Dynamic Variation of RAS on Silicotic Fibrosis Pathogenesis in Rats*

Bo-nan ZHANG[1, 2, 3†], Xin ZHANG[2†], Hong XU[2], Xue-min GAO[4], Gui-zhen ZHANG[2], Hui ZHANG[2], Fang YANG[2#]
[1]School of Public Health, [2]Hebei Key Laboratory for Organ Fibrosis, Medical Research Center, [3]Hebei Key Laboratory for Chronic Diseases, Tangshan Key Laboratory for Clinical and Basic Research on Chronic Diseases, School of Basic Medical Sciences, North China University of Science and Technology, Tangshan 063210, China
[4]Basic Medical College, Hebei Medical University, Shijiazhuang 050017, China

Summary: The dynamic variation of renin-angiotensin system (RAS) in silicosis remains unclear. Seventy Wistar rats were divided into 7 groups including control group, silicosis groups (inhaling SiO_2 for 2, 4, 8, 16 and 24 weeks, respectively) and Captopril (Cap) group. Rat lung primary fibroblasts were divided into control group, SiO_2-stimulated group (0, 0.5, 1, 3, 6, 12, 24 and 48 h) and Cap group. The silicotic nodules were formed and collagens were deposited gradually in silicosis group observed by haematoxylin and eosin (HE) staining and Van Gieson (VG) staining. Cap relieved the lung fibrosis and collagen deposition. Immunohistochemistry indicated the positive expression of α-smooth muscle actin (α-SMA) was increased gradually in silicotic rat lung tissue. Western blotting revealed the expression of collagen type Ⅰ (Col Ⅰ) and α-SMA was up-regulated in silicotic rat lung tissue and fibroblasts stimulated by SiO_2. Cap decreased the expression of Col Ⅰ and α-SMA in silicotic rat lung tissue and fibroblasts stimulated by SiO_2. Western blotting also demonstrated the expression of angiotensin-converting enzyme (ACE) and angiotensin Ⅱ type 1 receptor (AT1) was increased, and the expression of ACE2 and Mas was decreased gradually in silicotic rat lung tissue and fibroblasts stimulated by SiO_2. ELISA showed the serum levels of ACE and angiotensin Ⅱ (Ang Ⅱ) were also increased and ACE2 and Ang (1-7) were decreased in the silicosis group. Treatment with Cap decreased the expression levels of ACE, Ang Ⅱ and AT1, and increased the expression levels of ACE2, Ang (1-7) and Mas. These findings suggested that an imbalance between ACE-Ang Ⅱ-AT1 axis and ACE2-Ang (1-7)-Mas axis may participate in the development of silicosis.

Key words: silicosis; fibroblasts; SiO_2; renin-angiotensin system; α-smooth muscle actin

Silicosis caused by inhalation of silica dust is the most serious occupational disease in China and the incidence has increased severely in recent years[1, 2]. The characteristic pathological changes of silicosis are the formation of silicotic nodule and diffuse pulmonary fibrosis[3]. It had been reported that renin-angiotensin system (RAS) played an important role in the occurrence and development of pulmonary fibrosis[4, 5]. Clinical data indicated that the contents of serum angiotensin-converting enzyme (ACE) and angiotensin Ⅱ (Ang Ⅱ) were increased in silicotic patients[6]. Our research group had also found that the expression of ACE, Ang Ⅱ and angiotensin Ⅱ type 1 receptor (AT1) was up-regulated in lung tissue of silicotic rats[7]. Ang Ⅱ induces the proliferation of fibroblasts, differentiation of myofibroblasts and deposition of collagen, and promotes the development of pulmonary fibrosis through binding AT1, which manifests ACE-Ang Ⅱ-AT1 axis exerts important regulatory actions during silicosis. In addition, ACE2-Ang (1-7)-Mas, as another important target axis of RAS, plays the opposite regulatory action. ACE2 can hydrolyze Ang Ⅱ to Ang (1-7) and exert anti-pulmonary fibrosis effect through binding Mas[8]. Ang (1-7) or over-expression of ACE2 can inhibit the pulmonary fibrosis induced by bleomycin[9]. ACE2-Ang (1-7)-Mas axis is closely related to pulmonary fibrosis and whether the axis participates in the development of silicosis is still unknown. It has significant sense to research the role of ACE-Ang Ⅱ-AT1 and ACE2-Ang (1-7)-Mas axis in the development of silicosis. In the present study, we explored the dynamic variation of

Bo-nan ZHANG, E-mail: zhangbonan1982@126.com; Xin ZHANG, E-mail: 862648505@qq.com
†Both authors contributed equally to this work.
#Corresponding author, E-mail: fangyang_1955@126.com
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ACE-Ang Ⅱ-AT1 and ACE2-Ang (1-7)-Mas axis in the rat silicosis model and primary lung fibroblasts stimulated by SiO_2 in order to enrich the pathogenetic mechanism of silicosis.

