Therapeutic effect of ulinastatin on pulmonary fibrosis via downregulation of TGF-β1, TNF-α and NF-κB

DEJUN LI, HONGSHENG JI, BAO ZHAO, CHUNYANG XU, WENJUN XIA, LIHUI HAN, DONGQING YU, YUANRONG JU and CHANGJUN JIN

Surgical Intensive Care Unit, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250021, P.R. China

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Abstract. Pulmonary fibrosis is a chronic, progressive, lethal lung disease characterized by alveolar cell necrosis and dysplasia of interstitial fibrotic tissue, resulting in loss of lung function and eventual respiratory failure. Previously, glucocorticoid drugs were used to treat this lung disorder. However, positive responses were recorded in less than half of treated patients and the cytotoxicity caused by high dosage treatment is still a concern. The present study investigated whether ulinastatin, a typical urinary trypsin inhibitor that mitigates numerous inflammatory responses, could be a treatment option for lung fibrosis. The results demonstrated that ulinastatin had the ability to ameliorate interstitial fibrosis and alveolar exudates and to protect against lung diseases induced by smoke, irradiation or silica particles. The mechanism of ulinastatin resulted in the downregulation of inflammatory cascades: Transforming growth factor-β1, tumor necrosis factor-α and nuclear factor-κB, as demonstrated by western blotting and ELISA. Ulinastatin treatment with a high dose (100,000 U/kg body weight/day) resulted in an attenuated inflammatory response, and inhibited fibrosis formation in lungs, suggesting that ulinastatin may become a part of a clinical therapeutic strategy.

Introduction

Pulmonary fibrosis is a fatal disorder, considered as the outcome of many chronic pulmonary diseases (1). Fibrotic alveolar epithelial cells can develop for various reasons mainly due to overexposure to irradiation, smoke inhalation and disease of respiratory system. The early pathological lesion is demonstrated in a form of an acute alveolus inflammation, involving inflammatory cells, immune effector cells and cytokines, such as transforming growth factor-β1 (TGF-β1), tumor necrosis factor-α (TNF-α) and nuclear factor-κB (NF-κB). Pulmonary fibrosis is most prevalent among 50-70year-olds, and the estimated annual incidence among men and women is 10 and 7 per 100,000 individuals, respectively (2,3). The death rate of pulmonary fibrosis is gradually increasing and the risk is positively correlated with age (4). Traditionally, treatment of the disease relies on corticosteroids, but the response rate is not satisfactory and the drug is rarely used as a prophylaxis (5). Developing a therapeutic strategy against the disease is an urgent matter for patient who developed the symptoms of lung fibrosis.

Ulinastatin (UTI), usually used as a urinary trypsin inhibitor, is a 67 kDa glycoprotein normally purified from healthy human urine (6). UTI is a Kunitz-type protease inhibitor containing two active functional domains and no overlapping regions with an effective role against a broad range of enzymes (7). Previous studies demonstrated that UTI is able to inhibit numerous inflammatory proteases including trypsin, chymotrypsin, neutrophil elastase and plasmin (8). Since the number of proteases increases from the beginning of infection and inflammation, it is rational to use UTI as an effective anti-inflammatory molecule (9). Clinically, UTI has been widely used as a drug for the treatment of severe inflammatory responses, such as burn, sepsis and acute pancreatitis (10). No adverse toxicological effects of ulinastatin were observed during preliminary treatment. UTI was reported to be able to decrease the inflammatory reaction and mitigate the lung damage caused by smoke and lipopolysaccharide in rats subjected to the treatment of pulmonary disorder (11). Nonetheless, the mechanisms underlying the therapeutic process remain unclear, and the dosage-dependent effect has not been thoroughly investigated. Thus, the present study aimed to unravel the signaling pathway mediating the therapeutic effect of UTI in pulmonary fibrosis on a molecular level using Sprague-Dawley rats. In addition, correlation between UTI dosage and treatment efficacy was analyzed.

