Curcumin inhibits the proliferation of airway smooth muscle cells *in vitro* and *in vivo*

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Abstract. The inhibition of the proliferation of airway smooth muscle cells (ASMCs) is crucial for the prevention and treatment of asthma. Recent studies have revealed some important functions of curcumin; however, its effects on the proliferation of ASMCs in asthma remain unknown. Therefore, in this study, we performed *in vitro* and *in vivo* experiments to investigate the effects of curcumin on the proliferation of ASMCs in asthma. The thickness of the airway wall, the airway smooth muscle layer, the number of ASMCs and the expression of extracellular signal-regulated kinase (ERK) were significantly reduced in the curcumin-treated group as compared with the model group. Curcumin inhibited the cell proliferation induced by platelet-derived growth factor (PDGF) and decreased the PDGF-induced phosphorylation of ERK1/2 in the rat ASMCs. Moreover, the disruption of caveolae using methyl- β -cyclodextrin (M β CD) attenuated the anti-proliferative effects of curcumin in the ASMCs, which suggests that caveolin is involved in this process. Curcumin upregulated the mRNA and protein expression of caveolin-1. The data presented in this study demonstrate that the proliferation of ASMCs is inhibited by curcumin *in vitro* and *in vivo*; curcumin exerts these effects by upregulating the expression of caveolin-1 and blocking the activation of the ERK pathway.

Introduction

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play an important role (1). Patients with severe asthma with an incidence rate of 5-10% account for the high morbidity and health costs; thus, novel treatment strategies for this disorder are urgently required (2).

The airway obstruction in asthma is usually reversible; however, if left untreated, the obstruction may become irreversible due to airway remodeling (3-5). The process of airway remodeling in asthma includes subepithelial basement membrane fibrosis, epithelial goblet cell hyperplasia, an increase in the number of blood vessels and a proliferative state of the airway smooth muscle with increased mass due to hyperplasia and hypertrophy (5). Recent studies have indicated that the increased mass of airway smooth muscle cells (ASMCs) plays a critical role in the histopathological characteristics of airway remodeling (2-4). Thus, this may be a potential therapeutic target for asthma. Several classes of drugs that target the airway smooth muscle, including β -agonists, anti-cholinergics, antihistamines and anti-leukotrienes were developed decades ago (6). However, these life-long therapies only treat the symptoms, but have little or no effect on the structural alterations in asthma.

Curcumin [diferuloylmethane (C₂₁H₂₀O₆)], a polyphenol, is derived from the roots (rhizomes) of the plant, *Curcuma longa* (7). Curcumin has been used as a traditional medicine for the treatment of asthma in China and India since 1900 BC (8,9). Several studies have demonstrated that curcumin has wide range of properties, including anti-inflammatory, antioxidant, anticancer, chemopreventive and chemotherapeutic activities (7,9-12), affecting multiple signaling pathways, such as the extracellular signal-regulated kinase (ERK)1/2 pathway (12-16). The anti-proliferative effects of curcumin have been investigated in various cell lines (17-20). Studies have shown that curcumin inhibits vascular smooth muscle cell (VSMC) proliferation by restoring the expression of caveolin-1, blocking the activation of ERK1/2 (13,15,21-23). However, to our knowledge, whether curcumin inhibits ASMC proliferation in asthma has not been reported to date.

In this study, we investigated whether curcumin inhibits the proliferation of ASMCs *in vitro* and *in vivo*. Our results demonstrate that curcumin inhibits the proliferation of ASMCs by upregulating the expression of caveolin-1 and thus blocking the ERK signaling pathway; these results may promote the clinical application of curcumin in asthma.

