

Urotensin upregulates transforming growth factor- β 1 expression of asthma airway through ERK-dependent pathway

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Abstract Airway smooth muscle cells (ASMCs) play a key role in the process of asthma airway remodeling. Urotensin II (UII) and transforming growth factor (TGF)- β are potent mitogens for ASMCs proliferation. The study was aimed to determine whether UII-upregulated TGF- β -mediated ASMCs proliferation and extracellular signal-regulated kinase (ERK) was required for such an effect. OVA-sensitized rats were challenged to induce asthma. Lung morphology and airway dynamic parameters were monitored. ASMCs from control and asthma rats were purified for the measurement of UII and TGF- β 1 expression. In vitro experiments were conducted to determine the direct effect of UII on TGF- β 1 expression by ASMCs. Finally, U0126, an ERK inhibitor was used to examine the role of ERK pathway in UII mediated TGF- β 1 upregulation. We found that both UII and TGF- β 1 were upregulated in asthma lung tissues. In vitro study on ASMCs further revealed that UII may render its effect on ASMCs cells through the upregulation of TGF- β 1. Data also supported the conclusion that ERK pathway was required, but not sufficient in UII-induced TGF- β 1 upregulation. The current study provides new evidence that UII is involved in the TGF- β mediated mitogenic effect on ASMCs. UII, at least partially, uses ERK pathway to render such effect.

Keywords Asthma · Urotensin II · Extracellular signal-regulated kinase · Transforming growth factor · Airway

Introduction

The prevalence of asthma has increased remarkably in the last few decades in developed countries. Asthma has become one of the leading health burdens to modern society. Chronic airway inflammation and airway remodeling are main features of asthma pathology, which are seen in adult as well as childhood asthma. Treatment and prevention of airway remodeling appear to be the main focus of future asthma research [1]. Currently, effective tools are not available to either reverse or halt airway remodeling [2]. There is no doubt that better understanding of the underlying mechanism of asthma airway remodeling will facilitate the development of new treatment modality for asthma.

Urotensin II (UII) is a potent mitogen for airway smooth muscle cells (ASMCs). Studies have shown that UII induces ASMCs proliferation in a dose-dependent manner [3]. ASMCs play a key role in the process of asthma airway remodeling. The authors have previously reported that the changes of UII contents in serum and bronchoalveolar lavage fluid (BALF) may be associated with airway remodeling in asthmatic rats [4]. Transforming growth factor (TGF)- β is a potent regulator of ASMCs proliferation. Xie et al. [5] found that TGF- β 1 acts in an autocrine fashion to induce ASMCs hyperplasia. Dai et al. [6] showed that TGF- β 1 is upregulated in cardiac fibroblasts by UII. However, whether UII exerts its effect by upregulating TGF- β 1 remains unclear.

Studies have suggested that extracellular signal-regulated kinase 1 and 2 (ERK1 and ERK2), members of

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mitogen-activated protein kinase (MAPK) superfamily, play a key part in TGF- β 1 mediated cell proliferation. ASMCs proliferate in response to TGF- β 1, which is mediated by phosphorylation of p38MAPK and ERK1/2 [7]. Signaling molecules involved in TGF- β 1 upregulation include ERK, phosphatidylinositol 3-kinase (PI3 K), and c-Jun N-terminal Kinase (JNK) [5]. Matsusaka et al. [8] found that UII enhances the Human Aortic Smooth Muscle Cells (HASMC) migration through activation of an ERK-dependent pathway. Herein, we hypothesize that UII induces ASMCs proliferation in asthma animals is through ERK pathway.

Materials and methods

Reagents

Ovalbumin (OVA) was purchased from Sigma (USA). The UII and TGF- β 1 polyclonal antibodies from Abcam Inc. (USA). PCR Primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co Ltd (Shanghai, China). ERK inhibitor U0126 was purchased from Cell Signaling Technology (USA). Dulbecco's Modified Eagle Medium (DMEM) high glucose medium, 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) solution, and tissue culture grade fetal bovine serum were from Biowest (France). RNA enzyme inhibitor (RNasin), from Promega Corporation (USA). dNTP, DNase I, and Ex Taq HS were purchased from Takara Bio Inc (Japan). Reverse Transcriptase was purchased from Invitrogen Corporation (USA). SYBR I and 50 Calibration were purchased from Bio-Rad Laboratories, Inc (USA). TGF- β 1 ELISA kit was purchased from R&D Systems (USA). Cell Counting Kit-8 was obtained from Donjindo Laboratories (Kumamoto, Japan).