Materials and methods

Animals and reagents. A total of 90 male Sprague-Dawley rats used for all experiments were 9 weeks old and weighed

Correspondence to: Dr Changjun Jin, Surgical Intensive Care Unit, Shandong Provincial Hospital Affiliated to Shandong University, 5 Jing Road, Jinan, Shandong 250021, P.R. China
E-mail: changjunjin@163.com

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290-320 g (Super-B&K Laboratory Animal Corp, Shanghai, China). The materials used for this study included bleomycin (BLM; Tai He Pharmaceutical Co. Ltd., Tianjin, China) and UTI (Techpool Bio-Pharma Co. Ltd., Guangzhou, China). Pentobarbital sodium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in physiological saline and the concentration was adjusted to 10 mg/ml. Enzyme-linked immunosorbent assay (ELISA) kits specific to TGF-β1 (Abcam, cat. no. ab119558, USA), TNF-α (Abcam, cat. no. ab46070, USA) and NF-κB (Abcam, cat. no. ab28856, USA), and a western blotting kit (Amersham; GE Healthcare Lice Sciences, Little Chalfont, UK) were used in this study.

Rat model of pulmonary fibrosis. Rats were randomly allocated into one of 3 groups: Negative control (n=30), model (n=30) and ulinastatin treatment (n=30). All rats were bred and maintained in accordance with the 'Care and Use of Laboratory Animals' guidelines published by the National Institute of Health of China (12). For the model group, 250 μl BLM (4 g/l) was injected into lungs through the trachea, to achieve the dosage of 5 mg/kg body weight. Once BLM was injected, the animals were rotated immediately to ensure an equal distribution of the chemical in lungs. A total of 250 μl physiological saline solution was introduced into the lungs of rats as a control. In the UTI treatment group, rats were given different doses of UTI (high and low) by intraperitoneal injection one day following BLM administration. A total of 15 rats were treated with a high dose of UTI (100,000 U/kg body weight/day) and 15 rats were treated with a low dose of UTI (20,000 U/kg body weight/day). A total of 5 rats were treated with sodium pentobarbital sedative overdose at 7, 14 and 28 days in all three groups. A piece of lung tissue was collected from dead rats and stored at -70˚C for further analysis.

The present study received ethical approval from the Animal Ethics Committee of the Shandong University (Jinan, China).

Histopathological examination. Paraffin-embedded sections of pulmonary tissues were stained with hematoxylin-eosin (H&E) and Masson's trichrome stain for morphological studies (13). A piece of the left lung was fixed in 10% formaldehyde solution for 12 h. Blocks of the lung tissue were dehydrated and embedded in paraffin, cut into 5 μm slices, incubated at 60˚C overnight, dewaxed, then stained with H&E or Masson's trichrome for 5 and 10 min, respectively, at room temperature. Finally, the general pathological changes of the lung tissue and collagen fibrils were observed and captured using an optical microscope.

Alveolitis and pulmonary fibrosis of the experimental rats were classified according to the Szapiel's method (14). The alveolitis grades were: 0, no alveolitis; 1, mild alveolitis characterized by alveolar interval widening due to cell infiltration, with a lesion area <20% of the lung; 2, moderate alveolitis characterized by a lesion area of 20-50% of the lung; 3, diffuse alveolar inflammation characterized by a lesion area >50% of the lung.

The pulmonary interstitial fibrosis grades were: 0, no pulmonary fibrosis; 1, mild pulmonary fibrosis characterized by an involvement area <20% of the lung; 2, moderate lung interstitial fibrosis characterized by a disordered alveolar structure and an involvement area 20-50% of the lung; 3, severe pulmonary fibrosis characterized by an integrated alveolar, a disordered physical lung structure and an involvement area >50% of the lung.