1 MATERIALS and METHODS

1.1 Experimental Animals and Establishment of Silicosis Model

The animal use protocol was approved by the North China University of Science and Technology experimental animal ethics committee. The study was carried out in accordance with the Declaration of Helsinki. Seventy specific pathogen-free male Wistar rats (3 weeks of age) were obtained from Vital River Laboratory Animal Technology Co. Ltd. (SCXK 2009-0004, Beijing, China). The rats were housed in a temperature-controlled facility (25°C±1°C, 55%±10% humidity) with a 12 h light/dark cycle and received food and water regularly. A HOPE-MED 8050 exposure control apparatus supplied by HOPE Industry and Trade Co. Ltd. (China) was used to establish the silicosis model. This system can be set to a certain dust concentration and it is a non-invasive instrument for allowing animal inhalation. The parameters were set as follows: exposure chamber volume, 0.3 m^3; cabinet temperature, 20–25°C; humidity, 70%–75%; pressure, –50 Pa to +50 Pa; oxygen concentration, 20%; flow rate of SiO_2 (0.5–10 μm, approximately 80% between 1–5 μm, s5631, Sigma-Aldrich, USA), 3.0–3.5 mL/min; dust mass concentration in the cabinet, 40 μg/m^3. The rats were randomly divided into 7 groups: control group (not inhaling SiO_2); 2-week SiO_2 exposure group (inhaling SiO_2 for 2 weeks); 4-week SiO_2 exposure group (inhaling SiO_2 for 4 weeks); 8-week SiO_2 exposure group (inhaling SiO_2 for 8 weeks); 16-week SiO_2 exposure group (inhaling SiO_2 for 16 weeks); 24-week SiO_2 exposure group (inhaling SiO_2 for 24 weeks); Cap treatment group (inhaling SiO_2 for 16 weeks and treatment with Cap from 16th week). Cap, an ACE inhibitor (ACEI), was based on the peptide sequence of bradykinin-potentiating factor, which inhibited the conversion of Ang Ⅰ to Ang Ⅱ when it was perfused into pulmonary circulation. It has been shown to decrease fibrosis in experimental models of heart and kidney. Cap (30 μg/kg·day, C4042, Sigma-Aldrich, USA) was administered into the abdominal cavity via a mini-osmotic pump (Alzet, 200 μL, Durect, USA). Each group involved 10 rats and each animal inhaled SiO_2 for 3 h per day until the rats were sacrificed at the corresponding time point.

1.2 Histopathological Observation

The right lower lungs were fixed in 4% neutral formalin solution for 48 h. The samples were sequentially dehydrated, embedded in paraffin and cut into 4-μm thick sections. H.E. (BA4025, Baso Diagnostics Inc., China) staining was used to observe lung fibrosis. VG (BA4084, Baso Diagnostics Inc., China) staining was used to assess collagen deposition. The number and size/area of silicotic nodules were counted by cellSens Dimension software (Olympus Corporation, Japan)[10]. The silicotic area and the number of silicotic nodules were calculated by the total area of lung section.

1.3 Immunohistochemistry (IHC)

High-pressure method was used to repair antigen on dewaxed tissue sections and endogenous peroxidases were quenched with 0.3% H_2O_2. Samples were then incubated with primary antibodies against α-smooth muscle actin (α-SMA, 1:200, ab32575, Eptomics, USA) overnight at 4°C, followed by incubation with secondary antibody (PV-6000, Beijing Zhongshan Jinqiao Biotechnology Co. Ltd., China) at 37°C for 30 min. Immunoreactivity was visualized with DAB (ZLI-9018, ZSGB-BIO, China). Brown staining was considered positive.

1.4 Cell Culture

The lung tissue of newborn rat was removed. Lung fibroblasts were isolated from minced lung tissue and plated on 25-cm^2 plates in DMEM (BI-SH0019, BI, Kibbutz Beit-Haemek, Israel) medium containing 10% FBS (10099141, Gibco, Thermo Fisher Scientific, USA) and 1% penicillin-streptomycin. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. Cells at 80% confluence were cultured in FBS-free DMEM medium for 24 h, when most cells were quiescent. SiO_2 (50 μg/cm^2) was used to induce the lung fibroblasts to myofibroblasts at different time points (0, 0.5, 1, 3, 6, 12, 24 and 48 h) and cells were divided into 9 groups: control group (not stimulation with SiO_2); SiO_2-induced for 0.5 h group; SiO_2-induced for 1 h group; SiO_2-induced for 3 h group; SiO_2-induced for 6 h group; SiO_2-induced for 12 h group; SiO_2-induced for 24 h group; SiO_2-induced for 48 h group; Cap treatment group (10^{-6} mol/L, SiO_2-induced for 48 h and Cap was given 1 h before SiO_2 stimulation).

1.5 Western Blot

The lung tissue and cell lysates were extracted with RIPA buffer (BB-3201-1, BestBio, China) containing a protease inhibitor cocktail (Sigma-Aldrich). Protein concentration in the supernatant was quantified using the Bradford protein assay (PC0020, Solarbio, China). The proteins (20 μg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and electro-transferred onto PVDF membranes. The membranes were then blocked with 5% non-fat milk and incubated overnight at 4°C with primary antibody against Col Ⅰ (ab34710, Abcam, UK), α-SMA, ACE (sc-12187, Santa Cruz Biotechnology, USA), ACE2 (AF-3437, R&D systems, USA), AT1 (sc-1173, Santa Cruz Biotechnology, USA) and Mas (AAR-013, Alomone Laboratories, Ltd., Israel). The membranes were then

washed and incubated with 1:5000 diluted peroxidase-labeled affinity-purified antibodies to rabbit/mouse IgG (H + L) (074–1506/074–1806, Kirkegard and Perry Laboratories, USA). Target bands were visualized by the addition of ECL™ Prime Western Blotting Detection Reagent (RPN2232, GE Healthcare). The results were normalized with GAPDH (sc-25778, Santa Cruz Biotechnology, USA).