Rat asthma models

Animal protocols were approved by Institutional Animal Care and Use Committee (IACUC) of Wenzhou Medical College. Male Sprague–Dawley (SD) rats at the age of 4–6 weeks, weighing 100–120 g, were purchased from Shanghai Laboratory Animal Center (Shanghai, China) and kept in pathogen-free environment in the animal center of Wenzhou Medical College. Rat asthma models were established as reported [9, 10]. Male SD Rats were randomly divided into two groups, i.e., asthma group ($n = 8$) and control group ($n = 8$). The rats in asthma group were sensitized with intraperitoneal injection of 1 mg OVA with 100 μ g aluminum hydroxide dissolved in 1 ml saline on day 1 and day 8. They were then challenged with 1% OVA

aerosol every other day for 30 min from day 15 for 8 weeks. The control group received normal saline on the same schedule. The animals were sacrificed within 18–24 h after the final challenge. After adequate inflation, total lung tissues were fixed with 4% paraformaldehyde for 4 h. They were then dehydrated and embedded with paraffin for hematoxylineosin (HE) staining.

Morphological change of airways

Airway morphological changes were determined according to literature reports [9, 11]. Three to five small bronchus (1000–1500 μ m bronchial diameter) with complete cross-section from each rat were selected for observation. Basement membrane perimeter (Pbm), bronchial total area (At), lumen area (Ac), the outer edge of the tracheal smooth muscle area (AMe), the inner edge of the tracheal smooth muscle area (AMi) were measured by Imagepro-Plus analysis system. The following formulas were used to calculate bronchial wall thickness (Wat) and smooth muscle thickness (Wam): $Wat = (At - Ac) / Pbm$; $Wam = (AMe - AMi) / Pbm$.

Immunohistochemistry

Immunohistochemistry staining was performed according to manufacturer's instruction. Briefly, tissue sections, after dewaxing, were blocked by 3% hydrogen peroxide for 20 min and boiled for antigen retrieval using a pressure cooker for 4 min. The sections were then incubated by primary antibody (rabbit anti rat UII and mouse anti rat TGF- β 1) overnight at 4°C, followed by goat anti-rabbit (or mouse) IgG-HRP polymer antibody at for 30 min at 37°C. Slides were then counterstained with hematoxylin for 1 min and mounted with neutral gum prior to observation under microscope. Staining without primary antibody was served as negative control. The UII and TGF- β 1 were quantitated by measuring optical density with image-pro plus software system.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol according to manufacturer's instruction. One μ g RNA was reverse transcribed to cDNA by incubating with reverse transcriptase at 37°C for 1 h. Primers for rat UII and TGF- β 1 were chosen to cross introns to avoid amplification of genomic DNA. Primer sequences were listed below. PCR conditions were as follow: preheat at 95°C $\times$ 5 min, denaturation 95°C $\times$ 30 s, annealing 60.3°C $\times$ 30 s, extension 72°C $\times$ 30 s. A total 25 cycles were used which was followed by 5 min extension at 72°C.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product length (bp)
GAPDH	TGCTGAGTACGTGGAG	GTCTTCTGAGTGGCAGTGAT	288
TGF β 1	CCGCAACAACGCAATCTATG	AGCCCTGTATTCCGTCTCCTT	437
U11	ACGGACACTGGTGAAATG	AGCCCTGTATTCCGTCTCCTT	136

ASMCs culture and U11 treatment

ASMCs of asthmatic rats were purified by proteolytic enzyme digestion. Rat lung tissue was incubated with digestion buffer containing collagenase type IV (0.25 mg/ml), elastase (0.1 mg/ml) and trypsin (0.2 mg/ml) at 37°C for 40 min with gentle shaking. The isolated cells were collected by filtering through 200 μ m Nytex mesh, followed by centrifugation. The pellets were reconstituted in DMEM-Ham's F12 medium with 10% fetal bovine serum, penicillin (10,000 unit/ml) and streptomycin (10 mg/ml) in 25-cm² flask. Confluent cells were striped with a 0.25% trypsin solution containing 0.02% EDTA.