Materials and methods

Animals. Eighty female BALB/c mice, weighing approximately 18-22 g, were purchased from the Guangdong Medical Laboratory Animal Center, Foshan, China. The mice were divided into 5 groups (n=16 per group): the control group (normal

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saline-challenged mice treated with normal saline), model group [ovalbumin (OVA)-challenged mice treated with normal saline] and the curcumin-treated groups (OVA-challenged mice treated with 50, 100 and 150 mg/kg curcumin). All mice were kept in well-controlled animal housing facilities, and had free access to tap water and food pellets throughout the experimental period. All experimental procedures involving animals were carried out in accordance with the Guide for the Care and Use of Laboratory Animals and the Institutional Ethical Guidelines for experiments with animals.

Sensitization and challenge with OVA. Mice underwent OVA sensitization and challenge as previously described (24,25) with slight modifications. Mice in the model and curcumin-treated groups were immunized by an intraperitoneal (i.p.) injection of 0.2 ml of 50 μ g/ml OVA (Sigma-Aldrich Co., St. Louis, MO, USA) on days 0 and 12. The control group received the same volume of physiological saline at the same time. Mice in the model and curcumin-treated groups were then challenged once a day with 5% OVA (aerosolized for 30 min) via the airways between days 18 and 23, while normal saline was administered to the control group in a similar manner. Prolonged inflammation was induced by the subsequent exposure of mice to aerosolized 5% OVA 3 times a week for 30 min from day 26 onwards (chronic phase). Mice in the curcumin-treated groups were administered with an i.p. injection of curcumin 30 min prior to stimulation with OVA, while the others were administered normal saline. The mice were sacrificed on day 55.

Airway hyper-responsiveness (AHR). The activity of all the mice was observed closely following challenge with OVA. Airway responsiveness was measured indirectly by whole body plethysmography to calculate enhanced pause (Penh: Buxco Technologies, Petersfield, UK). Response to inhaled methacholine (Sigma-Aldrich Co.) at concentrations of 0.78, 1.56, 3.12, 6.25, 12.5, 25 and 50 mg/ml were measured for 1 min, as described previously (26). Airway responsiveness activities (Penh% values) were calculated by the formula ($P = m/s \times 100\%$, in which P, m and s represent the Penh% value, methacholine-stimulated Penh value and the saline-stimulated Penh value, respectively).

Histology and immunohistochemistry. Paraffin-embedded lung sections from mice of the 5 groups were stained with hematoxylin and eosin (H&E) to observe the pathological changes in the airways and lung tissue under an optical microscope. We performed immunohistochemistry using the monoclonal mouse antibody to α -smooth muscle actin (α -SMA) (1:100, Sigma-Aldrich Co.) and the polyclonal rabbit antibodies to ERK (1:100, Cell Signaling Technology, Inc., Danver, MA, USA). The sections from mice in the 5 groups were deparaffinized, and a 3% hydrogen peroxide solution was applied for 15 min to inhibit endogenous peroxidase activity. Antigen retrieval was performed with citrate solution for 45 min. After the sections were blocked with 10% goat serum in phosphate-buffered saline (PBS) for 20 min, they were incubated with the primary antibody overnight at 4°C. The horseradish peroxidase (HRP)-conjugated rabbit-anti-mouse immunoglobulin (Sigma-Aldrich Co.) and HRP-conjugated

goat-anti-rabbit immunoglobulin (Sigma-Aldrich Co.) were used as the secondary antibodies and 3,3-diaminobenzidine (DAB) (Sigma-Aldrich Co.) was used as the chromogen. Hematoxylin was used for the counterstaining of the sections. For the negative control, the primary antibody was replaced with PBS. The sections were observed under a microscope. The thickness of the airway wall, the thickness of the airway smooth muscle layer, the numbers of smooth muscle cells and the intensity of ERK staining were measured as an average optical density using Image-Pro Plus (IPP) software (Image-Pro Plus 6.0; Media Cybernetics, Inc., Rockville, MD, USA). A non-stained region was selected and set as the background.