1.6 Enzyme-linked Immunosorbent Assay (ELISA)

Commercial ELISA kits were used to detect the content of Ang (1-7) (E14241r, CUSABIO Biotechnology Co. Ltd, China), Ang Ⅱ (E04494r, CUSABIO Biotechnology Co. Ltd, China), ACE (E04490r, CUSABIO Biotechnology Co. Ltd, China) and ACE2 (E14308r, CUSABIO Biotechnology Co. Ltd, China) in rat blood serum according to the manufacturer's instructions.

1.7 Statistical Analysis

Statistical analysis was performed using SPSS 13.0 software (Inc., USA). One-way ANOVA for multiple comparisons was performed, followed by post-hoc analysis with the Bonferroni test. Data are presented as mean±SEM. The correlation of Ang Ⅱ and Ang (1-7) in serum of dynamic SiO_2-inhalation rats was analyzed by linear correlation. Differences with *P*-values <0.05 were considered statistically significant.

2 RESULTS

2.1 Pathological Morphology Changes of Lung Tissue in Silicosis Rats

H.E. and VG staining showed that the rat silicosis model was successfully established. As shown in fig. 1A and 1B, the structure of alveoli was clear, alveolar wall was thin and there were no inflammatory cells in control group. The alveolar wall became broadened, neutrophile granulocytes infiltrated in the lung tissue and inflammatory cells were oozed after 2-week exposure to SiO_2. Macrophages were visible in the lumen of the alveoli after 4-week exposure to SiO_2. Multiple cellular nodules, composed of macrophages, and collagen deposition were present in the lung tissue of rats after 8-week exposure to SiO_2. The silicotic nodules became larger with the duration of SiO_2 exposure; multiple fused nodules and collagen deposition were present in the lung tissue after 16-week exposure to SiO_2. Cellular fibrous nodules formed and collagen deposition accounted for about 50% of silicotic nodules area after 24-week exposure to SiO_2. The number and size/area of silicotic nodules were increased significantly at 16th and 8th week after exposure to SiO_2, respectively, compared to the control group (P<0.05, fig. 1C and 1D). Treatment with Cap relieved the lung fibrosis and collagen deposition. The number and size of silicotic nodules were also decreased obviously in Cap-treated group compared to the silicosis groups (fig. 2C and 2D).

2.2 Expression of Col Ⅰ and α-SMA in Rat Silicotic Lung Tissue and SiO_2-induced Fibroblasts

IHC staining showed that α-SMA was expressed on bronchial and vascular smooth muscle cells in the control group. In the rats exposed to SiO_2, the expression of α-SMA located on silicotic nodules and fibrosis lesions. The expression of α-SMA was

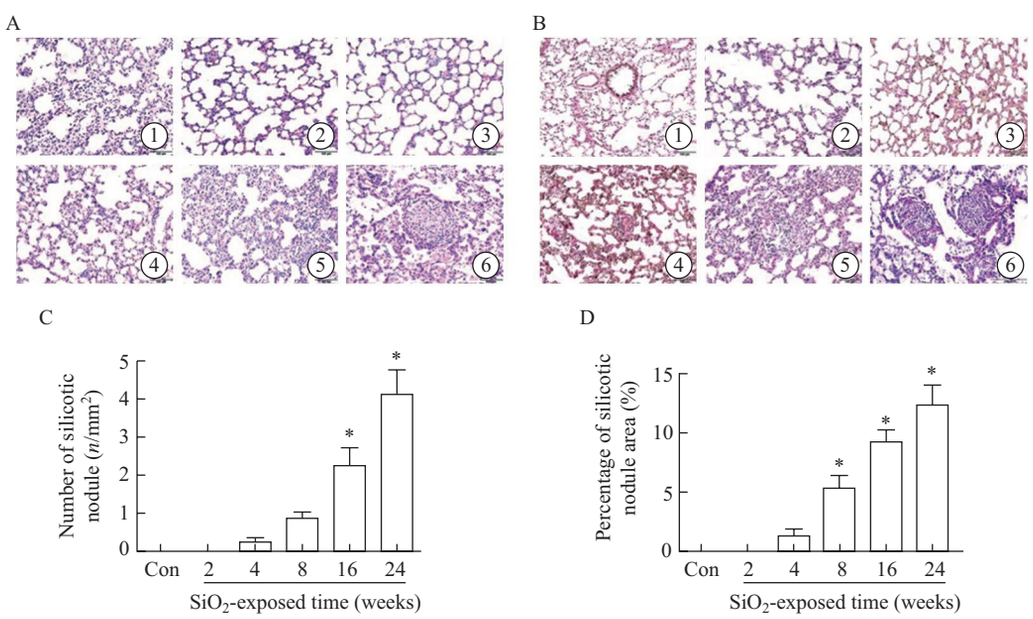

Fig. 1 Pathological morphology changes of lung tissue in silicotic rats for 0, 2, 4, 8, 16 and 24 weeks

A: H.E. staining; B: VG staining. Scale bars=100 μm. 1: control group; 2–6: SiO_2-exposed 2, 4, 8, 16, 24 weeks groups respectively; C: number of silicotic nodule; Con: control group. D: percentage of silicotic nodule area. Data are expressed as mean±SEM. $^*P$<0.05 *vs.* the control group (n=10)