Baseline expression of TGF- β 1 at protein and mRNA levels was determined in ASMCs of asthma rats. ASMCs were then incubated with U11 (100 nM) for 4–48 h (time course) and at variable concentrations (0.4–400 nM, dose-dependent study) to modulate the expression of TGF- β 1. The time point which showed maximal effect of U11 was used in subsequent experiments.

To explore the role of ERK in U11 mediated TGF- β 1 expression, ASMCs were pretreated with ERK inhibitor U0126 at 40 nM for 30 min prior to the addition of U11. TGF- β 1 was quantitated as mentioned above.

Real-time PCR

Total RNA was extracted and used for QuantiTect SYBR Green One-Step quantitative real-time PCR on Mx3000P cyclor. Primer sequences are listed as follow. Specific primers were used at a final concentration of 1 μ M. The reverse transcription was done at 50°C for 30 min. Cycling conditions are as follow: 95°C $\times$ 15 min, 45 cycles of denaturation at 94°C $\times$ 15 s, annealing at 60°C $\times$ 30 s, extension 72°C $\times$ 30 s. Melting curve analysis was used to confirm the amplicon specificity. Calibration curves were generated by measuring serial dilutions of stock cDNA to calculate the amplification efficiency. The relative amount of mRNA for each target gene was normalized to the value obtained for the housekeeping gene GAPDH.

Primers used for real-time polymerase chain reaction analysis of gene expression.

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product length (bp)
GAPDH	AGACAGCCGCATCTTCTTG	CTTGCCGTGGGTAGAGTCAT	206
TGF-beta1	ATACGCCTGAGTGGCTGTCT	TGGGACTGATCCCATTGATT	152

Sandwich ELISA assay for TGF- β 1

Supernatants from different experiments were collected and stored at –80°C prior to assay. TGF- β 1 concentrations were determined by sandwich ELISA according to manufacturer's instruction. Standard curve was created by using recombinant TGF- β 1. The low limit of detection was 2.5 pg/ml.

Cell proliferation assay [12]

ASMCs were plated into 96-well plates at 2.5×10^4 cells per well in DMEM/F12 medium containing 10% FCS. The cells were allowed to grow to be 80% confluent and switched to serum-free medium in the presence of U11 and U0126. After 24 h, they were harvested for CCK-8 determination.

Statistical analysis

All data were expressed as means $\pm$ SEM. Comparison among multiple groups was performed using a one-way ANOVA followed by post hoc Dunnett's T3 tests. For samples with unequal variances, Tamhane's T2 test was used instead. One-sample *t* test (two-tailed) was used to compare treated samples from their own basal levels. *P* < 0.05 was considered to be statistically significant.

Results

Histological change of airways

Rats from control and OVA-induced asthma were sacrificed and their lung tissues were subjected to HE staining. As demonstrated in Fig. 1, control group showed normal small airway and alveoli structure (Fig. 1a). In contrast, lung tissues from asthma group showed significant airway wall thickening, epithelial goblet cell metaplasia and damaged ciliated epithelial cells (Fig. 1b). In addition, significant infiltration of eosinophils and lymphocytes in the airway was also observed in asthma group (Fig. 1c).

