could effectively inhibit cell overproliferation caused by asthma.

Effect of TRPC1 and TRPC3 on ASMC proliferation

To evaluate the effect of TRPC1 and TRPC3 on ASMC cell proliferation, ASMCs were transfected with control vector, si-TRPC1, TRPC1 overexpression vector, or si-TRPC3, TRPC3 overexpression vector. As shown in **Figure 4A** and **4C**, the protein expression levels of TRPC1 and TRPC3 were significantly increased in ASMCs transfected with TRPC1 and TRPC3 overexpression vector. However, the protein expression



Figure 4. The effect of TRPC1 and TRPC3 on cell proliferation in ASMCs. A. The protein levels of TRPC1 in ASMCs transfected with control vector, si-TRPC1 and TRPC1 overexpression vector. B. The proliferation of ASMCs transfected with control vector, si-TRPC1 and TRPC1 overexpression vector. C. The protein levels of TRPC3 in ASMCs transfected with control vector, si-TRPC3 and TRPC3 overexpression vector. D. The proliferation of ASMCs transfected with control vector, si-TRPC3 and TRPC3 overexpression vector. *P<0.05, **P<0.01 vs. control group.



Figure 5. The effect of TRPC1 and TRPC3 on DNA synthesis in ASMCs determined by thymidine (³H-TdR) incorporation assay. A. DNA synthesis of cells transfected with control vector, si-TRPC1 and TRPC1 overexpression vector. B. DNA synthesis of cells transfected with control vector, si-TRPC3 and TRPC3 overexpression vector. *P<0.05 vs. control group.

levels of TRPC1 and TRPC3 were significantly decreased in ASMCs transfected with si-TRPC1 and si-TRPC3.

Then MTT assay was used to detect cell proliferation. Compared with the cells transfected

with control vector, the value of A570 was significantly increased in the ASMCs transfected with TRPC1 and TRPC3 overexpression vector. In contrast, knockdown of TRPC1 and TRPC3 dramatically decreased the value of A570 (**Figure 4B** and **4D**). Moreover, ³H-TdR incorporation was performed to identify the cell proliferation in ASMCs. As shown in **Figure 5A** and **5B**, compared with the cells transfected with control vector, cellular DNA synthesis quantity was significantly upregulated in the ASMCs transfected with TRPC1 and TRPC3 overexpression vector. Nevertheless, cellular DNA synthesis quantity was remarkably downregulated in the ASMCs transfected with si-TRPC1 and si-TRPC3 than that transfected with control vector. These results all demonstrated that both TRPC1 and TRPC3 played important roles in cell proliferation in ASMCs.

Discussion

In the present study, we investigated the role of TRPC1 and TRPC3 in cell proliferation of airway smooth muscle cells. Firstly, we established the asthma models induced by ovalbumin. Secondly, the increased expression of TRPC1 and TRPC3 in asthmatic rats was observed. Thirdly, we found SKF96365 could effectively inhibit cell over-proliferation caused by asthma. Finally, si-TRPC1 and si-TRPC3 abrogated the increasing cell proliferation of ASMCs. However, overexpression of TRPC1 and TRPC3 showed the opposite tendency. These results demonstrated that targeting TRPC1 and TRPC3 may be a good method to suppress the proliferation of ASMCs.

Airway remodeling is considered as an important pathogenic alteration in the asthmatic airway. A number of typical pathological characteristics are linked to the remodeling response, such as, epithelial hypertrophy, increase in basal membrane thickness, myocyte hyperplasia and hypertrophy, subepithelial fibrosis in the lungs [24, 25]. Moreover, smooth muscle remodeling is associated with a reduction in lung function, which gives rise to a more severe asthma phenotype [26]. Thus, it is essential to study the molecular mechanisms of ASMC proliferation in asthma.

In order to elucidate the molecular mechanisms of ASMC proliferation, we focused on the members of the TRPC family and examined the expression of TRPC1 and TRPC3 in asthmatic and non-asthmatic rats. And higher mRNA and protein levels of TRPC1 and TRPC3 were observed in asthmatic rats. Therefore, TRPC1 and TRPC3 might contribute to the ASMC proliferation in asthma.

It has been demonstrated that membrane depolarization exists in ovalbumin-sensitized ASMCs [27]. Subsequently, membrane depolarization can cause extracellular Ca2+ influx [28]. As TRPCs participates in controlling [Ca²⁺] in SMCs, they may play a key role in asthma [29]. TRPC1 is a transmembrane protein expressed in smooth muscle, endothelium and salivary gland cells [30]. Moreover, many biological roles of TRPC1 are found, such as regulation of smooth muscle cell proliferation [31], salivary gland secretion [32], neuronal differentiation [33], growth cone turning [34] and so on. In previous studies, it has been demonstrated that the expression of TRPC1 increased significantly in the sensitized rats and si-TRPC1 abrogated the increasing proliferation of ASMCs [35]. Besides, TRPC1 knockdown repressed the proliferation of pulmonary artery smooth muscle cells [31]. Furthermore, the expression of TRPC1 increased in proliferating ASMCs [36]. Our results were in agreement of the previous studies.

TRPC3 is a major molecular component of native non-selective cation channels (NSCCs) to increase [Ca2+], in freshly isolated airway SMCs [15]. White et al. revealed that mRNA and protein expression of TRPC3 were significantly increased in pass aged human airway SMCs after treatment with TNFa, an important asthma mediator [37]. Chen et al. found TRPC3mediated Ca2+ entry resulted in LPS-induced ASMC proliferation [38]. Song et al. suggested allergens stimulated the increase of TRPC3 channel expression and activity, which caused the increase in proliferation of ASMCs, leading to airway remodeling, and ultimately asthma [39]. In our studies, we showed the similar results that the mRNA and protein expression of TRPC3 significantly increased in asthmatic rats. And overexpression of TRPC3 increased the proliferation of ASMCs.

The inhibitory effect of SKF 96365 on Ca²⁺ entry has been reported. Because SKF 96365 could inhibit the endoplasmic reticulum (ER) Ca²⁺ pump in epithelial cells [40]. Besides, SKF 96365 is an inhibitor of multiple TRP channels [41]. Furthermore, proliferating cell nuclear antigen (PCNA) changes throughout the cell cycle, but it is dramatically elevated in a narrow time during cell division, hence, it is a marker of cell proliferation [42]. In present studies, we investigated the effect of SKF 96365 on the protein expression of PCNA and absorbance of MTT assay in asthmatic rats. And the results showed SKF 96365 could reduce the cell proliferation in asthma.

In conclusion, we proposed that the proliferation of airway smooth muscle cells was regulated by the expression of TRPC1 and TRPC3. These findings provide a novel insight into the role of TRPC1 and TRPC3 in asthma and propose a novel therapeutic strategy for asthma.

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Disclosure of conflict of interest

None.

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