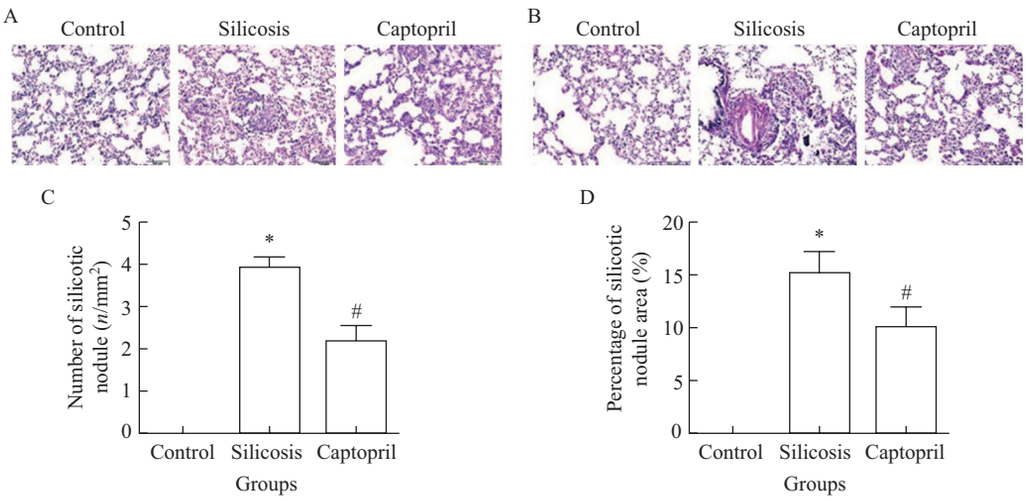

Fig. 2 Effect of Captopril on pathological morphology changes of lung tissue in silicotic rats
A: H.E. staining; B: VG staining. Scale bars=100 μm. C: number of silicotic nodule; D: percentage of silicotic nodule area. Data are expressed as mean±SEM. $^{*}P<0.05$ *vs.* the control group, $^{\#}P<0.05$ *vs.* the silicosis group (n=10)

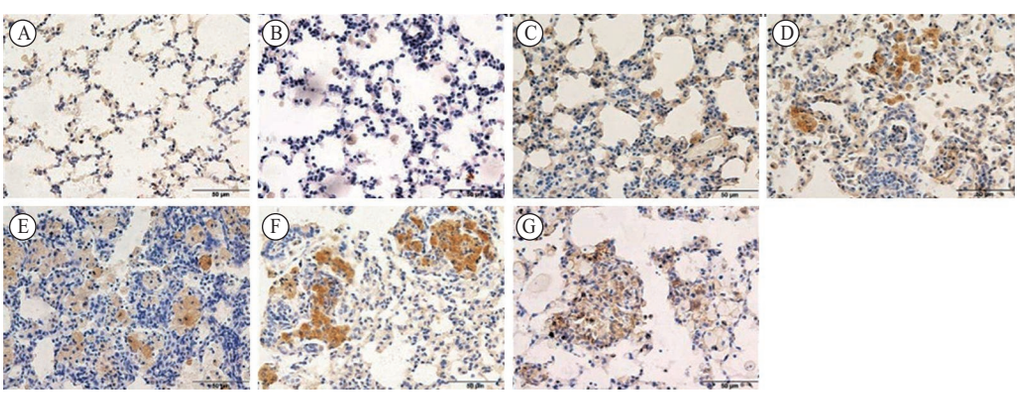

Fig. 3 Expression of α-SMA in silicotic rat lung tissue observed by immunohistochemistry
A: control group; B–F: SiO_2-exposed 2, 4, 8, 16 and 24 week groups respectively; G: captopril-treated group. Scale bars=50 μm

decreased in Cap-treated group (fig. 3). Western blot indicated that the expression of Col Ⅰ and α-SMA was increased gradually in lung tissue and fibroblasts with the duration of SiO_2 exposure. The expression of Col Ⅰ and α-SMA was increased significantly at 8th week and 6th h, respectively, in lung tissue and fibroblasts compared to the control group. Cap decreased the expression of Col Ⅰ and α-SMA in silicotic rat lung tissue and SiO_2-induced fibroblasts (P<0.05, fig. 4).

2.3 Protein Expression of ACE, AT1, ACE2 and Mas in Rat Silicotic Lung Tissue and SiO_2-induced Fibroblasts

The protein expression of ACE, AT1, ACE2 and Mas was examined by Western blot. The results showed that with the time extension of SiO_2 exposure, ACE and AT1 were up-regulated in rat silicotic lung tissue and SiO_2-induced fibroblasts gradually accompanied with down-regulation of ACE2 and Mas compared to the control group (P<0.05, fig. 5). The expression of ACE and AT1 was decreased, and that of ACE2 and Mas was increased in Cap-treated group (P<0.05, fig. 6).

2.4 Level of Serum ACE, Ang Ⅱ, ACE2 and Ang (1-7) in Rats Examined by ELISA

The serum contents of circular RAS in rats were examined by ELISA and the results indicated that the levels of ACE and Ang Ⅱ were increased significantly at 8th week, and those of ACE2 and Ang (1-7) were decreased significantly at 8th week compared to control group (P<0.05, fig. 7). Cap decreased the contents of ACE and Ang Ⅱ and increased the contents of ACE2 and Ang (1-7) compared to the silicosis groups (P<0.05, fig. 8).

