

Effect of TRPV1 channel on the proliferation and apoptosis in asthmatic rat airway smooth muscle cells

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ABSTRACT

Background: Hyperplasia of airway smooth muscle cells (ASMC) is a major contributor to airway remodeling in asthma. Transient receptor potential vanilloid 1 (TRPV1) is an important channel to mediate Ca^{2+} influx. This study explores the expression of TRPV1 channel and its effect on the proliferation and apoptosis in rat ASMC, in order to find a new target to treat airway remodeling in asthma. **Methods:** Rats were sensitized and challenged with ovalbumin to replicate asthmatic models. Proliferating cell nuclear antigen (PCNA) was detected by immunohistochemistry. Reverse transcriptase-polymerase chain reaction, immunocytochemistry, and Western blot were used to detect the mRNA and protein expression of TRPV1 channel. Intracellular calcium ($[\text{Ca}^{2+}]_i$) was detected using confocal fluorescence Ca^{2+} imaging. $[\text{H}^3]$ thymidine incorporation and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay were used to observe the DNA synthesis and proliferation. TUNEL assay was used to detect the apoptosis of ASMC. **Results:** (1) The expression of PCNA was significantly increased in intact asthmatic rat ASMC. (2) The expression of TRPV1 channel was significantly increased in asthmatic rat ASMC. (3) $[\text{Ca}^{2+}]_i$ in ASMC of the asthmatic group was significantly increased. After treatment with TRPV1 agonist capsaicin (CAP), $[\text{Ca}^{2+}]_i$ was further increased, whereas $[\text{Ca}^{2+}]_i$ was decreased after administration of TRPV1 antagonist capsazepine (CPZ) in ASMC of the asthmatic group. (4) The DNA synthesis and absorbance of MTT were significantly increased, while apoptosis was significantly decreased in asthmatic ASMC. CAP further enhanced proliferation and decreased apoptosis. CPZ significantly inhibited the effect of CAP in asthmatic ASMC. **Conclusion:** TRPV1 channel was involved in the regulation of proliferation and apoptosis in asthmatic ASMC.

KEYWORDS airway smooth muscle cells (ASMC), apoptosis, asthma, proliferation, transient receptor potential vanilloid 1 (TRPV1)

INTRODUCTION

Bronchial asthma is a highly prevalent chronic respiratory disease that is seriously hazardous to human health. Because the pathogenesis of asthma has not been clear, the current treatment can only achieve symptom control. Therefore, further clarifying the pathogenesis of asthma and looking for more targeted treatment can help in better asthma management. Airway remodeling is an important pathological feature of asthma, which is the pathological basis of irreversible airflow obstruction in asthma. As a major

component of airway wall, the proliferation of airway smooth muscle cells (ASMC) is one of the important reasons of airway remodeling [1]. Transient receptor potential vanilloid (TRPV) is an important channel that plays the key role in cell proliferation and differentiation, especially for TRPV1 [2, 3]. Some studies have found that genetically determined level of TRPV1 channel is relevant for asthma pathophysiology. Recasens et al. [4] had reported that the loss of function of TRPV1 genetic variant was associated with lower risk in active childhood asthma. However, whether TRPV1 was involved in the proliferation regulation of rat ASMC and its effect on airway remodeling have not been reported. Therefore, this study is going to further explore the effect of TRPV1 channel on intracellular Ca^{2+} concentration, proliferation and apoptosis in asthmatic rat ASMC in order to find a new target to treat airway remodeling of asthma in clinics.

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MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), bovine serum albumin (BSA), 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 0.25% trypsin-EDTA, fetal bovine serum (FBS), TRIZOL and ethylene glycol tetraacetic acid (EGTA) were obtained from Gibco (USA). Alexa Fluor 488-conjugated chicken polyclonal to mouse IgG, Fluo 4-AM and ER-Tracker Green were from Invitrogen (USA). Omniscript reverse transcriptase kit, TRPV1 primer (QT00193907) and β -actin (QT00193473) and polymerase chain reaction (PCR) reaction kit were obtained from Qiagen (USA). Primary mouse monoclonal anticalponin antibody (ab700), rabbit polyclonal antibody specific to TRPV1 (ab63083) and β -actin (ab75186) and goat antirabbit horseradish peroxidase (HRP)-conjugated secondary antibody (ab97069) were from Abcam (UK). Primary rabbit polyclonal antibody specific to α -actin (sc-130619) and proliferating cell nuclear antigen (PCNA; sc-7907), secondary antirabbit antibody conjugated with fluorescein isothiocyanate (FITC) fluorophore (sc-2012) and Texas Red (sc-2780) and 4',6-diamidino-2-phenylindole (DAPI) were all from Santa Cruz Biotechnology Inc. (USA). Capsaicin (CAP), capsaizipine (CPZ), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ovalbumin (OVA), aluminum hydroxide, dimethyl sulfoxide (DMSO) and [^3H] thymidine were obtained from Sigma (USA). Inactivated bordetella pertussis vaccine was from Chengdu Institute of Biological Product (China). Hematoxylin and eosin (HE) staining kit was obtained from Wuhan Boster Biological Engineering Co., Ltd. (China). TUNEL assay kit was from Roche (USA).

Establishment of Chronic Rat Asthmatic Model

Twenty male Sprague-Dawley rats (5–6 weeks old, 180–200 g body weight) were randomly divided into normal control group and chronic asthmatic group. The rats were kept in specific pathogen-free animal research facilities and received food and water *ad libitum*, and all procedures performed on the animals were in compliance with the Chinese Council of Animal Care Guidelines (approved by Zhengzhou University Animal Care and Use Committee). The chronic asthmatic model was replicated referring to reported method [5], rats were actively sensitized with intraperitoneal injection of 10 mg OVA, together with 200 mg aluminum hydroxide in 1 mL saline, and

1 mL inactivated bordetella pertussis vaccine (6×10^9 heat-killed baciUin) was administered intraperitoneally on the first day (Day 1). The rats were sensitized again on the eighth day (Day 8). Then the next 6 weeks, the rats were exposed to aerosolized 2% OVA in saline, which was generated by an ultrasonic nebulizer with 3 mL/minute output for 30 minutes and repeated three times a week. After challenged, the rats showed symptoms of asthmatic attack such as agitation, bucking, tachypnea, cyanosis, etc. In the normal control group, the rats were sensitized and challenged with the same volume of saline instead of OVA. The rats were sacrificed within 18–24 hours after the final challenge. Take the lower lobe of left lung for mid-sagittal sections (5 μm in thickness), and then HE staining, pathomorphological image analysis and immunohistochemistry staining were done. The right lung tissue was used for ASMC culture.