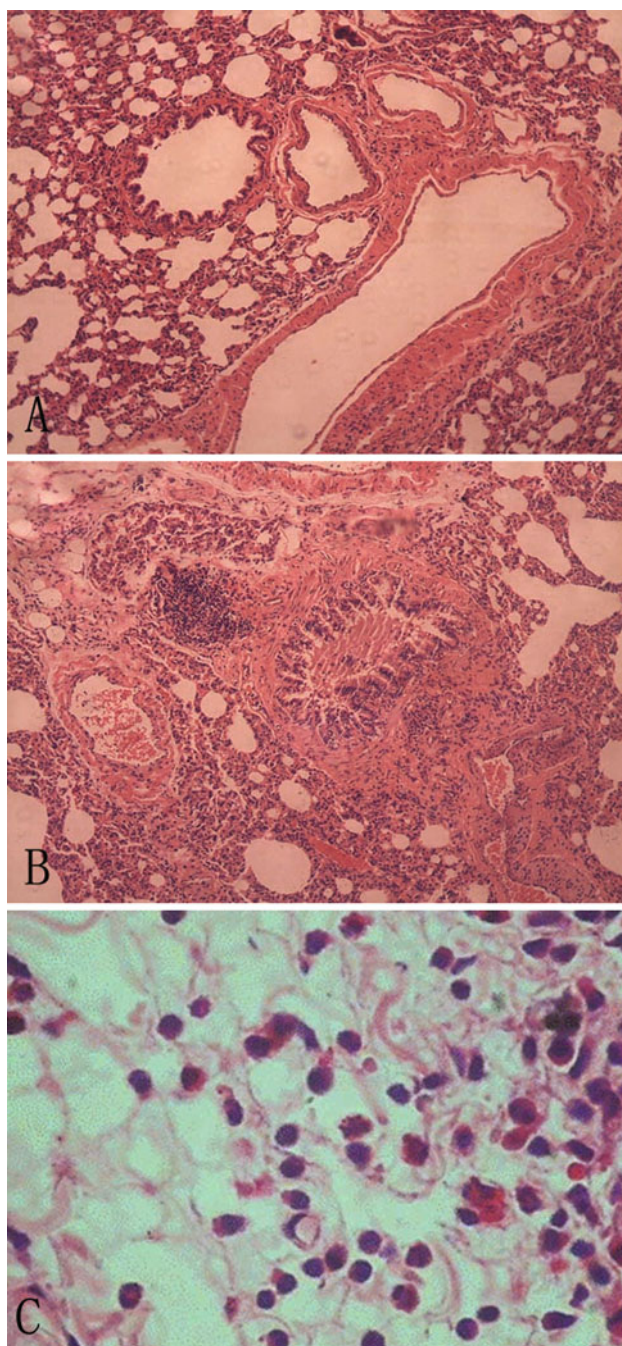


Fig. 1 Histology change of airway. Lung tissues from control (a) and OVA-induced asthma rats (b) were stained with HE as described in “Materials and Methods” section. The infiltration of eosinophils of asthma group (c). Data presented is the representatives of eight animals from each group with similar results. Magnification = 400

Wat and Wam

Next, we determined the Wat and Wam of asthma and control groups. As clearly illustrated in Fig. 2, asthma animals showed remarkable increased Wat than control

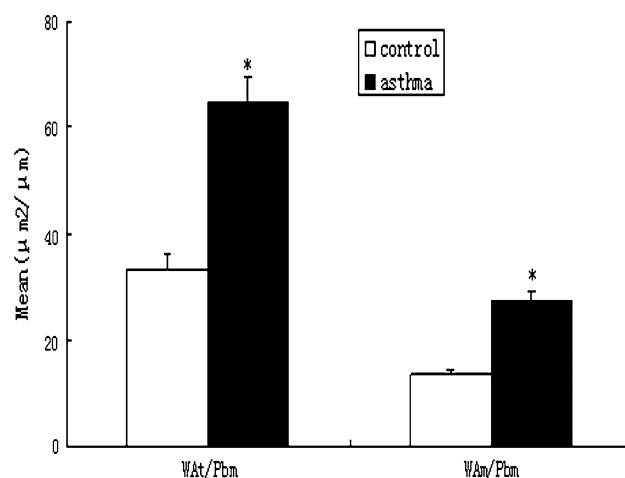


Fig. 2 Wat and Wam. Lung tissues from control and asthma rats were obtained and analyzed for Wat and Wam using Image pro-Plus analysis system. Data presented as mean ± SEM. * $P < 0.01$ comparing to control group

($64.00 \pm 2.57 \mu\text{m}^2/\mu\text{m}$ and $32.17 \pm 1.24 \mu\text{m}^2/\mu\text{m}$, respectively, $P < 0.01$). Similarly, asthma group demonstrated greater Wam than control ($27.97 \pm 2.13 \mu\text{m}^2/\mu\text{m}$ and $13.11 \pm 1.27 \mu\text{m}^2/\mu\text{m}$, $P < 0.01$), indicating significant airway remodeling.