2.5 Correlation Analysis of Serum Ang Ⅱ and Ang (1-7) in Rats

The results of correlation analysis revealed that Ang Ⅱ and Ang (1-7) showed negative correlation in serum of dynamic SiO_2-inhalation rats (r=–0.966, P=0.002).

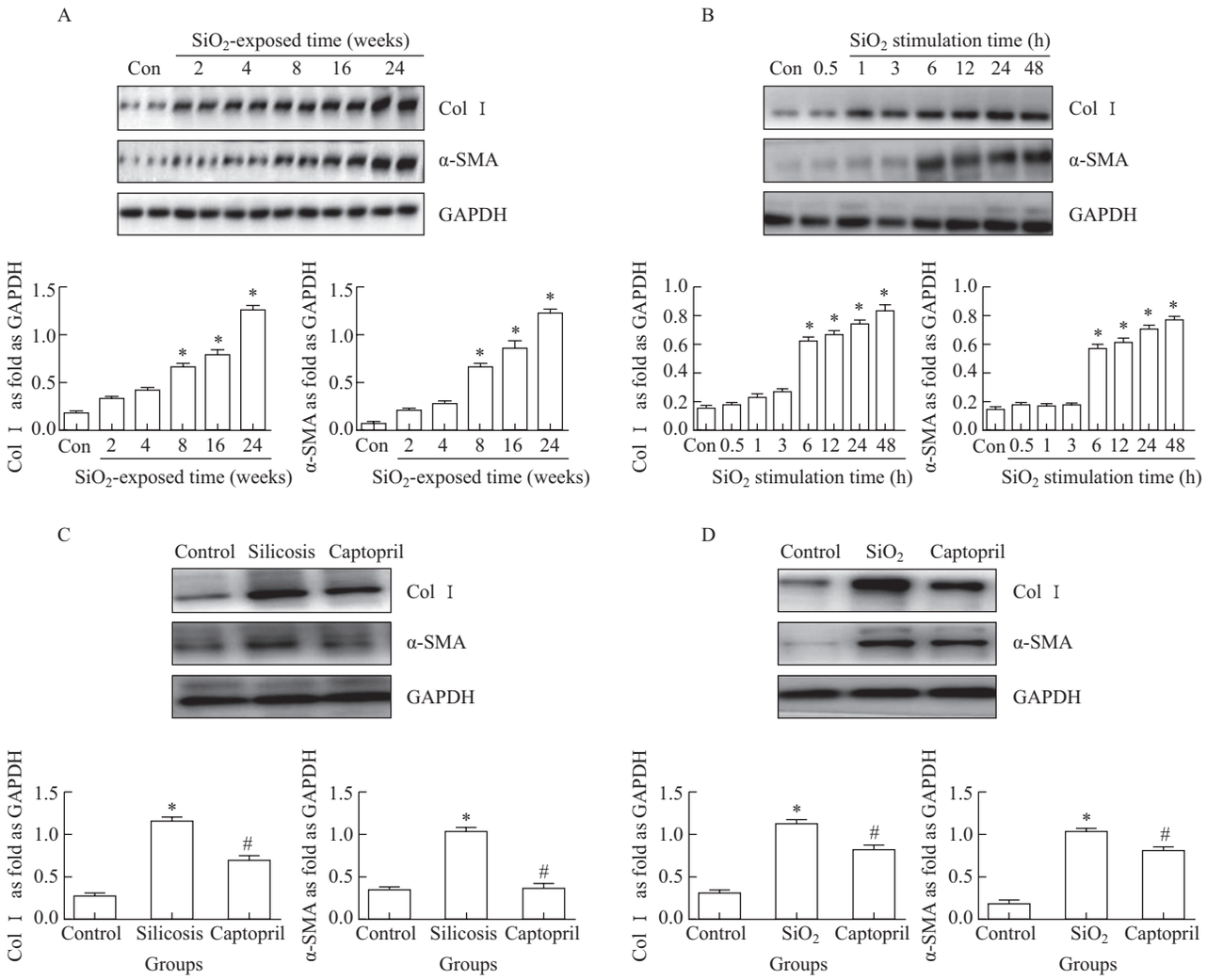

Fig. 4 Protein expression of Col Ⅰ and α-SMA measured by Western blot

A: the protein expression of Col Ⅰ and α-SMA in rat lung tissue exposed to SiO_2 for 0, 2, 4, 8, 16 and 24 weeks; B: the protein expression of Col Ⅰ and α-SMA in rat lung fibroblasts stimulated by SiO_2 for 0, 0.5, 1, 3, 6, 12, 24 and 48 h; C: effect of Captopril on the protein expression of Col Ⅰ and α-SMA in silicotic rat lung tissue; D: effect of Captopril on the protein expression of Col Ⅰ and α-SMA in lung fibroblasts stimulated by SiO_2. The results were normalized with GAPDH. Data are expressed as mean±SEM. Con: control group. $^{*}P<0.05$ *vs.* the control group, $^{\#}P<0.05$ *vs.* the silicosis/SiO_2 group (n=4)