Western blot analysis. Lung samples from three mice per group were randomly selected from each group and protein expression levels were determined by western blot analysis. For tissue lysate preparation, frozen lung tissues were homogenized at 4˚C with 100 ~200 μl radioimmunoprecipitation assay (RIPA) lysis buffer (cat. no. P0013B, Beyotime Institute of Biotechnology, Haimen, China) supplemented with 1% protease and phosphatase inhibitor cocktail (Beyotime Institute of Biotechnology, cat. no. P1005), followed by centrifuged at 10,000 x g for 10 min at 4˚C. Total tissue lysate was collected as a supernatant. Equal amounts of protein lysates (30 μg/lane) were resolved on a SDS-PAGE gel (12%), and the separated bands were transferred onto a 0.22-μm nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a transfer tank (Bio-Rad Laboratories, Inc.). Subsequently, the membrane was blocked at room temperature for 2 h in PBS containing 0.1% Tween-20 (Sigma-Aldrich; Merck KGaA) and 5% skimmed milk. The blocked negative control was then incubated overnight at 4˚C with rabbit polyclonal primary antibodies (anti-TGF-β1, anti-TNF-α and anti-NF-κB, Santa Cruz Biotechnology, Inc.). An antibody specific toglyceraldehyde-3-phosphate dehydrogenase, a house keeping protein, served as an internal reference. After the incubation, the membranes were washed in a dilution buffer containing 1XTris-buffered saline (TBS, Beyotime Institute of Biotechnology, cat. no. P0233) and 0.1% Tween-20 (Beyotime Institute of Biotechnology, cat. no. ST825) and 5% BSA (cat. no. A8020, Beijing Solarbio Science and Technology, Co., Ltd., Beijing, China) and incubated for 1 h with horseradish peroxidase (HRP)-coupled developing antibody (HRP-conjugated anti-rabbit, diluted 1:8,000, Santa Cruz Biotechnology, Inc.). After the final wash with the dilution buffer (3 times, 10 min each) the blots were immunodetected with enhanced chemiluminescence (Cell Signaling Technology, Inc., Danvers, MA, USA). The grayscale value for each band representing the concentration of the corresponding protein was measured using a molecular imager (Bio-Rad Laboratories, Inc.).

ELISA analysis. The frozen lung tissue was thawed and homogenized at 4˚C, followed by centrifugation at 10,000 x g at 4˚C for 20 min. Subsequently, 20 μl supernatant was pipetted and transferred into an Eppendorf tube for future use. The calibrator diluent with different concentrations of standard TGF-β1, TNF-α and NF-κB, and triplicate samples were added to individual polystyrene plate wells. Tissue was incubated for 2 h at 37˚C. Between each step of the procedure, the plates were washed three times using TBS (pH 7.4). PBS was used as a negative control. A total of 100 μl antigen-specific biotin conjugate (Cell Signaling Technology, cat. no. L27A9) was added to each well of the plate, and then the plate was incubated at room temperature for 40 min, followed by an addition of 100 μl streptavidin-HRP conjugate. The plate was incubated at room temperature for 30 min, 100 μl 3, 3, 5, 5-Tetramethylbenzidine (TMB) substrate solution was added to each well, and then the plate was incubated at room temperature for 30 min in the dark. Finally, 100 μl stop

solution was added to each well, and the plate was measured at a wavelength of 450 nm using a 680-microplate reader (Bio-Rad Laboratories, Inc.).

Statistical analysis. All statistical analyses were performed using Statistical Package for the Social Sciences 13.0 software (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation from three separate experiments. Comparison of multiple groups was performed using analysis of variance followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Histopathological characteristics of the lung tissues. The comparison between lung tissues from different groups was presented in Fig. 1. The control group demonstrated a thin interstitial matrix and regular alveolar lumen (Fig. 1A). 7 days following the experiment, a mild alveolitis with localized fibrosis became evident, indicating that BLM caused rat lung injury (Fig. 1B). Symptoms such as hemorrhage, widened alveolar septa and infiltration of numerous macrophages occurred following more than 14 days of the experiment and represented a severe alveolitis and pulmonary fibrosis (Fig. 1C and D). When the injured rats were administered UTI treatment, typical inflammatory symptoms, such as hemorrhage, alveolar exudates and neutrophil accumulation were evidently alleviated compared with the injury model group (Fig. 1E and F) indicating that UTI is therapeutic to pulmonary fibrosis and alveolitis. As demonstrated by the results from the histopathology grading (Table I), UTI treatment mitigated alveolitis and lung fibrosis in all three modeling periods, with recovery rates >40%. UTI cured >65% interstitial fibrosis rats induced by 7 days of the experimental injury, demonstrating therapeutic potential of UTI in the treatment of pulmonary disorders.