Primary cell culture. The ASMCs were cultivated as an explant culture as previously described with slight modifications (27). Briefly, primary rat airway smooth muscle was isolated from the bronchial smooth muscle from 10-12-week-old specific-pathogen-free (SPF) Sprague-Dawley (SD) male rats and the smooth muscle was isolated, cut into sections of 1-2 mm in cubic size, and placed on culture flasks with Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) in an incubator at 37°C, 5% CO₂. The ASMCs migrated from the tissue explants and approached confluence around the explants. The culture medium was changed every 3 days. When the cells reached 90-100% confluence, they were passaged with 0.25% trypsin-EDTA (Gibco). The cells at passage 3 to 6 were used in the following experiments.

Immunofluorescence *in vitro*. The primary ASMCs were characterized by microscopy and their purity was verified by the immunofluorescence staining of α -SMA. The location of caveolin-1 in the ASMCs was also detected by immunofluorescence. After the cells grew to approximately 50% confluence, they were fixed in the culture plate using 4% paraformaldehyde for 10 min, permeabilized with 0.3% Triton X-100, blocked with 5% bovine serum albumin (BSA) at 37°C for 1 h, incubated with monoclonal mouse antibodies against α -SMA (1:100, Sigma-Aldrich Co.) and rabbit anti-rat caveolin-1 (1:500, Abcam, Cambridge, UK) respectively overnight at 4°C, and then labeled with fluorescein isothiocyanate (FITC) secondary anti-mouse or anti-rabbit antibody (1:100, Sigma-Aldrich Co.) at 37°C for 1 h in the dark. The nuclei were counterstained with 20 μ g/ml propidium iodide (PI) and 1 μ g/ml DAPI in methanol for 5 min. The culture plate was washed 3 times between each step for 5 min in 0.1 M PBS (pH 7.4). The cells were examined and images were acquired using a fluorescence microscope.

Cell proliferation assay. The ASMCs were dispensed in a 96-well culture plate at a cell density of 2×10^4 /ml and incubated at 37°C, 5% CO₂. The cells were divided into 5 groups: i) negative control group (DMEM/F-12 containing 2.5% FBS), ii) positive control group (DMEM/F-12 containing 10% FBS), iii) proliferation group [25 ng/ml platelet-derived growth factor (PDGF) in DMEM/F-12 supplemented with 2.5% FBS], iv) curcumin-treated group with caveolin-1 (25 μ mol/l curcumin with 25 ng/ml PDGF in DMEM/F-12 2.5% FBS),

and v) curcumin-treated group without caveolin-1 [the cells were pre-treated with 5 μ mol/l methyl- β -cyclodextrin (M β CD) for 60 min, then 25 μ mol/l curcumin were added with 25 ng/ml PDGF in DMEM/F-12 containing 2.5% FBS]. Cell Counting Kit-8 (CCK-8) assays (Dojindo Laboratories, Kumamoto Japan) were used to measure cell proliferation according to the manufacturer's instructions. After the cells were treated with the culture medium for 6, 12, 24 and 48 h, they were incubated in 10% CCK-8 diluted in normal culture medium at 37°C for 2 h and the absorbance at 450 nm (OD at 450 nm) was measured. Each group was analyzed in triplicate. The results were expressed as OD values at 450 nm.

Real time PCR. Total RNA was extracted from the ASMCs in each group at 5 time points (0, 4, 8, 12 and 24 h) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration, purity and amount of total RNA were determined by ultraviolet spectrometry (ND-1000 spectrophotometer; NanoDrop Technologies, Wilmington, DE, USA). Reverse transcriptase PCR was carried out using the Prime Script™ RT reagent kit (Takara Bio Inc., Otsu, Japan). The sense and antisense primers for caveolin-1 (GenBank ID: NM_133651) and β -actin (GenBank ID: NM_031144.2) were synthesized by Takara Bio Inc. The primers used are listed in Table I.

Real-time PCR analyses were performed using SYBR Premix Ex Taq (Takara Bio Inc.). Amplification was performed in an ABI PRISM 7500 Real-Time PCR System with the following temperature profile: a pre-denaturation step of 30 sec at 95°C, extended at 60°C for 34 sec, and denaturation for 5 sec at 95°C for 40 cycles. The data were calculated using the $2^{-\Delta\Delta C_t}$ method normalized to β -actin. Each experiment was performed 3 times.