The expression of UII and TGF- $\beta 1$ from asthma and control rats

The expression of UII was determined by immunohistochemistry method. Lung tissue from control animals showed minimal expression of UII (Fig. 3a). In contrast, significant elevation of UII was observed in tissues from asthma rats (Fig. 3b). The result of TGF- $\beta 1$ expression demonstrated in the same pattern, i.e., a remarkable upregulation noticed in asthma animals (Fig. 3d) comparing to control group (Fig. 3c).

RT-PCR was employed to detect the expression of UII and TGF- $\beta 1$ at mRNA levels. The results, as shown in Fig. 4a–c demonstrated a significant increase of UII and TGF- $\beta 1$ expression in lung tissues of asthma group.

UII upregulates TGF- $\beta 1$ expression of ASMCs

In vitro studies were conducted next, to demonstrate the direct effect of UII on ASMCs TGF- $\beta 1$ expression. Purified ASMCs were incubated with UII for 4, 8, 12, 24, and 48 h. TGF- $\beta 1$ was determined by real-time PCR for messenger level and ELISA for protein level. RNA levels of TGF- $\beta 1$ started increase as early as 4 h after UII treatment. By 48 h, it decreased to baseline levels which were no different from control (Fig. 5a). Interestingly, protein levels of TGF- $\beta 1$ started rise at 12 h, peaked at 24 h and sustained up to 48 h (Fig. 5b). We next examined the dose-dependent effect of

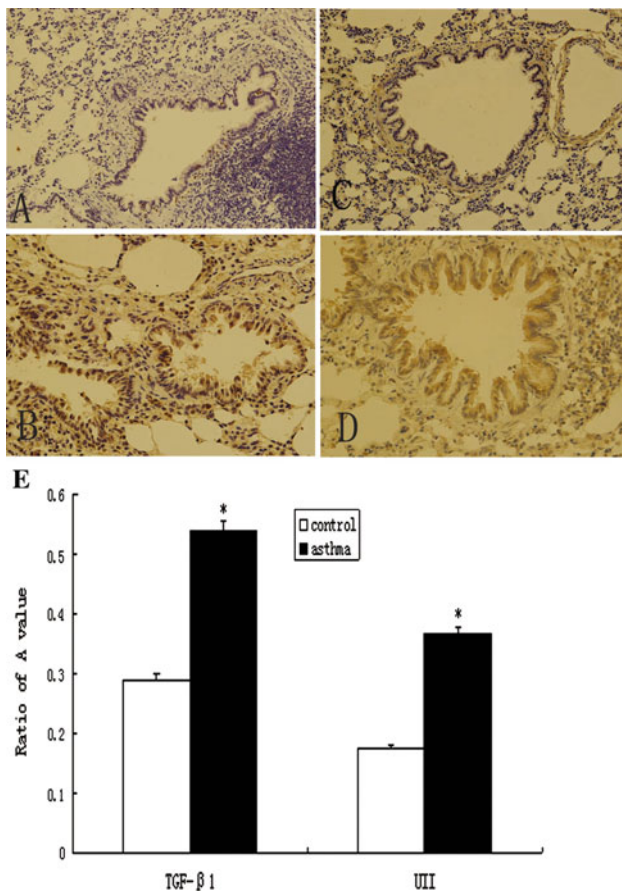
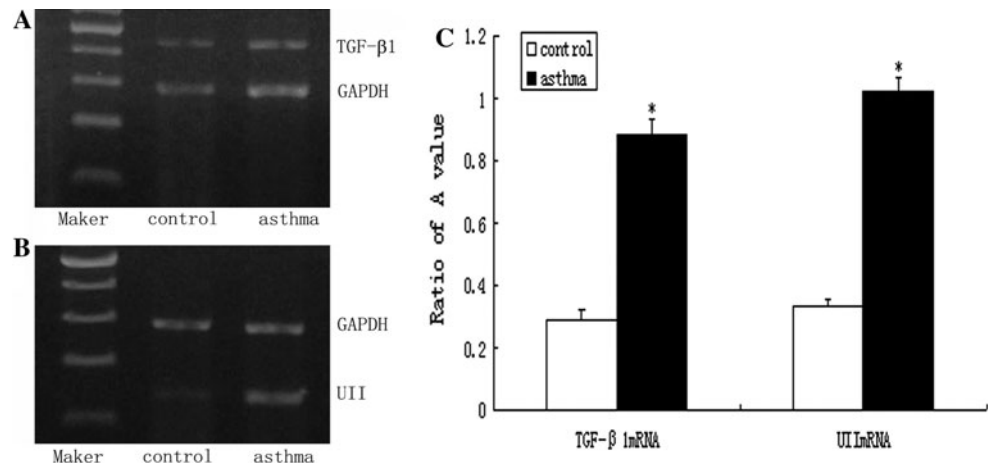