Pathomorphological Image Analysis of Airway Remodeling

Lung tissue frozen sections stained with HE staining kit were selected and analyzed with 422BLK8079 High-resolution Medical Colorful Image Analyzing System (Yiming Technology Development Co., Ltd., China). The airway internal perimeter (P_i), wall area (WA), external perimeter (P_e), the area of bronchial smooth muscle (S) and the number of bronchial smooth muscle nucleus (N) were measured. Then the above measured values were standardized by P_i and WA/P_i , S/P_i , N/P_i were represented, respectively.

Immunohistochemistry Staining for PCNA Expression

Lung tissue frozen sections were washed three times with physiological saline solution (PSS). After blocked with 1% BSA and 0.5% Triton X-100 for 1 hour, the tissues were incubated with the 1:100 primary rabbit polyclonal antibody specific to α -actin and PCNA overnight at 4°C. Then the tissues were washed and incubated with the 1:500 secondary antirabbit antibodies for α -actin conjugated with an FITC fluorophore (green fluorescence) and for PCNA conjugated with Texas (red fluorescence) for 2 hours at room temperature in the dark. Fluorescence images were obtained using a laser-scanning confocal microscope (Olympus, Japan). Excitation of FITC was by illumination with the 488 nm line and 543 nm for Texas Red. The ASM layer present green fluorescence for α -actin. The positive staining for PCNA is the red fluorescence in the nuclei. All images were analyzed using the Olympus microscope 4.0 analyzing software. For the measurement

of PCNA expression, the mean fluorescence intensity was quantified. The fluorescence intensity was normalized to the normal control group.

ASMC Isolation and Culture

Using the reported method to culture ASMC with some improvement [6, 7], Sprague-Dawley rats were deeply anesthetized with pentobarbital sodium (5 mL/kg ip) and euthanized by exsanguination according to a protocol approved by the Institutional Animal Care and Use Committees of Zhengzhou University. Large bronchi that were dissected from the surrounding parenchyma were washed three times in precooled D-Hanks' balanced salt solution (in mM): 118 NaCl, 4.7 KCl, 1.2 NaH₂PO₄, 25.5 NaHCO₃ and pH value adjusted to 7.4 with 1 mM NaOH, containing antibiotics (penicillin G 100 U/mL and streptomycin 100 U/mL). Airways without cartilages were selected and pure airway smooth muscle bundles were dissected free from surrounding tissues with the aid of a dissecting microscope. Then the airways were cut open and the epithelial cells were removed by gently stripping the luminal surface with a blade. The minced tissue (1 mm³ fragments) was seeded into sterile 25 mL culture flasks and 2–3 mL DMEM containing 20% (v/v) FBS was added. The flasks were placed in a 5% CO₂ humidified incubator at 37°C. The culture medium was replaced regularly (2–3 days) until cell confluence occurred (usually between 8 and 10 days). Then the cells were digested with 0.25% trypsin-EDTA and subcultured in DMEM with 10% FBS. The cells were used for experiment between passages 3 and 5.

Immunocytochemistry for Calponin to Identify Smooth Muscle Cells

The cells were fixed with 4% formaldehyde in PSS for 10 minutes and permeabilized with 0.3% Triton X-100 before blocking with 2% BSA was done for 30 minutes. The samples were incubated with primary antibody (1:300 in PSS with 2% BSA and 0.3% Triton X-100) overnight at 4°C. An Alexa Fluor 488-conjugated chicken polyclonal to mouse IgG (green) was used as secondary antibody at 1:400 dilution. DAPI was used to stain the cell nuclei (blue).

Grouping of ASMC

ASMC were cultured in DMEM with 10% FBS for 24 hours, and then the medium was changed to serum-free DMEM for a further 24 hours to synchronize the cells. Then ASMC of the two groups were randomly divided into four groups: (1) blank control

group: ASMC were cultured in 10% FBS group without any intervention; (2) CAP group: ASMC were cultured in 10% FBS with 1 μ M CAP; (3) CPZ group: ASMC were cultured in 10% FBS with 1 μ M CPZ; and (4) CAP+CPZ group: ASMC pretreated with 1 μ M CPZ for 1 hour and then 1 μ M CAP was added. Culture lasted another 24 hours. Both CAP and CPZ were dissolved into DMSO. The effective doses of CAP and CPZ were determined by our previous studies, and these concentrations were consistent with those reported in Ref. [8].

Reverse Transcriptase-Polymerase Chain Reaction to Detect the Expression of TRPV1 mRNA

Total RNA was isolated and purified using TRIzol reagent according to the manufacturer's instructions; mRNA was synthesized into cDNA with the aid of an omniscrypt reverse transcriptase using 100 ng of RNA per reaction. PCR amplification (40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 68°C for 1 minute; 72°C for 5 minutes) was performed with 5 μ L of cDNA using the specific TRPV1 primer and β -actin, yielding a product of 104 bp and 145 bp, respectively. Amplified PCR products were confirmed by electrophoresis on 1.5% agarose gel containing ethidium bromide. The straps of amplified products were analyzed with MUVB-20 gel analysis system (Ultralum Corporation, USA). The optic density was normalized to β -actin.

Immunocytochemistry to Localize the TRPV1 Channel

ASMC were fixed with 4% formaldehyde for 10 minutes, permeabilized with methanol (–80°C), blocked with 2% BSA and incubated with 1:250 primary rabbit polyclonal antibody specific to TRPV1 overnight at 4°C. Cells were washed and incubated with 1:1000 fluorescent antirabbit secondary antibody conjugated with Texas Red for 2 hours at room temperature in the dark. For specific sarcoplasmic reticulum (SR) membrane staining of TRPV1, cells were incubated in ER-Tracker™ Green in PSS for 30 minutes at 37°C. Then DAPI was used to stain the cell nuclei. Excitation of FITC was by illumination with the 488 nm line, 543 nm for Texas Red and 405 nm for DAPI. All images were analyzed using the Olympus micro-image 4.0 analyzing software.

Western Blot to Detect the Expression of TRPV1 Protein

Rat ASMC were washed with cold *phosphate buffered saline* (PBS) and detached with 0.25% trypsin.