Western blot analysis. The cells were lysed in cell lysis buffer (Cell Signaling Technology, Inc.). Total protein was extracted by sonication and centrifugation of the cell lysates at 12,000 x g for 10 min at 4°C. Whole-cell extracts were fractionated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred onto a nitrocellulose membrane (Millipore, Bedford, MA, USA) using a wet transfer apparatus. For immune detection, the membranes were washed and incubated with rabbit anti-rat ERK1/2 polyclonal antibody (1:4,000), rabbit-anti-rat phospho-ERK1/2 polyclonal antibody (1:4,000), anti-GADPH polyclonal antibody (1:2,000; all from Cell Signaling Technology, Inc., USA), rabbit-anti-rat caveolin-1 polyclonal antibody (1:500, Abcam) and anti- β -actin monoclonal antibody (1:2,000, Sigma-Aldrich Co.) overnight at 4°C. The signals were amplified using the appropriate HRP-conjugated secondary antibody, and visualized by enhanced chemiluminescence (Pierce Biotechnology, Inc., Rockford, IL, USA).

Statistical analysis. All statistical analyses were performed using SPSS for Windows v.13.0 (SPSS, Chicago, IL, USA). All data are expressed as the means $\pm$ SD. Statistical analyses were carried out using one-way ANOVA, the rank-sum test or least significant difference tests where appropriate. A P-value <0.05 was considered to indicate a statistically significant difference.

Table I. Oligonucleotide sequences of primers used for real-time PCR.

Gene name	Sense/antisense primers
Caveolin-1	5'-GGGCAACATCTAGAAGCCCAACAA-3' 5'-CTGATGCACTGAATTCCAATCAGGAA-3'
β -actin	5'-CTTCCTTCCTGGGTATGGAATC-3' 5'-GAGATGATCTGGGGGTCTGA-3'

Results

Curcumin decreases the airway responsiveness in the mouse model of airway remodeling. Positive symptoms, such as dysphoria, tachypnea and abdominal spasms were observed in the mice in the model group 5 to 10 min following challenge with OVA, whereas less severe symptoms, including mild dysphoria and tachypnea were observed in the mice treated with curcumin; all the symptoms soon dissipated.

The mice with asthma (model group), the mice with asthma treated with curcumin (curcumin-treated groups) and the untreated normal mice (control group) began to suffer from breathing difficulties after inhaling methacholine at concentrations of 1.56, 6.25 and 25 mg/ml. The airway responsiveness (Penh%) of the model group and the curcumin-treated groups (treated with various concentrations of curcumin) significantly increased compared with the normal group when methacholine was inhaled at the concentrations of 3.12, 6.25, 12.5 and 25 mg/ml ($P < 0.05$) and at the concentration of 50 mg/ml ($P < 0.01$) (Fig. 1). The airway responsiveness (Penh%) of the curcumin-treated groups significantly decreased compared with the model group when methacholine was inhaled at the concentrations of 3.12, 6.25, 12.5, 25 mg/ml ($P < 0.05$) and at the concentration of 50 mg/ml ($P < 0.01$) (Fig. 1).

Effects of curcumin on OVA-induced histopathological changes in lungs. To assess the anti-remodeling effects of curcumin, histopathological experiments were performed. Using H&E staining, inflammatory cell infiltration in the peribronchial and perivascular areas was observed in OVA-challenged mice. Treatment with curcumin (50, 100 and 150 mg/kg) markedly reduced the degree of inflammatory cell infiltration in the peribronchial and perivascular areas (Fig. 2, upper panel). OVA induced the proliferation of ASMCs. In the curcumin-treated groups, curcumin inhibited the OVA-induced ASMC proliferation compared with the model group (Fig. 2, middle panel). The expression of α -SMA and ERK was detected by immunohistochemistry and the expression of ERK was increased in mice with asthma (Fig. 2, lower panel; ERK protein is stained brown). Curcumin decreased the expression of ERK in the curcumin-treated groups compared with the model group. The bronchial wall thickness (Wai/Pi), the thickness of the smooth muscle layer (Wam/Pi), the number of smooth muscle cells (N/Pi) and the ERK gray values in the ASMCs in each group were measured using IPP software (Table II). In the model group, the thickness of the airway wall, the thickness of the airway smooth muscle layer and the numbers of smooth muscle cells were significantly increased compared with the control group