3 DISCUSSION

Silicosis is the most common occupational lung disease and caused by long-term inhalation of free crystalline silica dust. Animal model is extensively used to study the possible mechanism of the occurrence and development of silicosis. Tracheal instillation of silica dust is one of the most common methods to establish silicosis model over the past decade[11, 12], but this method has several disadvantages including higher rat mortality, complex operation and inconformity with human silicosis pathogenetic process. In the present study, the rat silicosis model was established using silica dust inhalation from a HOPE-MED 8050 exposure control apparatus, which allowed convenient control and was similar to the development of human silicosis. H.E. and VG staining indicated that inflammatory reaction appeared at 2nd week, isolated silicotic nodules were visible in lung tissue at 4th week and more cellar silicotic nodules formed at 8th week. The silicotic nodules were fused at 16th week and fibrous-cellular silicotic nodules with diffuse interstitial fibrosis were observed at 24th week in rats. Treatment with Cap could alleviate the lung fibrosis and collagen deposition. Myofibroblasts, α-SMA-positive expressing cells[13, 14], play a pivotal role in wound healing; however, the unusual persistent proliferative and migratory properties of myofibroblasts lead to an excessive accumulation of extracellular matrix and lung fibrosis. IHC revealed that α-SMA-positive expressing myofibroblasts surrounded macrophages and were irregularly distributed in interstitial fibrotic

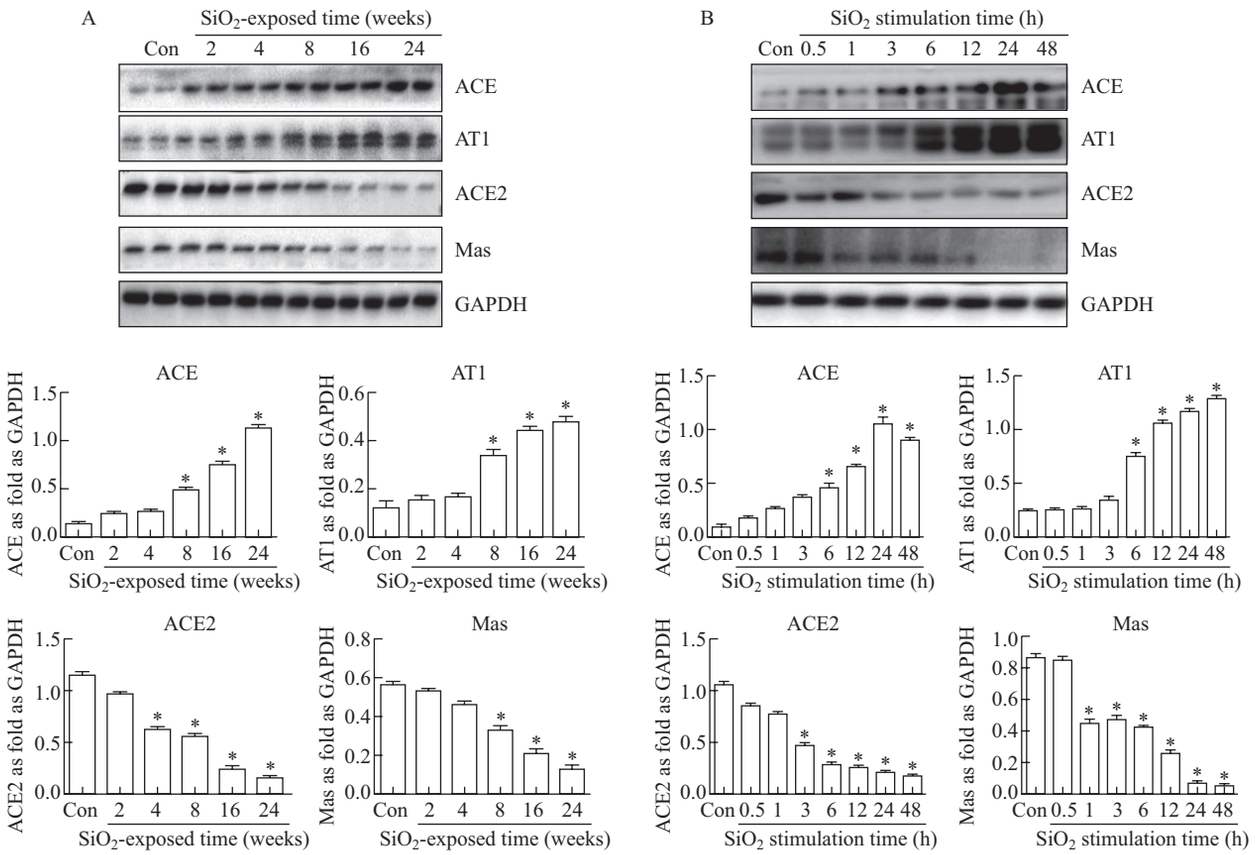

Fig. 5 Protein expression of ACE, AT1, ACE2 and Mas measured by Western blot

A: the protein expression of ACE, AT1, ACE2 and Mas in rat lung tissue exposed to SiO_2 for 0, 2, 4, 8, 16 and 24 weeks; B: The protein expression of ACE, AT1, ACE2 and Mas in rat lung fibroblasts stimulated by SiO_2 for 0, 0.5, 1, 3, 6, 12, 24 and 48 h. The results were normalized with GAPDH. Data are expressed as mean±SEM. $^*P<0.05$ *vs.* the control group (n=4)

areas. Further characterization with Western blot demonstrated that the expression of Col Ⅰ and α-SMA was increased significantly in silicotic lung tissue at 8th week and SiO_2-induced fibroblasts at 6th h compared to the control group. Treatment with Cap could down-regulate the expression of Col Ⅰ and α-SMA in rat lung tissue and fibroblasts. These findings suggested that a silicosis model was successfully established using a HOPE-MED 8050 dynamic dust system, and Cap inhibited the silicotic fibrosis.