Whole cell protein was extracted on ice for 30 minutes with lysis buffer (50 mM Tris-HCl [pH 8.0], 0.15M NaCl, 0.1% sodium dodecyl sulfate [SDS], 100 $\mu\text{g/mL}$ phenylmethylsulfonyl fluoride, 1 $\mu\text{g/mL}$ aprotinin, 0.02% sodium azide, 0.5% sodium deoxycholate and 1% Nonidet P-40). Samples were centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatants were collected and protein concentrations were measured using the BCA protein assay kit (Pierce Chemical Company, USA). Equal amounts of protein extracts (30 μg) were combined with a 5 $\times$ gel loading buffer (10% SDS [w/v], 50% glycerol [v/v], 0.25 M Tris-HCl [pH 6.8], 0.025% bromophenol blue [w/v] and 0.5 M dithiothreitol) and boiled for 10 minutes before cooling to room temperature. Proteins were subjected to SDS-polyacrylamide gel electrophoresis (12% w/v) at 110 V for 2 hours in a buffer consisting of 0.1% SDS, 25 mM Tris and 250 mM glycine (pH 8.4) and then transferred onto a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline (TBS: 10 mM Tris and 150 mM NaCl, pH 7.6) containing 0.05% Tween 20 (TTBS) and 5% nonfat milk at room temperature for 2 hours. After a brief rinse with TTBS, the membrane was incubated overnight at 4°C with rabbit primary polyclonal antibody to TRPV1 and β -actin (1:500) in TTBS containing 5% dried skimmed milk. Then the membrane was incubated with antirabbit HRP-conjugated secondary antibody (1:5000 in TTBS containing 5% dried nonfat milk) for 1 hour. The membrane was then washed again and developed with an ECL assay kit (Bio-Rad Laboratories, Inc., USA) before the exposure to Kodak film (Alao Scientific, USA). The bands of interest were confirmed by comparison with

molecular mass markers. The blots were quantified by the Quantity One imaging system (Bio-Rad). Densitometry data were normalized to β -actin values.

Confocal Fluorescence Ca^{2+} Imaging to Detect $[\text{Ca}^{2+}]_i$ With Fluo 4-AM

Referring the reported method to detect intracellular Ca^{2+} [9], Fluo 4-AM was chosen as an intracellular free Ca^{2+} fluorescent probe for the analysis of Ca^{2+} concentration under a confocal fluorescence microscope. ASMC were loaded with 4 μM Fluo 4-AM for 20 minutes at 37°C, 5% CO_2 in the dark. Then cells were washed with and imaged in physiological HEPES-buffered solution (in mM): 146 NaCl, 4.7 KCl, 2.5 CaCl_2 , 0.6 MgSO_4 , 0.15 NaHPO_4 , 0.1 ascorbic acid, 8 glucose and 10 HEPES (pH 7.4). The system was built around an inverted Olympus IX-70 microscope equipped with an Olympus PlanApo $\times 60$ oil-immersion lens and a Nikon camera. The protocol was Zarine-488 FITC 150 with 100 ms exposure time. All experiments were performed at room temperature (22°C–25°C). $[\text{Ca}^{2+}]_i$ was represented with the mean fluorescence intensity. The fluorescence intensity was normalized to the blank control of the normal group.

Detecting Proliferation of ASMC

$[\text{H}^3]$ thymidine incorporation to detect DNA synthesis

$[\text{H}^3]$ thymidine incorporation was performed as described in Ref. [10]. Cells were plated into

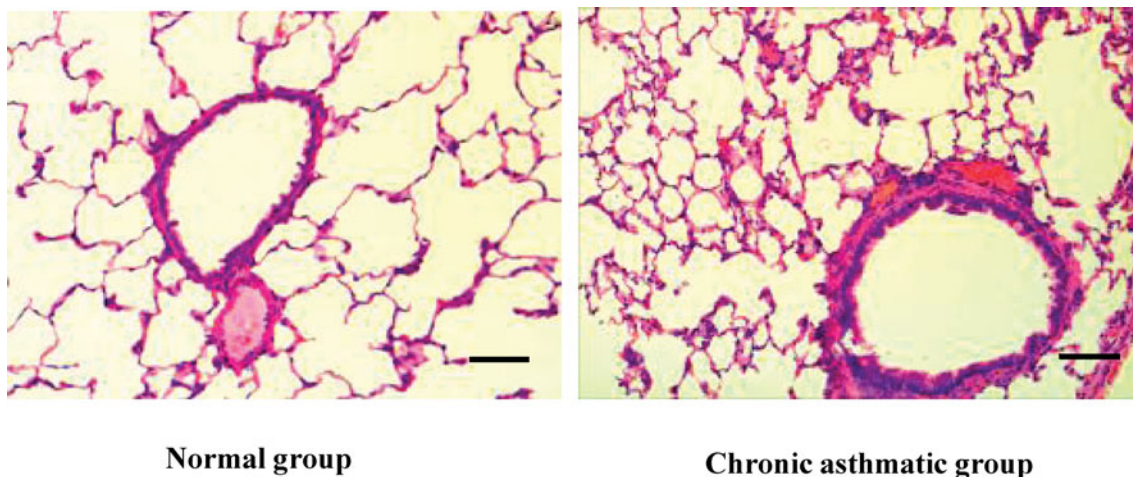


FIGURE 1. The airway of control and asthmatic groups (HE staining). In chronic asthmatic airway, the mucosal fold membranes were increased and broken. A lot of eosinophils were infiltrated into the airway submucosa and the thickness of airway wall and bronchial smooth muscle was significantly increased (bar = 20 μm).

96-well plates. Following 6 hours of incubation with $1 \mu\text{Ci/mL}$ [^3H] thymidine ($3.7 \times 10^7 \text{ kBq/mL}$) at 37°C , the cells were washed twice with cold PBS and three times with ice-cold 10% trichloroacetic acid. Then the cell lysis was obtained with 1M NaOH and harvested onto scintillation vial, and 500 μL scintillation fluids were added. The radioactivity was monitored with a Beckman multifunction liquid scintillation counting meter (Beckman Coulter, USA) and counts per minute (cpm) were determined. The results were expressed as cpm/ 10^6 cells.