Expression of TGF-β1, TNF-α and NF-κB in lungs of the experimental rats. Expression levels of TGF-β1, TNF-α and NF-κB were investigated using western blotting to determine specific functional pathways of UTI activity on lung fibrosis. As presented in Fig. 2, expression of all three inflammation-associated cytokines increased in the model group from day 7 to 28, compared with the control group. This increase indicated that the inflammatory response was activated by BLM and contributed to histopathological injuries (Fig. 1). When UTI treatment was applied, the expression of these cytokines was significantly reduced. The expression of TNF-α and NF-κB was restored to the normal level, indicating that the likely mechanism of UTI action on pulmonary fibrosis is downregulation of inflammatory regulators contributing mostly to the migration, proliferation, and differentiation of resident mesenchymal cells (15).

UTI-mediated mitigation of pulmonary fibrosis demonstrated by ELISA. To quantify the number of cytokines expressed during alveolitis and fibrotic formation, ELISA using HRP/TMB system chromogenic agent was used. Fig. 3 summarizes the changes in expression levels of TGF-β1, TNF-α and NF-κB. UTI significantly inhibited these cytokines in the treatment groups, compared with the negative control group.

Table I. Histopathological grade system for the assessment of pulmonary alveolitis and interstitial fibrosis.

Parameter	Group	n	Grade		
			7 d	14 d	28 d
Pulmonary Alveolitis	Control	10	0±0	0±0	0±0
	Modeling	10	2.4±0.5	2.6±0.4	2.0±0.4
	Ulinastatin	10	1.3±0.2	1.2±0.3	1.0±0.2
Interstitial Fibrosis	Control	10	0±0	0±0	0±0
	Modeling	10	0.9±0.3	2.3±0.2[a]	2.7±0.4[a]
	Ulinastatin	10	0.3±0.1	1.2±0.3[a]	1.2±0.5[a]

[a]$P<0.05$ vs. the 7-day grade.

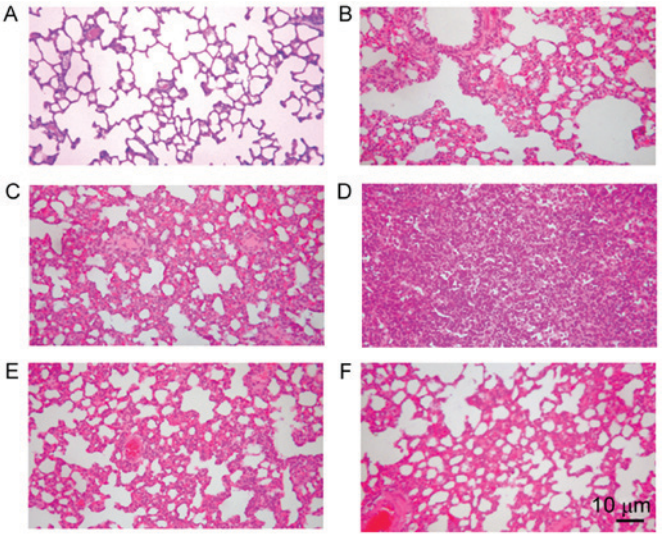

Figure 1. Representative rat lung tissue histological sections from control, model and ulinastatin-treated groups. (A) Normal lung tissue with no fibrosis; (B) minimal lung fibrosis and inflammation following7 days of modeling with BLM; (C) moderate pneumonia with contracted alveolar cavity following 14 days of modeling with BLM; (D) severe pulmonary fibrosis with thickened alveolar walls and inflammatory cells infiltration following 28 days of modeling with BLM; (E) low dosage (20,000 U/kg body weight/day) ulinastatin treatment, f ibrosis mitigated and alveolar interval narrowed; (F) High dose (100,000 U/kg body weight/day) ulinastatin treatment, volume of alveolar cavity expanded and number inflammatory cells reduced. Magnification, x100. BLM, bleomycin.

Additionally, expression levels of TGF-β1, TNF-α and NF-κB in groups with both high and low dose UTI treatment were significantly reduced 4 weeks post injury compared with the modeling, untreated group.