Table II. Lung pathology of the mice in the 5 groups.

Group	WAI/Pi (μm)	WAm/Pi (μm)	N/Pi (/mm)	ERK gray-scale value
Control	5.6 $\pm$ 0.5	1.2 $\pm$ 0.5	12.4 $\pm$ 1.2	68.5 $\pm$ 6.4
Model	14.6 $\pm$ 3.6 ^b	6.4 $\pm$ 1.4 ^b	35.3 $\pm$ 4.4 ^b	97.6 $\pm$ 15.2 ^b
50 mg/kg curcumin	7.7 $\pm$ 1.7 ^a	4.2 $\pm$ 1.5 ^a	25.8 $\pm$ 2.5 ^a	90.5 $\pm$ 15.8 ^a
100 mg/kg curcumin	5.6 $\pm$ 1.5 ^a	3.2 $\pm$ 1.3 ^a	16.5 $\pm$ 1.6 ^a	80.6 $\pm$ 10.3 ^a
150 mg/kg curcumin	5.6 $\pm$ 0.7 ^a	1.5 $\pm$ 0.7 ^a	12.5 $\pm$ 1.7 ^a	74.5 $\pm$ 7.1 ^a

The data are expressed as the means $\pm$ SD (n=16). ^aP<0.05, compared with the model group and ^bP<0.01, compared with the control group. Pi, internal perimeter; N, number of smooth muscle cells; WAI, inner wall area; WAm, smooth muscle area.

Figure 1. Effect of curcumin on airway hyper-responsiveness (AHR) in mice. The AHR of mice was measured by the whole body plethysmography system. The airway responsiveness (Penh%) of the curcumin-treated groups was significantly decreased compared with the model group. All data are expressed as the means $\pm$ SD (n=16 per group), *P<0.05, compared with control group, **P<0.01, compared with control group, [†]P<0.05, compared with curcumin-treated groups, [‡]P<0.01, compared with curcumin-treated groups.

(P<0.01). In the curcumin-treated groups, the thickness of the airway wall, the thickness of the airway smooth muscle layer and the numbers of smooth muscle cells were significantly decreased compared with the model group (P<0.05).

Correlation between ERK expression and ASMC proliferation. Semi-quantitative image analysis demonstrated that the expression level of ERK in the airways positively correlated with the thickness of the airway smooth muscle layer (r=0.745, P<0.05), and positively correlated with the number of ASMCs (r=0.821, P<0.05).

Rat primary ASMC identification and location of caveolin-1 in ASMCs. The ASMCs generally became adherent within 2 days, and assumed a stretched, spindle-shaped valley-like growth patterns after 4-5 days (Fig. 3A). The ASMCs grew to approximately 90% confluence after 8-10 days; they were then digested by trypsin and passaged for immunofluorescence staining (Fig. 3B). The cytoplasmic filaments of positive cells were stained green. The percentage of pure ASMCs was approximately 94.32% using Image Pro Plus 6.0 software.

Caveolin-1 was detected in the ASMC plasma membrane under a fluorescence microscope (Fig. 3C-E).