MTT assay for cell proliferation

ASMC were seeded in 96-well plates with the concentration of $1 \times 10^4/\text{mL}$ in replicates of six. After the cells were intervened for 24 hours, the medium was removed and replaced it with 100 μL fresh DMEM. MTT (12 mM) was added to the wells (20 $\mu\text{L}/\text{well}$). After a 4-hour incubation at 37°C , the medium was removed from the wells and DMSO was added (150 $\mu\text{L}/\text{well}$). The plates were agitated gently at room temperature for 10 minutes. Then the absorbance at 490 nm wavelength (value of A_{490}) was

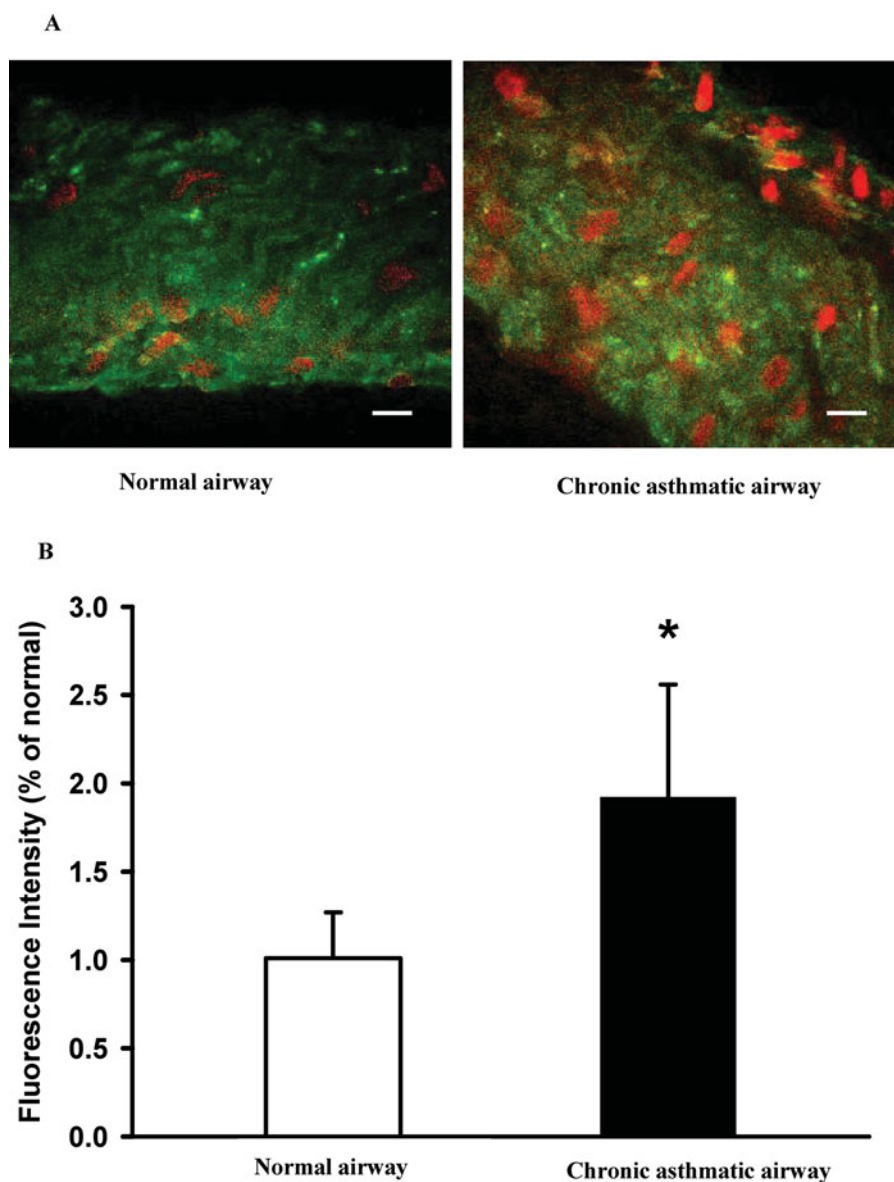


FIGURE 2. Expression of PCNA in epithelium cells and smooth muscle cells of intact rat airway. The ASM layer presents green fluorescence for α -actin. (A) The positive expression of PCNA presents red fluorescence in the nuclei of epithelium cells and smooth muscle cells of the airway. (B) The fluorescence intensity of PCNA in asthmatic ASMC significantly increased compared with the normal ASMC (* $P < .05$ vs. the normal group; $N = 8$).

TABLE 1. Comparison of WA/P_1 , S/P_1 and N/P_1 of Airway in Each Group (Means $\pm$ SEM)

Group	WA/P_1 ($\mu\text{m}^2/\mu\text{m}$)	S/P_1 ($\mu\text{m}^2/\mu\text{m}$)	N/P_1 (n/ μm)
Control group	7.03 ± 0.29	2.55 ± 0.26	0.0179 ± 0.0047
Chronic asthmatic group	$12.46 \pm 1.29^*$	$3.73 \pm 0.51^*$	$0.0391 \pm 0.0174^*$

* $P < .05$ vs. the control group. $N = 10$.

read with an enzyme-linked immunosorbent assay reader (Bio-tek ELX800, Japan). Absorbance value is proportional to the number of the living cells.

TUNEL assay to detect ASMC apoptosis

DNA 3'-OH ends of apoptotic nuclei were detected by terminal transferase-mediated dUTP (deoxyuridine) nick end labeling, operating strictly according to the instructions. The cells were fixed with 4% paraformaldehyde for 30 minutes. Then the endogenous peroxidase were inactivated by 3% H_2O_2 . Triton X-100 (0.1%) was added to permeabilize the cells, and the cells were incubated with TUNEL reaction solution for 1 hour at 37°C . Then the cells were developed with alkaline phosphatase (AP). The cover slide was sealed with neutral gum, and observed under the

light microscopy. That was set as the negative control which PBS was instead of terminal dUTP transferase enzyme solution. The dark blue stainings of the cell nuclei were the positive cells (apoptotic cells). Six fields were randomly selected and the apoptotic cells were counted. Then apoptotic index (AI) was calculated using $\text{AI} = \text{apoptotic cells}/\text{total cells} \times 100\%$.

Statistical analysis

All data were expressed as means $\pm$ standard error of mean (SEM). Statistical significance between the two groups was evaluated using Student's t -test. Differences among several groups were determined with the one-way ANOVA. Differences were considered significant when $P < .05$. Sigma Plot v11.0 was used to make the figures.