Masson staining to measure healing of the fibrotic lung tissues. Masson trichrome staining was used to evaluate the degree of pulmonary fibrosis by calculating the proportion of the blue-fibrosis stain to the whole area (16). As presented in Fig. 4A, fibrous tissue in a normal range (<20%) was observed in the lung of rats from the control/untreated group. BLM modeling resulted in the formation of interstitial collagens depositions leading to dysplastic fibrosis of the lung (area >50%). When UTI

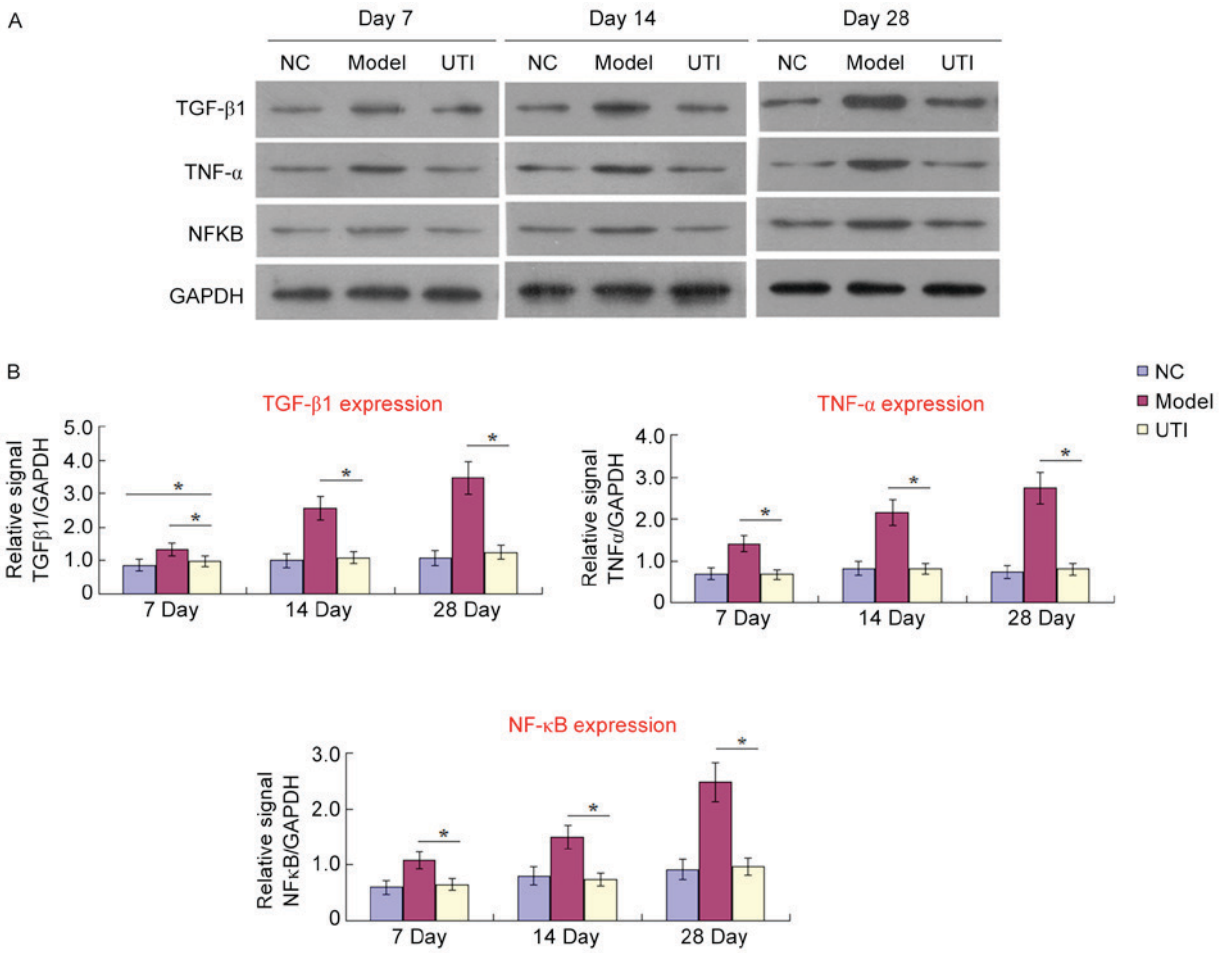

Figure 2. Cytokine expression. (A) Western blotting results representing expression of TGF-β1, TNF-α and NF-κB in rat pulmonary tissue from the negative control, model and ulinastatin treatment groups. GAPDH was used as a reference to evaluate relative expression of TGF-β1, TNF-α and NF-κB. (B) Quantitative comparison of TGF-β1, TNF-α and NF-κB expression in different groups at different time points. Data are presented as the mean ± standard deviation. *P<0.05. NC, negative control; UTI, ulinastatin; TGF-β1, transforming growth factor-β1; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α.

treatment was applied, both in a low and high dose, the area of fibrotic tissue was significantly reduced compared with the BLM modeling group, indicating that progression of pulmonary fibrosis was inhibited by UTI, which could have also contributed to the replacement of fibrotic collagen and the dissipation of inflammatory cells. The fibrosis area in UTI group was larger than that of the control group (Fig. 4B) possibly due to a short course of treatment. Longer treatment with UTI and a refined protocol may cure pulmonary fibrosis.

Discussion