Fig. 3 Immunohistochemistry staining of TGF- β 1 and UII. Lung tissues from control (a, c) and OVA induced asthma rats (b, d) were stained for UII (a, b) and TGF- β 1 (c, d) as described. Data presented is representative of 8 animals from each group with similar results. Magnification = 400. Quantitation of TGF- β 1 and UII expression. * $P < 0.01$ comparing to control (e)

UII on ASMCs TGF- β 1 production. UII caused significant increase of TGF- β 1 in a dose-dependent manner from 4–40 nM. They reached plateau after 40 nM (Fig. 5c, d).

Fig. 4 mRNA expression of UII and TGF- β 1. Total RNA was extracted and reverse transcribed into cDNA. Twenty-five cycles of PCR was performed using specific primers. The products were visualized on 1% agarose gel. Graph shown are representative of eight animals from each group. a TGF- β 1; b UII; c densitometry data. * $P < 0.01$



The effect of UII on TGF- β 1 is ERK-dependent

ERK inhibitor U0126 was used next to determine whether ERK is involved in UII-induced TGF- β 1 production. The result, as illustrated in Fig. 6a, b showed that U0126 caused significant decrease on UII-induced TGF- β 1 production at mRNA and protein levels, indicating ERK pathway is required for such up a regulation. However, ERK is probably not sufficient since the block is not complete. This data imply that other pathways may participate the process of UII-induced TGF- β 1 production.

UII increases ASMCs proliferation in vitro

To test if UII regulates ASMCs proliferation, CCK-8 assay was performed on cells treated with or without UII in vitro. The data, seen in Fig. 7, clearly illustrated the increase in cell proliferation by UII. Of note, such regulation is partially ERK-dependent since proliferation index was significantly, but not completely abolished by ERK inhibitor U0126.

Discussion

By using an OVA-based rat asthma model, we have demonstrated the upregulation of TGF- β 1 and UII in airways of asthma animals. Furthermore, in vitro studies illustrated that increase of TGF- β 1 in ASMCs is mediated, by UII, partially via ERK pathway.

The TGF- β superfamily consists of structurally related polypeptide growth factors. These can be phylogenetically divided into three families: TGF- β s, activins, and bone morphogenetic proteins (BMPs). They regulate development through their effects on cell proliferation, differentiation, and migration. TGF- β proteins also are involved in inflammation, immunity, fibrosis, and angiogenesis, as well

Fig. 5 TGF- β 1 expression by UII treated ASMCs in vitro. Purified ASMCs at concentration of 5×10^6 /ml were treated with UII (100 nM) for 4, 8, 12, 24, and 48 h. At each time point, TGF- β 1 mRNA (a) and protein (b) levels were determined by real-time PCR and ELISA respectively. Dose-dependent response of UII on TGF- β 1 was also performed to show at mRNA (c) and protein (d) levels as well. Data presented are mean $\pm$ SEM of 4 independent experiments with similar results. * $P < 0.01$, # $P > 0.05$ versus control