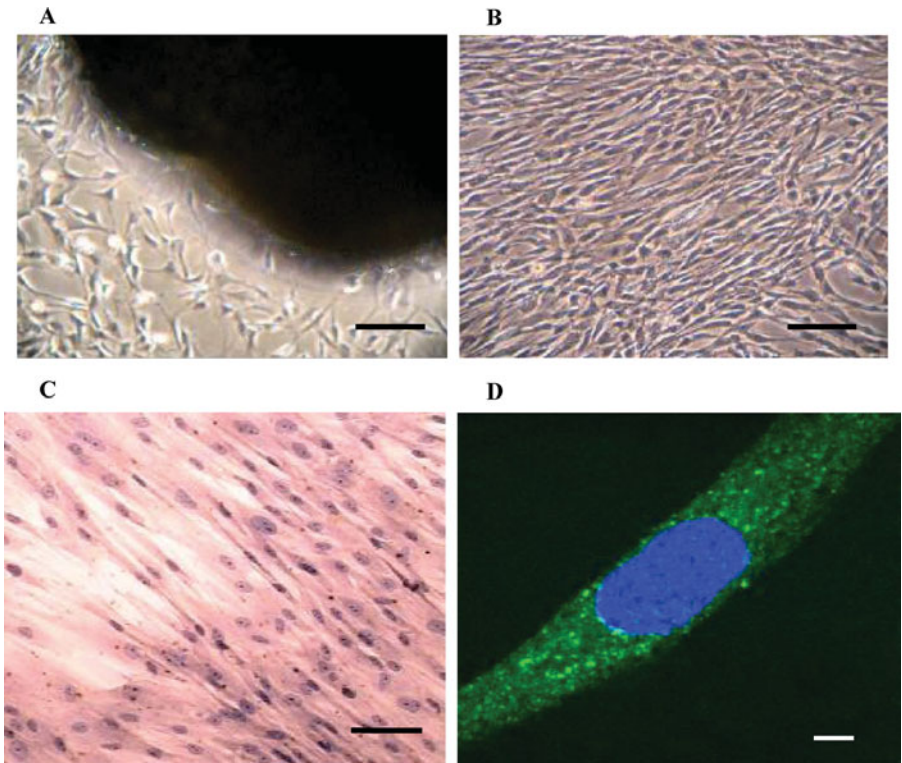


FIGURE 3. Primary rat ASMC. (A) Cells growing around the muscle tissues with spindle-shaped cell body and central oval nuclei in 2–3 days (phase-contrast microscopy; bar = $100\ \mu\text{m}$). (B) Cells confluence occurring in 8–10 days (phase-contrast microscopy; bar = $100\ \mu\text{m}$). (C) Typical “hill and valley” appearance in ASMC (HE staining; bar = $50\ \mu\text{m}$). (D) Immunofluorescence staining for calponin showed positive green fluorescence in cytoplasm using a confocal microscopy (bar = $10\ \mu\text{m}$).

RESULTS

Pathomorphological Images of Airway Remodeling

As shown in Figure 1, in chronic asthmatic rats, the mucosal fold membranes of the airway were increased and broken. A lot of eosinophils were infiltrated into the airway submucosa and the thickness of airway wall and bronchial smooth muscle was significantly increased. WA/P_i , S/P_i and N/P_i of the normal control and chronic asthmatic groups were shown in Table 1.

Expression of PCNA in the Intact Rat Airways

The ASM layer presents green fluorescence for α -actin. The positive expression of PCNA presents red fluorescence in the nuclei of epithelium cells and smooth muscle cells of the airway (see Figure 2A). The fluorescence intensity of PCNA in asthmatic ASMC (1.92 ± 0.64) significantly increased compared with the normal ASMC (1.01 ± 0.26 , $P < .05$; see Figure 2B).

Characteristics and Identification of Rat ASMC in Primary Culture

Under phase-contrast microscopy, cells emerged from the explants (2–3 days) with spindle-shaped cell body and central oval nuclei. On 8–10 days, the cells began to merge and displayed the typical “hill and valley” morphology at confluence (see Figure 3A, B, and C). Immunofluorescence staining for calponin using a confocal microscopy showed positive green fluorescence in cytoplasm (see Figure 3D). The cultured cells were identified as smooth muscle cells.

Expression of TRPV1 Channel in Rat ASMC

Expression of TRPV1 channel mRNA

As shown in Figure 4, compared with the blank control of normal rats (1.01 ± 0.09), the mRNA expressions of TRPV1 (1.83 ± 0.40) were manifestly elevated in ASMC in the blank control of chronic asthmatic rats ($P < .05$). Compared with the blank control of chronic asthmatic rats, the expression of TRPV1 mRNA was manifestly elevated in the CAP group (2.54 ± 0.6) and manifestly reduced in the CPZ group (0.92 ± 0.21 ; $P < .05$), while there was no significant difference in the CPZ+CAP group (1.81 ± 0.35).

Expression of TRPV1 channel protein

TRPV1 channel protein was detected by immunocytochemistry and Western blot. Immunocytochem-

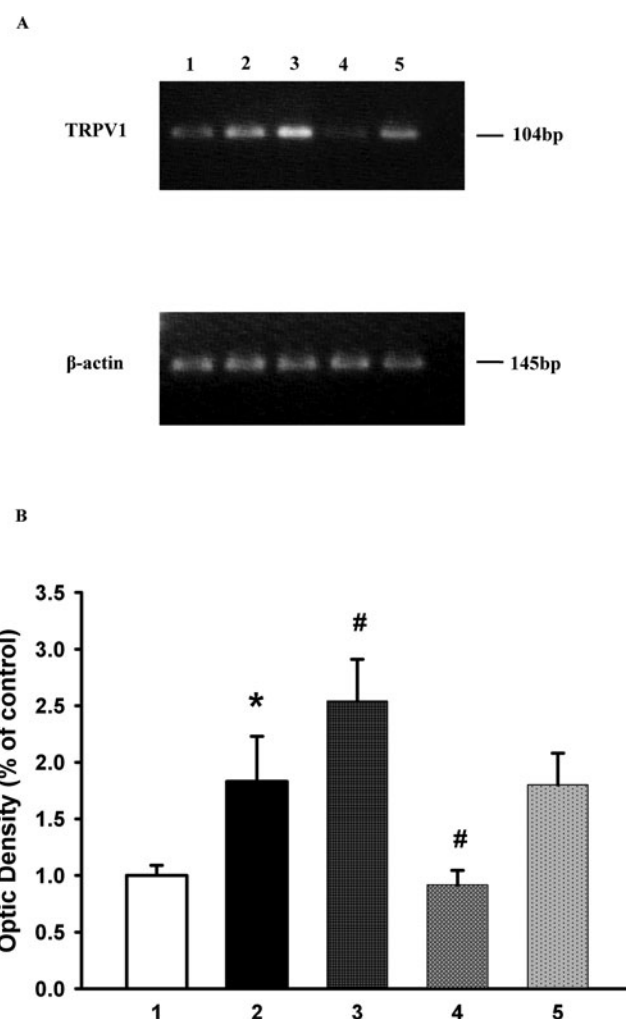


FIGURE 4. RT-PCR for TRPV1 mRNA expression in rat ASMC of each group ($n = 8$). 1: The blank control of normal rats; 2: the blank control of chronic asthmatic rats; 3: the CAP group of chronic asthmatic rats; 4: the CPZ group of chronic asthmatic rats; 5: the CPZ+CAP group of chronic asthmatic rats (* $P < .05$ vs. the blank control of the normal group. # $P < .05$ vs. the blank control of the chronic asthmatic group).

istry in conjunction with membrane dyes specific for SR showed that TRPV1 channels (red) are localized to SR membrane (green) in the ASMC. At the same time, TRPV1 channels are also present at the plasmic membrane. Therefore, TRPV1 channels play a role in regulating intracellular Ca^{2+} in ASMC. Compared with the normal ASMC, the fluorescence intensity of TRPV1 in asthmatic ASMC was increased (see Figure 5A).