Pulmonary fibrosis is a progressive, irreversible, and usually lethal lung disease. Its incidence and prevalence increase markedly with age, and the median survival is 3 years following diagnosis (17). Research into the etiology of this devastating lung injury revealed that the cause of pulmonary fibrosis is heterogeneous but probably arising from the interplay between genetic and environmental factors (18). Although the disease received tremendous attention, the exact mechanisms of pathogenesis is still unclear, leading to limited availability of treatment options for patient with both idiopathic and induced pulmonary fibrosis. Traditionally, glucocorticoids, immunosuppressors and cytotoxic agents are used to treat the lung disorder; however, positive responses are recorded in 10-30% of the treated patients, and long-term use of these drugs may cause undesirable side effects (19). Therefore, developing a novel therapeutic strategy with low cytotoxicity and high efficacy is needed.

The present study investigated the effect of UTI treatment on BLM-induced pulmonary fibrosis in rats. UTI treatment significantly mitigated pulmonary injury as demonstrated by the histological reduction of inflammatory exudates and collagen deposition compared with the model group with chronic fibrosing alveolitis. UTI treatment resulted in a more efficient lung gas exchange, less pulmonary microvascular leakage, and decreased tissue injury and fibrosis formation. Similar results are also reported by other research teams focusing on the anti-inflammatory effects of UTI (20-22). It was also demonstrated that high dose UTI (100,000 U/kg body weight/day) more effectively prevented BLM-induced pulmonary injury during the acute inflammation phase, than the traditional therapeutic strategy. Rats from the modeling group suffered more pronounced inflammation (several died following 28 days of modeling) than those in the treatment groups, suggesting that UTI elicited a protective effect. In addition, acute inflammatory symptoms in the control group were more severe compared with both low and high dose UTI treatment group, suggesting that pretreatment with high dose