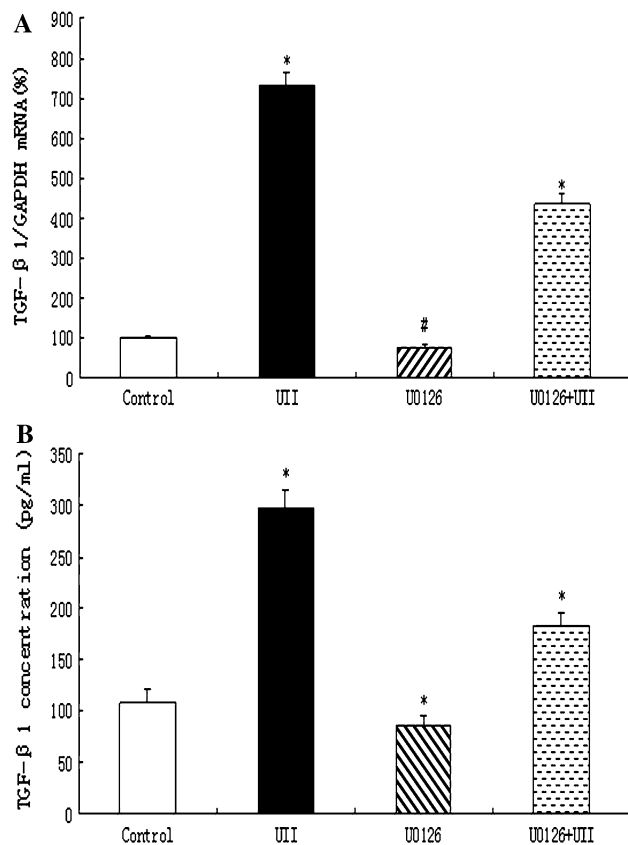
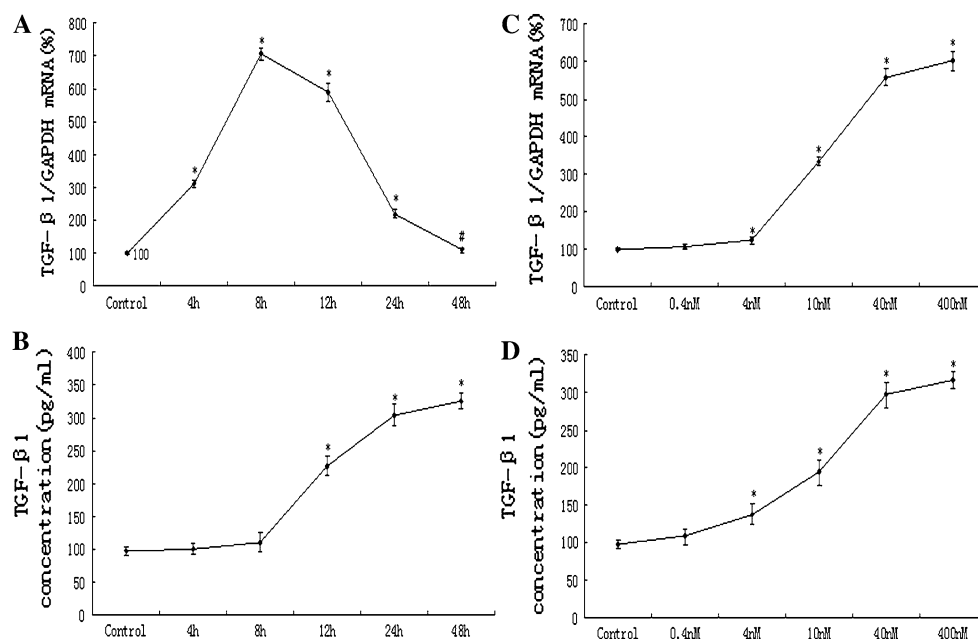


Fig. 6 UII upregulates TGF- β 1 production partially through ERK pathway. Purified ASMCs were incubated with UII at 40 nM for 12 and 24 h in the absence or presence of ERK inhibitor U0126. TGF- β 1 at mRNA (a) and protein (b) levels were determined by real-time PCR and ELISA. ASMCs alone and U0126 treatment alone were served as control. Data expressed as mean $\pm$ SEM of 4 independent experiments with similar results. * $P < 0.01$, # $P < 0.05$ when comparing to control