The expression of TRPV1 protein was quantified by Western blot. Compared with the blank control of normal rats (0.99 ± 0.21), there was a significant increase of TRPV1 protein in the blank control of chronic asthmatic rat ASMC (2.26 ± 0.53 ; $P < .05$). Compared with the blank control of chronic

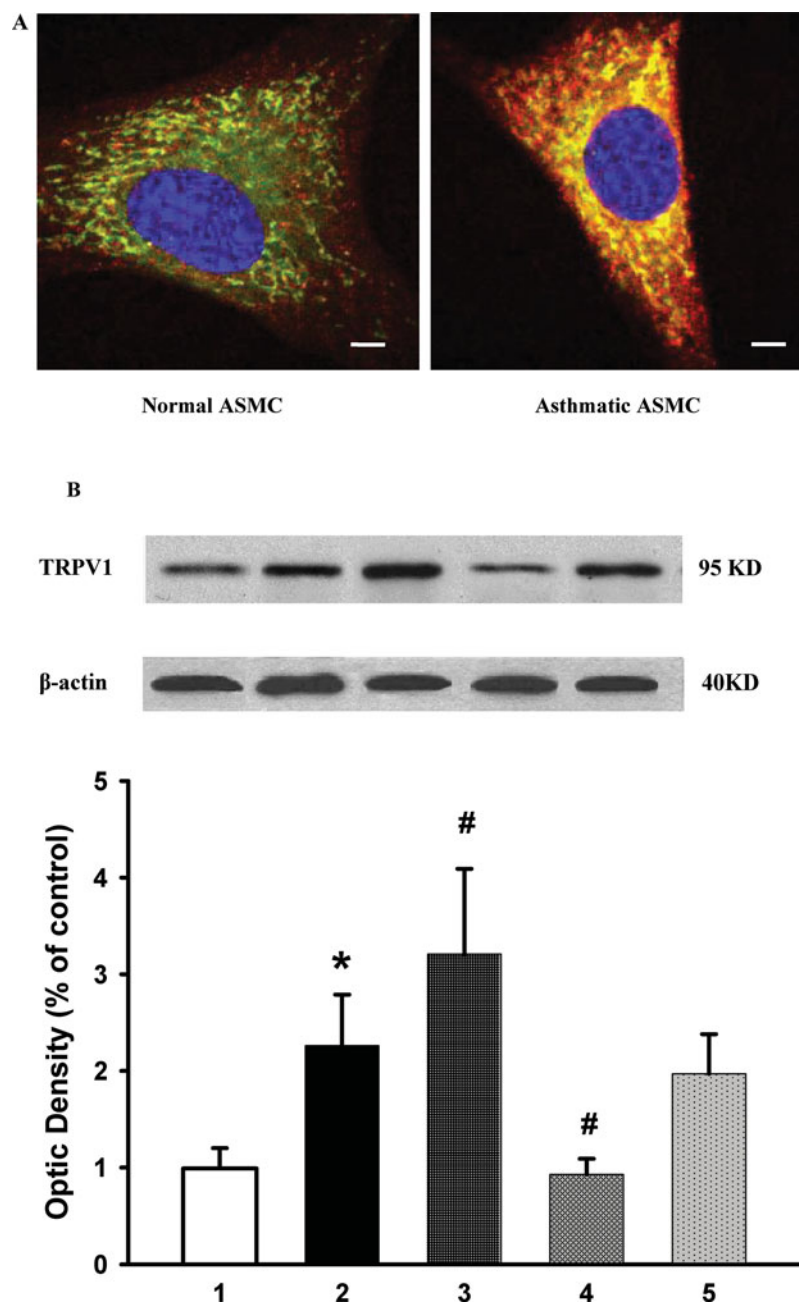


FIGURE 5. Immunocytochemistry and Western blot for TRPV1 protein expression in rat ASMC of each group ($n = 8$). (A) Immunolabeled for TRPV1 channels (red) and stained for SR membrane using ER-Tracker Green (green). Nuclei were dyed with blue by DAPI. The representative images showed that TRPV1 channels are localized to the plasmic membrane and SR membrane in primary ASMC. Compared with the normal ASMC, the fluorescence intensity of TRPV1 in asthmatic ASMC was increased (bar = 10 μ m). (B) Densitometric evaluation of Western blot for the expression of TRPV1 protein in rat ASMC. Densitometry data were normalized to β -actin values. 1: The blank control of normal rats; 2: the blank control of chronic asthmatic rats; 3: the CAP group of chronic asthmatic rats; 4: the CPZ group of chronic asthmatic rats; 5: the CPZ+CAP group of chronic asthmatic rats (* $P < .05$ vs. the blank control of normal group. # $P < .05$ vs. the blank control of chronic asthmatic group).

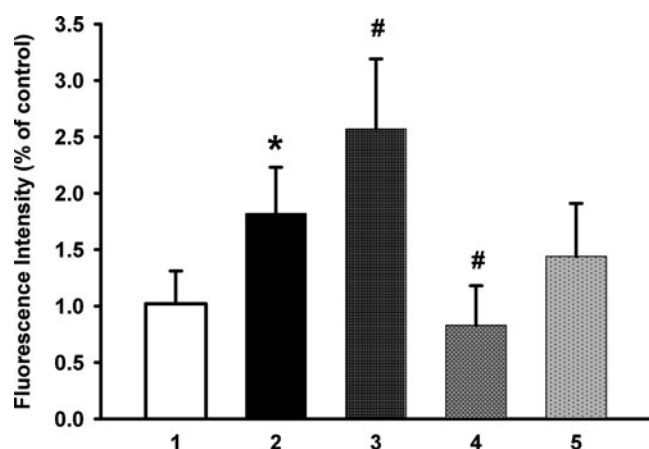


FIGURE 6. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat ASMC of each group. $[Ca^{2+}]_i$ was represented with the mean fluorescence intensity. The relative fluorescence intensity of each group was normalized to the blank control of the normal group. 1: The blank control of normal rats; 2: the blank control of chronic asthmatic rats; 3: the CAP group of chronic asthmatic rats; 4: the CPZ group of chronic asthmatic rats; 5: the CPZ+CAP group of chronic asthmatic rats (* $P < .05$ vs. the blank control of the normal group, # $P < .05$ vs. the blank control of the chronic asthmatic group. $N = 26-32$).

asthmatic rats, the expression of TRPV1 protein was significantly increased in the CAP group (3.21 ± 0.88) and significantly decreased in the CPZ group (0.93 ± 0.16 ; $P < .05$), while there was no significant difference in the CPZ+CAP group (1.97 ± 0.41 ; see Figure 5B).