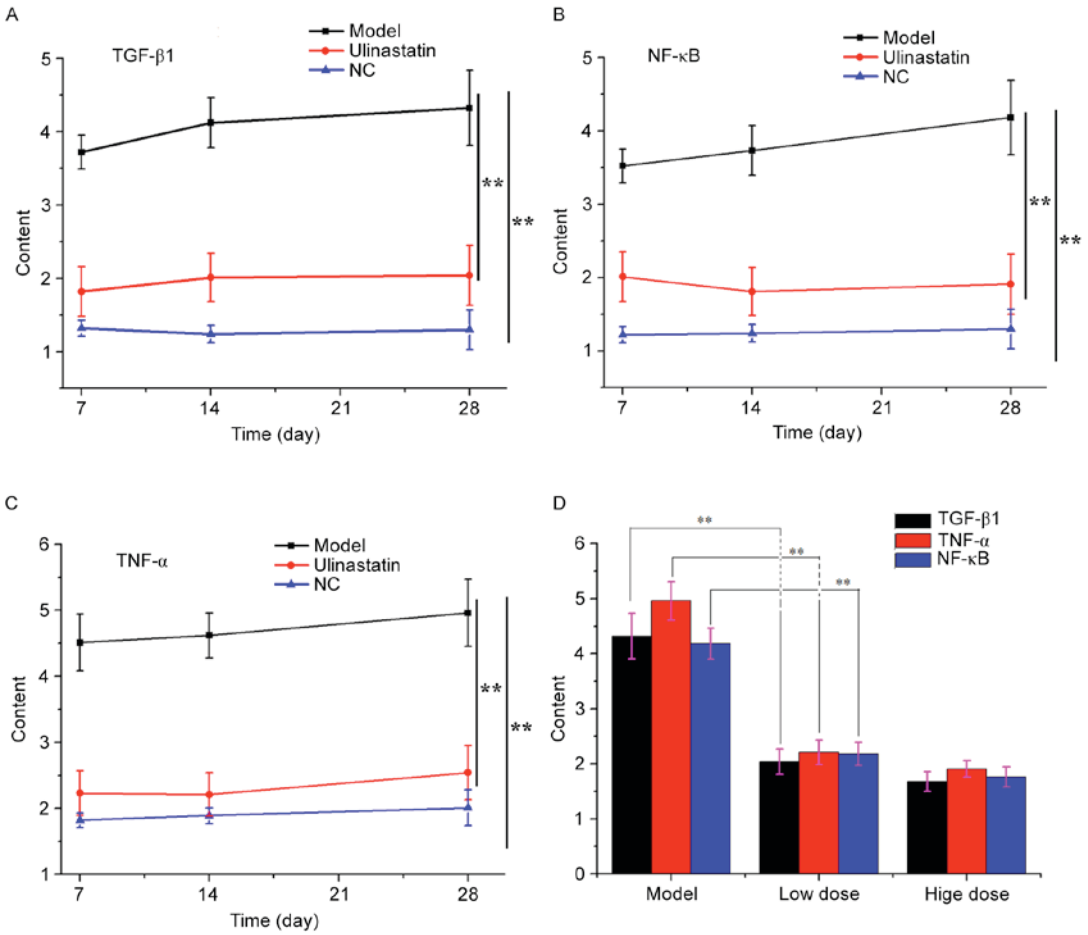

Figure 3. Changes in the expression of TGF-β1, NF-κB and TNF-α throughout the experiment. Expression of (A) TGF-β1, (B) NF-κB and (C) TNF-α in the negative control, model and ulinastatin treatment group at 7, 14 and 28 days. **P<0.01 (D) Comparison of TGF-β1, NF-κB and TNF-α between the modeling group, low dose UTI treatment group (20,000 U/kg body weight/day) and high dose treatment group (100,000 U/kg body weight/day). Data are presented as the mean ± standard deviation. **P<0.01 vs. the control group. UTI, ulinastatin; NC, negative control; TGF-β1, transforming growth factor-β1; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α.

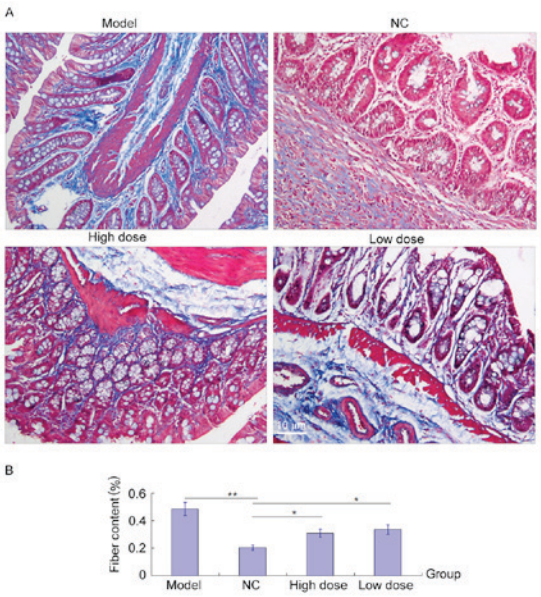

Figure 4. Masson staining. (A) Lung tissues from the control, modeling and ulinastatin low dose (20,000 U/kg body weight/day) and high dose (100,000 U/kg body weight/day) group (magnification, x400). (B) Percentage of collagen fibrils within the field of vision. Three parallel tissue sections were evaluated. Data are presented as the mean ± standard deviation. *P<0.05, **P<0.01. NC, negative control.