Activation of ACE-Ang Ⅱ-AT1 axis is associated with tissue fibrosis[15, 16]. Ang Ⅱ stimulates extracellular matrix accumulation and collagen deposition through the induction of mitogen activated protein kinases (MAPKs) *in vivo* and *in vitro*[17]. It has been suggested that Ang Ⅱ, acting via the AT1 receptor, modulates pro-fibrotic downstream effects including inflammatory cell recruitment, cellular proliferation and the accumulation of extracellular matrix[5]. Studies had reported that the expression of ACE in bronchoalveolar lavage fluid was increased[20] and the angiotensinogen gene expression was higher in the lung tissues of patients with pulmonary fibrosis[21]. Clinical research indicated the serum contents of ACE were increased obviously in silicotic patients compared to the control patients, and the serum contents of ACE in progressive silicotic patients were also higher than those in non-progressive silicotic patients[22, 23]. Animal experiments showed that the systolic blood pressure was elevated and serum Ang Ⅱ was also increased in rats after intratracheal instillation with SiO_2[24], which may indicate that the RAS was activated in silicotic rats to induce hypertension. Cell experiments also indicated that Ang Ⅱ induced lung fibroblasts to myofibroblasts through activating p-Smad2/3 signaling and increased the Col Ⅰ expression[25]. Ang Ⅱ could also promote TGF-β secretion and generate Ang Ⅱ-TGF-β crosstalk to induce silicosis. All the researches above manifested that ACE-Ang Ⅱ-AT1 axis may be activated in silicosis. Cap is a kind of ACEI which decreases the generation of Ang Ⅱ. It had been reported that ACEI could inhibit lung fibrosis induced by bleomycin and paraquat[26]. ACE2 acts as an endogenous counter-regulator of ACE[27]. In contrast to ACE, which cleaves Ang Ⅰ into Ang Ⅱ, ACE2 primarily hydrolyzes Ang Ⅱ into Ang (1-7). Ang (1-7) counteracts the effects of Ang Ⅱ via