Comparison of $[Ca^{2+}]_i$ in ASMC of each group

Compared with the blank control of normal rats (1.02 ± 0.29), the $[Ca^{2+}]_i$ in the blank control of chronic asthmatic rat ASMC (1.82 ± 0.41) was significantly increased. Compared with the blank control of chronic asthmatic rats, the $[Ca^{2+}]_i$ was significantly increased in the CAP group (2.57 ± 0.62 ; $P < .05$) and significantly decreased in the CPZ group (0.83 ± 0.35 ; $P < .05$), whereas there was no significant change in the CPZ+CAP group (1.44 ± 0.47 ; see Figure 6).

Effect of TRPV1 Channel on ASMC Proliferation

$[^3H]$ thymidine incorporation in ASMC

As shown in Table 2, compared with the blank control of normal rats, cellular DNA synthesis quantity was significantly increased in the blank control of chronic asthmatic rats ($P < .01$). In both the normal and chronic asthmatic rats, DNA synthesis quantities were significantly increased in the CAP group ($P < .01$) and significantly decreased in the CPZ group compared with the corresponding blank control group ($P < .05$), while there were no significant changes in the CPZ+CAP group.

MTT assay in ASMC

The absorbance of each group was shown in Table 2. Compared with the blank control of normal rats, the value of A_{490} was significantly increased in the blank control of chronic asthmatic rats ($P < .05$). In both the normal and chronic asthmatic rats, the values of A_{490} were significantly increased in the CAP group ($P < .01$) and significantly decreased in the CPZ group compared with the corresponding blank control group ($P < .05$), while there were no significant changes in the CPZ+CAP group.

TUNEL to detect apoptosis in ASMC

The main signs for apoptosis cells were nuclei stained dark blue by AP and karyopyknosis cracking (see Figure 7).

Compared with the blank control of normal rats, AI was significantly decreased in the blank control of chronic asthmatic rats ($P < .01$). No matter in the normal or chronic asthmatic rats, AI was significantly decreased in the CAP group ($P < .05$) and significantly increased in the CPZ group compared with the corresponding blank control group ($P < .01$), while there were no significant differences in the CPZ+CAP group (see Table 2).

TABLE 2. Effect of TRPV1 Channel on ASMC Proliferation and Apoptosis (Means $\pm$ SEM)

Groups	^3H -TdR(cpm/ 10^6 cells)		A_{490}		AI	
	Normal	Asthmatic	Normal	Asthmatic	Normal	Asthmatic
Control	1142.33 $\pm$ 79.42	1511.24 $\pm$ 101.61**	0.296 $\pm$ 0.049	0.414 $\pm$ 0.072*	9.68 $\pm$ 0.65	5.25 $\pm$ 0.54**
CAP group	1408.15 $\pm$ 121.73##	2016.44 $\pm$ 137.69##	0.513 $\pm$ 0.108##	0.664 $\pm$ 0.097##	5.31 $\pm$ 0.33##	3.63 $\pm$ 0.34#
CPZ group	902.27 $\pm$ 88.73#	910.61 $\pm$ 77.92##	0.176 $\pm$ 0.025#	0.255 $\pm$ 0.061#	12.68 $\pm$ 1.12##	10.45 $\pm$ 0.99##
CPZ+CAP group	1138.46 $\pm$ 68.85	1623.53 $\pm$ 128.13	0.282 $\pm$ 0.040	0.397 $\pm$ 0.052	9.4 $\pm$ 0.72	6.38 $\pm$ 0.71
Vehicle	1121.46 $\pm$ 57.46	1468.53 $\pm$ 99.83	0.277 $\pm$ 0.043	0.402 $\pm$ 0.061	9.82 $\pm$ 0.55	6.06 $\pm$ 0.44

* $P < .05$, ** $P < .01$ vs. the normal group; # $P < .05$, ## $P < .01$ vs. the control group. $N = 10$.