UTI might have more protective effect than after-treatment with either a low or high dose.

UTI is an anti-inflammatory agent used for treatment of many inflammation disorders such as pancreatitis, arthritis, nephritis and associated disorders (23-25). Previous studies demonstrated that UTI can inhibit the accumulation of pro-inflammatory cytokines (26-28), and the present study investigated the expression of cytokines in response to UTI administration. The results of the present study demonstrated that UTI can markedly reduce the expression levels of TGF-β1, a key mediator of fibrosis formation. Fibroblasts, myoblasts and macrophages have been proposed as TGF-β1 effectors in the progression of lung fibrosis (29,30). Therefore, reducing the production of TGF-β1 should inhibit the abnormal growth of fibroblasts and myoblasts, which are responsible for excessive collagen deposition and alveolar membrane collapse, resulting in the amelioration of fibrosis in lungs. TNF-α (also known as cachectin), secreted by macrophages, is able to induce the differentiation and proliferation of fibroblasts, leading to the formation of fasciculate collagen fibers in extracellular matrix (31). The way UTI acts on TNF-α signaling pathway is similar to the effect it has on TGF-β1. Inflammatory components were not demonstrated to be involved in TNF-α and TGF-β1 signaling pathways, and this may explain the low

efficacy of glucocorticoids in the treatment of chronic fibrosing alveolitis. In addition, since UTI is not cytotoxic, it may be used to prevent lung fibrosis in high genetic or environmental risk patients.

NF-κB is a transcription factor that regulates genes responsible for both innate and adaptive immune responses. It was reported that NF-κB directly or indirectly controls the expression of several cytokines, such as TGF-β1 and TNF-α which were investigated in the present study (32). Normally, NF-κB is in a relatively inactivate state and promotes hardly any gene expression. Upon exogenous stimulation and phosphorylation, it can promote expression of certain genes. Previous studies demonstrated that during inflammatory response NF-κB initiates mRNA synthesis of TGF-β1 and TNF-α, leading to elevated expression of these cytokines in serum and plasma (33), suggesting that NF-κB regulates the expression of TGF-β1 and TNF-α in inflammatory cells. In the present study, pulmonary fibrosis was positively associated with the enhanced activity of NF-κB, and production of TGF-β1 and TNF-α. Lung injury was attenuated with the downregulation of NF-κB, TGF-β1 and TNF-α upon UTI application, as demonstrated by ELISA. Since NF-κB regulates the expression of TGF-β1 and TNF-α, one possible mechanism of UTI action on lung fibrosis is that UTI inhibits NF-κB, causing decreased expression of TGF-β1 and TNF-α and mitigation of the inflammatory reaction and renewal of fibrotic tissues. As described in the present study, the activity of NF-κB, and expression of TGF-β1 and TNF-α decreased simultaneously upon UTI administration, indicating that UTI simultaneously acts on three factors and downregulates them in the pulmonary cells exhibiting inflammatory symptoms.

In conclusion, the present study demonstrated that UTI can significantly ameliorate the symptoms of pulmonary injury and the subsequent development of pulmonary fibrosis in a rat model. The functional mechanism of UTI is likely a simultaneous downregulation of NF-κB, TGF-β1 and TNF-α along with their associated signaling pathways. High dose UTI treatment (100,000 U/kg body weight/day) may in the future demonstrate a therapeutic effect for lung fibrosis in high risk people exposed to radiation, smoke and silica particles. Based on the data presented in this study, UTI represents a promising therapeutic strategy for pulmonary fibrosis and inflammatory disorders. Evaluation of long-term side-effects and dosage-related cytotoxicity of UTI needs to be performed before initiation of clinical applications.

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