Effect of curcumin on PDGF-induced ASMC proliferation. Cell proliferation was determined using the CCK-8 assay as shown in Fig. 4. At 12, 24 and 48 h, the absorbance at 450 nm was significantly higher in the PDGF + curcumin group compared with the negative control group (P<0.05). Of note, the ASMC proliferation induced by PDGF in group B peaked at 48 h, compared with the negative control group (P<0.01). Curcumin (25 $\mu\text{mol/l}$) significantly decreased the PDGF-induced the proliferation of ASMCs at 24 h (P<0.05) (group C compared with group B). After caveolae was disrupted by M β CD, the PDGF-induced proliferation of ASMCs in group D was markedly increased compared with group C. This suggests that the anti-proliferative effects of curcumin are weakened without caveolin-1; thus, caveolin-1 may play an important role in the anti-proliferative effects of curcumin on ASMCs.

Effect of curcumin on the expression of ERK1/2 protein in ASMCs. After the 4th passage ASMCs were treated with or without curcumin (25 $\mu\text{mol/l}$) for 30 and 60 min, total protein was extracted for western blot analysis to detect the expression of total ERK1/2 protein and the phosphorylation of ERK1/2 protein. A control group was set up. Treatment with PDGF increased ERK1/2 phosphorylation compared with the control group. The expression of PDGF-induced phosphorylated-ERK was significantly decreased following treatment with curcumin (Fig. 5), and phosphorylated ERK could not be detected at 60 min. Curcumin had no effect on the expression of total ERK1/2.

mRNA and protein expression of caveolin-1 following treatment with curcumin. After the rat ASMCs were treated with curcumin (25 $\mu\text{mol/l}$) for 0, 4, 8, 12 and 24 h, the mRNA expression of caveolin-1 in the ASMCs was measured by real-time PCR. The result revealed that following treatment with PDGF with or without curcumin for 4 h, the caveolin-1 mRNA expression was increased (compared with the control group) (P<0.01). The mRNA expression of caveolin-1 was significantly increased in the PDGF + curcumin-treated group compared with the PDGF group (P<0.05) (Fig. 6A). After the 4th passage ASMCs were treated with PDGF or curcumin and PDGF for 24 h, total protein was extracted for western blot

Figure 2. Hematoxylin and eosin (H&E) staining, α -smooth muscle actin (α -SMA) and ERK immunohistochemical staining. (magnification, x400). The small arrows in α -SMA staining figures refer to the airway smooth muscle layer, the black triangles refer to the increased mucosal folds and the large arrow refers to the fallen-off bronchial mucosa. In the control group: alveolar walls had structural integrity, the thickness of the alveolar walls was normal, alveolar epithelial cells were arranged in order, smooth muscle layer was discontinuous and the expression of ERK was not observed. In the model group: eosinophilic infiltration was detected around the bronchi and blood vessels; the number of bronchi mucosal folds was increased and some of the bronchial mucosa fell off; a thicker alveolar wall and continuous smooth muscle layer were observed; the expression of ERK was markedly increased. In the curcumin-treated groups: compared with the model group, treatment with curcumin decreased the pathological changes, the thickness of smooth muscle layer and the expression of ERK in the lung sections of the ovalbumin (OVA)-challenged mice with asthma.

Figure 3. Identification of primary airway smooth muscle cells (ASMCs) and the location of caveolin-1 on the ASMC membrane. (A) ASMCs assumed a 'peak and valley' growth pattern, as observed under a light microscope (magnification, x100). (B) Mouse anti-rat α -SMA antibody and FITC-labeled secondary anti-mouse IgG were used for the identification of rat ASMCs, and the cells were observed under a fluorescence microscope; the cytoplasm showed green positive staining. The nuclei were stained with propidium iodide (PI) and showed orange positive staining under a fluorescence microscopy. (magnification, x400). (C) Rabbit anti-rat caveolin-1 antibody and FITC-labeled secondary goat anti-rabbit IgG was used for detecting the location of caveolin-1 in the rat ASMCs; caveolin-1 showed green positive staining under a fluorescence microscope (magnification, x200). (D) The nuclei were stained with DAPI and showed blue positive staining under a fluorescence microscope (magnification, x200); (C and D) were merged into (E) (magnification, x200).

analysis to detect the expression of caveolin-1 protein. The results are shown in Fig. 6B. Compared with the PDGF group, the protein expression of caveolin-1 in the cucumin + PDGF group was significantly increased ($P < 0.01$).