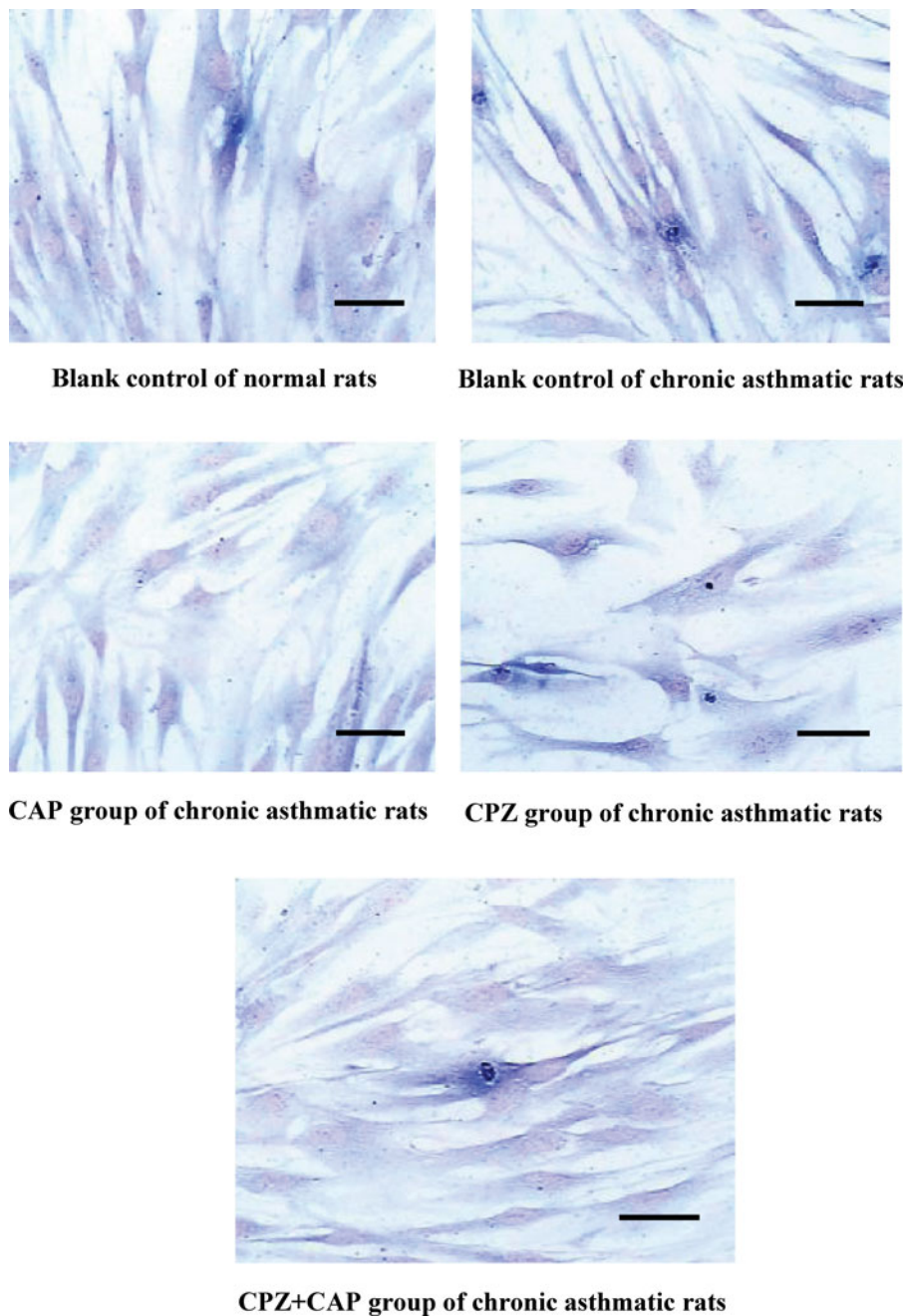


FIGURE 7. Representative images of apoptosis in rat ASMC of each group detected by TUNEL assay. The main signs for apoptosis cells were nuclei stained dark blue by alkaline phosphatase (AP) and karyopyknosis cracking. Apoptosis cells were significantly decreased in the blank control of chronic asthmatic rats. CAP further decreased the apoptosis cells. CPZ significantly inhibited the effect of CAP and increased the apoptosis cells in asthmatic ASMC (bar = 50 μ m).

DISCUSSION

In recent years, airway remodeling is considered to be an important part in the pathogenesis of asthma. This role in the pathogenesis and treatment of asthma has received more and more attention [11, 12]. Airway

remodeling is a series of chronic injury and repair processes characterized by the changes of airway wall cellular and molecular composition, quantity and structure, mainly with the performance of the deposition of extracellular matrix and basement membrane thickening, hyperplasia and hypertrophy of ASMC,

etc. [13, 14]. As the characteristic pathological changes of asthma, airway remodeling was the pathological basis of chronic, persistent, severe asthma, irreversible airway obstruction and also the pathological basis of steroid-resistant asthma [15, 16]. Therefore, prevention and treatment of airway remodeling are very important, which is the true sense of treatment and control of asthma. As a major component of the airway wall, hyperplasia and hypertrophy of ASMC is one of the important contributors to airway remodeling.

Cell apoptosis, programmed cell death, is a form of ubiquitously physiological cell death. Together with cell proliferation, they regulate the size and structure of the tissues [17]. Therefore, the airway remodeling is closely related to the imbalance between cell proliferation and apoptosis of ASMC. For this reason, the mechanism of ASMC proliferation and apoptosis is currently a hot topic in the research of asthmatic airway remodeling.

As a member of transient receptor potential superfamily, TRPV channel consists of six members (TRPV1–6). Various subtypes of TRPV are distributed in the skin, brain, cardiovascular system and the respiratory system with different degrees [18–21]. TRPV1 channels can be activated by high temperature, extracellular low pH values, inflammatory cytokines, certain chemical poisons and even more to wild herbs molecules (including CAP) [22–25]. Some studies have found that TRPV1 had the important influences on bronchoconstriction, protein secretion, airway mucosal edema and inflammatory cell chemotaxis [26–28]. In addition, the expression of TRPV1 receptor was increased in the patients with chronic cough, and the reason may be a reaction to the chronic inflammation [29–31]. However, whether TRPV1 channel exerted a function on the airway smooth muscle tissue remodeling induced by asthma is not clear. Therefore, this study is going to further explore the change in TRPV1 expression and intracellular Ca^{2+} concentration in ASMC, so as to clarify the effect of TRPV1 channel on the proliferation and apoptosis in asthmatic rat ASMC.

We replicated the chronic rats asthmatic model and found that the expression of TRPV1 channel was significantly increased in rat asthmatic ASMC, which mediated the increase of intracellular Ca^{2+} concentration. We also found that the cell proliferation enhanced and the apoptosis reduced, which induced hyperplasia of ASMC and airway remodeling formed. In order to further elucidate the role of TRPV1 in regulating ASMC proliferation, TRPV1 selective agonist CAP and inhibitor CPZ were used to treat ASMC. We found that CAP could increase the expression of TRPV1 and intracellular Ca^{2+} concen-

tration, and then the cell proliferation was enhanced and cell apoptosis was attenuated by CAP. On the contrary, CPZ could inhibit cell proliferation and increase apoptosis by diminishing the expression of TRPV1 and intracellular Ca^{2+} concentration. These findings suggested that the increase in the expression and activity of TRPV1 channel was involved in the hyperplasia in asthmatic ASMC. Therefore, inhibiting the hyperplasia of ASMC induced by asthma would be realized by attenuating the expression and activity of TRPV1 in ASMC. Thus the exact mechanism of CAP-induced increase in the expression of TRPV1 is unclear. The possible contributory mechanisms are that CAP could induce the increase in the release of substance P (SP) [32], calcitonin gene-related peptide (CGRP) [33] and other inflammatory factors, and then SP and CGRP cause the increase in the expression of TRPV1.

It had been reported that chronic hypoxia could significantly increase the expression of TRPV1 and intracellular Ca^{2+} concentration in human pulmonary artery smooth muscle cells (PASMC). PASMC proliferation was also enhanced in chronic hypoxia. Thus the researchers suggested that TRPV1 may be an important pathway or a regulating factor in these changes caused by hypoxia [34]. Our results are similar to that report. We also found that TRPV1 channel was an important regulating factor and pathway to affect ASMC proliferation, hypertrophy and airway remodeling induced by asthma. Our findings have an important significance in studying the pathophysiology of asthma and provide a new target to prevent and treat asthma.

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