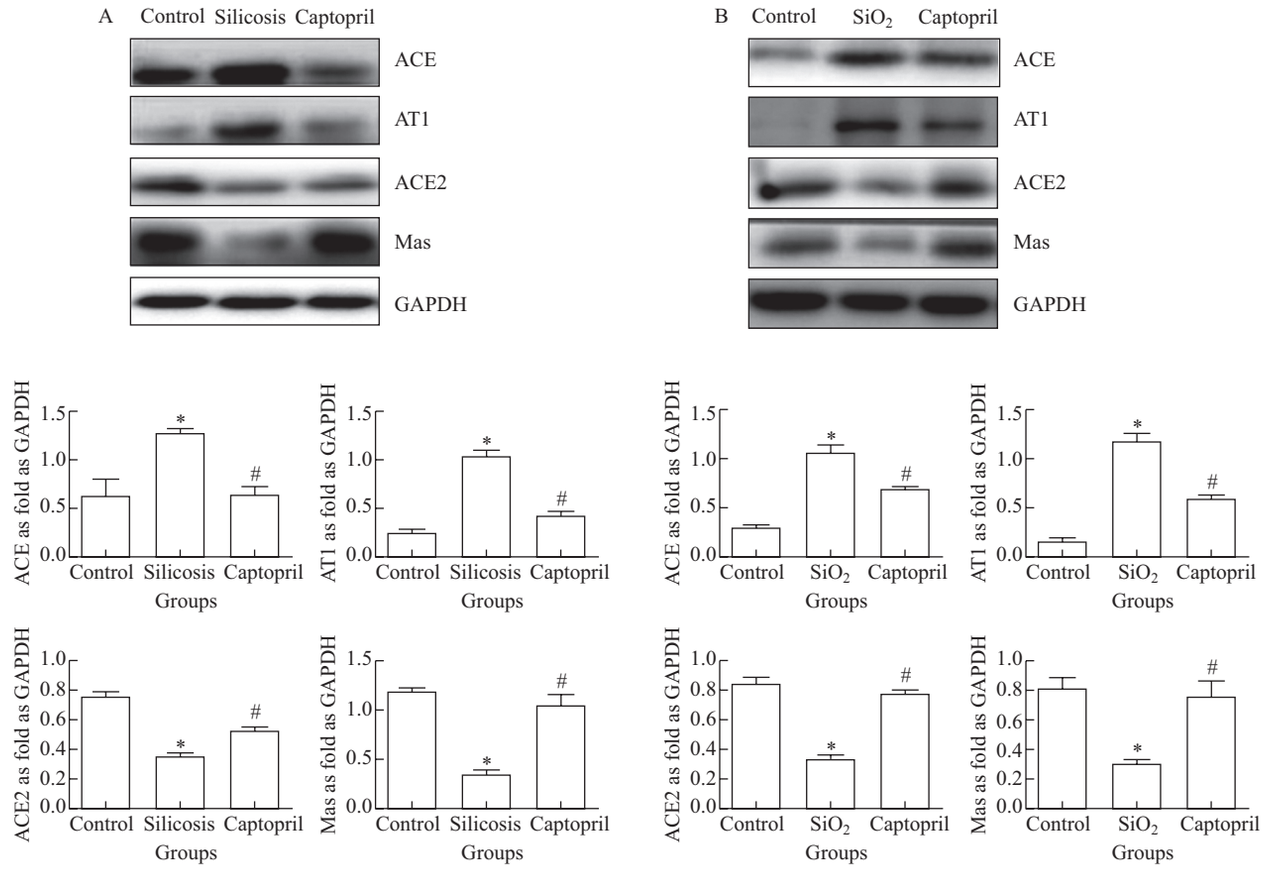

Fig. 6 Protein expression of ACE, AT1, ACE2 and Mas measured by Western blot
A: effect of Captopril on the protein expression of ACE, AT1, ACE2 and Mas in rat silicotic lung tissue; B: effect of Captopril on the protein expression of ACE, AT1, ACE2 and Mas in rat lung fibroblasts stimulated by SiO_2. The results were normalized with GAPDH. Data are expressed as mean±SEM. $^{*}P<0.05$ *vs.* the control group, $^{\#}P<0.05$ *vs.* the silicosis/SiO_2 group (n=4)

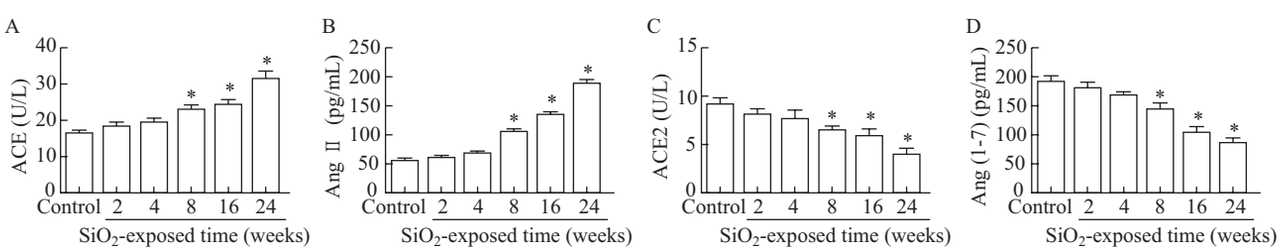

Fig. 7 Levels of serum ACE (A), Ang Ⅱ (B), ACE2 (C) and Ang (1-7) (D) in silicotic rat measured by ELISA
Data are expressed as mean±SEM. $^{*}P<0.05$ *vs.* the control group (n=10)

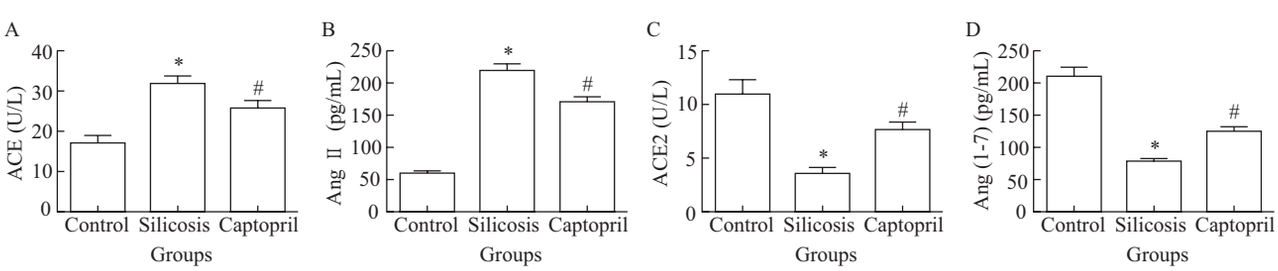

Fig. 8 Effect of Captopril on the levels of serum ACE (A), Ang Ⅱ (B), ACE2 (C) and Ang (1-7) (D) in silicotic rat measured by ELISA
Data are expressed as mean±SEM. $^{*}P<0.05$ *vs.* the control group, $^{\#}P<0.05$ *vs.* the silicosis group (n=10)

its G protein-coupled receptor Mas[28]. Ang (1-7) could inhibit the apoptosis of alveolar type Ⅱ epithelial cells induced by Ang Ⅱ through inhibiting the activation of JNK signaling and decreasing the expression of caspase-3 and caspase-9[29–31]. Experimental studies indicated that ACE2 and Ang (1-7) had beneficial effects on lung injury[32]. Meanwhile, studies had further concluded that ACE2 could protect against bleomycin-induced lung fibrosis[9]. Therefore, the balance of ACE and ACE2 influences the endogenous ratio of Ang Ⅱ to Ang (1-7) and consequently contributes to the regulation of the organ fibrosis[33]. In the present study, Western blot results demonstrated that the protein expression of ACE and AT1 was up-regulated gradually accompanied with down-regulation of ACE2 and Mas in silicotic rat lung tissue and SiO_2-stimulated fibroblasts compared to the control group. ELISA also indicated that the level of ACE and Ang Ⅱ was increased gradually accompanied with the decrease of ACE2 and Ang (1-7) level in serum of rats exposed to silica compared to the control group. Treatment with Cap decreased the expression of ACE, Ang Ⅱ and AT1, thus increased the expression of ACE2, Ang (1-7) and Mas. These findings revealed that the circular and local RAS may participate in the development of silicosis.

Conflict of Interest Statement

We declare that there are no relevant financial or non-financial conflicts of interest in the study.

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