Discussion

Asthma is traditionally defined as a chronic disease characterized by airway inflammation, AHR and airway remodeling. In

Figure 4. Curcumin inhibits the platelet-derived growth factor (PDGF)-induced proliferation of airway smooth muscle cells (ASMCs). The cell viability of the ASMCs treated with curcumin was examined using CCK-8 at 6, 12, 24 and 48 h. The data are expressed as the means $\pm$ SD, $n=3$. ** $P<0.01$, compared with the control group; $^{\dagger}P<0.05$, compared with group B; and $^{\ddagger}P<0.05$, compared with group C.

Figure 5. Curcumin inhibits the platelet-derived growth factor (PDGF)-induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) protein in airway smooth muscle cells (ASMCs). The 4th passage ASMCs were treated with or without curcumin for 30 and 60 min. Western blot analyses were performed using whole-cell fractions of cell lysates. Results are representative of 3 separate experiments. p-ERK1, phosphorylated-ERK1; p-ERK2, phosphorylated-ERK2; t-ERK1, total ERK1; t-ERK2, total ERK2.

a previous study, Chung examined patients with asthma using airway histopathology and lung function dynamic observation and indicated that a series of structural changes could be found in the airway wall in the patients with asthma (5). Among the histopathological characteristics of airway remodeling, previous studies have indicated that the increased mass of ASMCs plays a critical role (2,4,5,28,29). Due to their important role in airway remodeling, ASMCs may be a potential therapeutic target for the treatment of patients with asthma.

Curcumin has been used in indigenous medicine for the treatment of a variety of inflammatory conditions and chronic diseases (9,13,14,23) and studies have mainly focused on the effects of curcumin on inhibiting bronchial inflammation (30-34). However, limited studies have investigated the effects of curcumin on structural changes in asthma, such as ASMC proliferation in airway remodeling. Therefore, in this study, we performed *in vitro* and *in vivo* experiments to examine the effects of curcumin on the proliferation of ASMCs and to elucidate the underlying mechanisms. Our findings demonstrate that curcumin inhibits the proliferation of ASMCs both *in vitro* and *in vivo*.

In *in vivo* experiments, we used OVA-challenged mice to establish a model of chronic asthma airway remodeling; the curcumin-treated groups were administered various doses of

Figure 6. Curcumin increases the mRNA and protein expression of caveolin-1 in airway smooth muscle cells (ASMCs). (A) After the ASMCs were treated with curcumin for 0, 4, 8, 12 and 24 h, the mRNA expression of caveolin-1 in the ASMCs was measured by real-time PCR. The data are expressed as the mean $\pm$ SD, $n=3$. ** $P<0.01$, compared with the control group; $^{\dagger}P<0.05$, compared with the platelet-derived growth factor (PDGF) group and $^{\ddagger}P<0.01$, compared with the PDGF group. (B) Total proteins from the 4th passage ASMCs were used for western blot analysis to detect the protein expression of caveolin-1. The data are expressed as the means $\pm$ SD, $n=3$, $^{\#}P<0.01$, compared with the PDGF group.

curcumin by an i.p. injection prior to challenge with OVA. The mouse model of airway remodeling induced by OVA has previously been used in a number of studies; however, the majority of these studies focused on the inflammatory infiltration in this model. Our results were identical with those from previous studies (30-33). Our findings indicated that the symptoms of an asthma attack dissipated following treatment with curcumin and that curcumin significantly reduced the airway responsiveness. In addition, we paid particular attention to the changes in airway smooth muscle. We found that following treatment with curcumin, the thickness of the airway wall and the bronchial smooth muscle layer became thinner and the number of smooth muscle cells decreased; the most significant changes were observed in the high-dose curcumin-treated group. These results demonstrate that the administration of curcumin inhibits the proliferation of ASMCs *in vivo*.