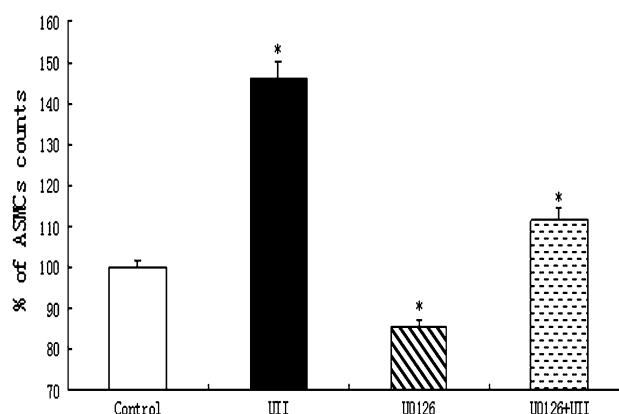


Fig. 7 UII increased ASMCs proliferation. ASMCs were incubated with UII at 40 nM for 48 h in the absence or presence of ERK inhibitor U0126. Samples were collected and subjected to CCK-8 assay as described in the [Materials and Methods](#) section. Data expressed as mean $\pm$ SEM of 4 independent experiments with similar results. * $P < 0.01$ comparing to control

as pathological process including autoimmune/inflammatory diseases, atherosclerosis, fibrosis-associated disorders, and cancer in humans [13, 14]. TGF- β 1 is known to mediate airway remodeling, a signature change in asthma pathogenesis [15]. In agreement with literature reports, data presented herein showed elevation of TGF- β production in asthma animals. Reduction of TGF- β production may facilitate asthma control.

UII is a somatostatin-like cyclic peptide synthesized by proteolytic cleavage from a precursor molecule, prepro-UII, and has been identified as one of the most potent vasoconstrictors in mammals [16]. Hay [17] first reported the contractile activity of human UII (hUII) in airways and

suggested that the potential pathophysiological role of this peptide in lung diseases. UII and its receptor are expressed by human lung adenocarcinoma A549 cells [18] and lungs tissues with lymphangioleiomyomatosis [19]. Our in vivo data revealed a significant increase of UII mRNA and protein expressions by lung tissues in asthma rats than that of the control. The in vitro experiments in the current study, by using purified ASMCs, demonstrated the direct relationship between UII and overexpression of TGF- β 1. Chen et al. [3] reported that UII can serve as a potent mitogen for ASMCs. It induced ASMCs proliferation in a dose-dependent manner [3]. Animal studies also showed that UII can lead to portal venous pressure at a dose-dependent manner. High-dose infusion UII in experimental rats resulted in an increase of TGF- β in hepatic cells [20]. TGF- β 1, which is upregulated in cardiac fibroblasts by UII, is profibrotic [6]. Here, we demonstrated that UII contributes to airway remodeling by regulating TGF- β 1 expression in ASMCs.

Previous study from our group has showed that ERK is involved in asthma airway remodeling [21]. Several studies have pointed to the effect of ERK on ASMCs proliferation and airway remodeling [5, 7, 22–24]. The study of Xie et al. [25] reveals a key role of the ERK1/2 signaling pathway in the phenotypic modulation of ASMCs in chronic asthmatic rats. Growing evidence indicates an overlap between signal transduction pathways used by ERK and UII. Matsusaka et al. [8] finds that UII affects the migration of HASMC through the activation of an ERK-dependent pathway. ERK pathway may also be involved in the central pressor action of UII in conscious rats [26]. Using ERK/MAPK kinase inhibitor PD98059, several studies have suggested that ERK may be involved in UII-induced proliferation of vascular smooth muscle cells (VSMCs). Activation of integrin-mediated signaling pathways plays a critical role in UII-induced phosphorylation of ERK, leading to proliferation of VSMCs [27]. hUII is an autocrine/paracrine growth factor for renal epithelial cells which activates both ERK1/2 and protein kinase C (PKC) pathways as well as Ca^{2+} influx via voltage-dependent Ca^{2+} channels [28]. The finding of Zou et al. [29] shows that UII induces hypertrophic responses in cardiac myocytes via the activation of ERK. Meanwhile, Our results confirmed the effect of UII on TGF- β 1 expression from ASMCs in rat asthma model. By using specific inhibitor U0126, we further provided evidence that ERK pathway is required, but not sufficient in UII-induced TGF- β 1 expression. This result, along with other reports, suggests the involvement of other signaling molecules. The direct observation of cell proliferation, as reflected from CCK-8 assay, added another layer of evidence that UII mediates ASMCs proliferation, thus, leading to airway smooth muscle thickening, a key feature of airway remodeling.

In summary, the current study provided new evidence that UII is involved in the TGF- β mediated mitogenic effect on ASMCs. The UII, at least partially, uses ERK pathway to render such effect.

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