In *in vitro* experiments, we stimulated the proliferation of primary rat ASMCs by PDGF and provided a proliferative experimental model for the study of asthma at the cellular and molecular level. Our results revealed that curcumin inhibited the PDGF-induced proliferation of ASMCs *in vitro*.

The mitogen-activated protein kinase (MAPK) signaling cascade has been shown to play an important role in the activation of various cells (35-38). It is activated by the 3-tiered sequential phosphorylation of MAPK kinase, MAPK/ERK kinase (MEK) and MAPK. There are 3 major groups of MAPK in mammalian cells, including ERK, p38 MAPK and c-Jun N-terminal kinase. Accumulating evidence has shown that ERK plays a crucial role in human ASMC growth. A previous study demonstrated that ERK activity in the lungs of asthmatic mice was significantly higher compared with normal mice (39). ERK activation is necessary for the proliferation of ASMCs (4). In addition, PDGF and other growth factors are promoters of ERK1/2 (p42/44 MAPK) expression and the activation of phosphorylation, which are closely associated with the proliferation of smooth muscle cells (27,40-44). Thus, the ERK signaling pathway plays a crucial role in the pathological process of airway remodeling in asthma. The results of our study are in accordance with these findings; we found that the phosphorylated-ERK1/2 protein levels were significantly decreased following the treatment of ASMCs with curcumin. This suggests that the inhibition of the activation of ERK1/2 by curcumin is involved in the inhibition of ASMC proliferation. We provide evidence that curcumin inhibits ASMC proliferation and that caveolin-1 plays a crucial role in this process; the involvement of caveolin-1 is partly due to the fact that it has the ability to regulate the ERK 1/2 pathway.

We found that curcumin significantly inhibited the PDGF-induced proliferation of ASMCs and that this effect was significantly attenuated by M β CD. The expression of caveolin-1 was significantly increased in the curcumin-treated group as compared with the PDGF group in our study. Caveolae are small vesicular invaginations of the cell membrane. It is within this organelle that cells perform transcytosis and signal transduction. Caveolae are composed of a mixture of lipids and proteins (45-50). The chief structural proteins of caveolae are caveolins (caveolin-1, caveolin-2 and caveolin-3), and caveolin-1 appears to be an essential component of caveolae (51-57). Evidence suggests that caveolin-1 regulates the ERK1/2 pathway during cell proliferation (55,58-60). Buitrago and Boland found that when proliferating mouse skeletal myoblastic cells were pre-treated with M β CD, a caveolae-disrupting agent, the 1 α ,25(OH) $_2$ D $_3$ -dependent activation of ERK1/2, p38 MAPK and c-Src was suppressed (58). Furthermore, studies on human ASMCs have shown that caveolae and caveolin-1 coordinate PDGF receptor signaling, leading to myocyte proliferation, and inhibit the constitutive activity of p42/p44 MAPK, sustaining cell quiescence (55). These data are in accordance with those in the study by Peterson *et al*, who showed that the treatment of VSMCs with PDGF for 24 h resulted in a loss of caveolin-1 protein expression and plasma membrane-associated caveolae (21). The data mentioned above indicate that caveolin-1 is an important negative regulator of cell proliferation and PDGF signaling events. Our results therefore suggest that caveolin-1 plays a crucial role in the anti-proliferative effects of curcumin through the ERK1/2 pathway.

In conclusion, we demonstrate that curcumin inhibits the proliferation of ASMCs *in vitro* and *in vivo*; the possible mechanisms behind the inhibitory effects of curcumin may be the upregulation of the expression of caveolin-1 and the blocking of the ERK pathway, thereby inhibiting the proliferation of

ASMCs. Our findings provide new insight into the application of curcumin in the prevention and treatment of asthma, particularly airway remodeling in severe asthma.

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