

# Targeting thrombin with hirudin alleviates paraquat-induced pulmonary fibrosis via the PAR-1-mediated TGF- $\beta$ 1 pathway

Xiao Hu<sup>1</sup>, Weijuan Liu<sup>2</sup>, Guowen Zheng<sup>3</sup>, Zhaojun Song<sup>4</sup> and Zike Zhang<sup>1</sup>

<sup>1</sup>Department of Laboratory Medicine, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, <sup>2</sup>Department of Laboratory Medicine, Linfen People's Hospital, Linfen, <sup>3</sup>Department of Clinical Laboratory, The Fourth Affiliated Hospital, Zhejiang University School of Medicine and <sup>4</sup>Laboratory Animal Center, The Fourth Affiliated Hospital, Zhejiang University School of Medicine, Yiwu, China

**Summary.** Background. Paraquat (PQ)-induced pulmonary fibrosis (PF) is a serious disease without specific antidotes. Thrombin is important for promoting fibrosis development. We aimed to explore whether thrombin promotes PQ-induced PF by activating the protease-activated receptor-1 (PAR-1)-mediated transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) pathway.

**Methods.** Male Sprague Dawley rats received PQ treatment, either alone or with thrombin or hirudin (a thrombin inhibitor) (n=9 in the control, model, thrombin, and hirudin groups). After 7 or 14 days of treatment, oxidative stress (OS) indicators and histopathological damage in the lungs were detected. PF degree was evaluated using Masson staining and hydroxyproline levels in lung tissues and collagen levels in bronchoalveolar lavage fluid. Furthermore, type I collagen (Col-1), TGF- $\beta$ 1, and PAR-1 expression, as well as extracellular signal-regulated kinase 1/2 (ERK1/2) and mothers against decapentaplegic homolog 3 (Smad3) phosphorylation in the lungs, were assessed. To investigate the mechanism of thrombin on PQ-induced PF, rats treated with PQ and thrombin further received SCH79797 (a PAR-1 inhibitor) alone or combined with SRI-011381 (a TGF- $\beta$  agonist) (n=9 in the thrombin+SCH79797 and thrombin+SCH79797+SRI-011381 groups). In addition to Masson staining and detection of the above-mentioned genes and proteins, alpha-smooth muscle actin ( $\alpha$ -SMA), TGF $\beta$  receptor type I (T $\beta$ RI), and type II (T $\beta$ RII) expression in the lungs were also detected.

**Results.** Both 7 and 14 days of thrombin treatment triggered OS, exacerbated lung histopathological

damage, and promoted PF in PQ-stimulated rats. Furthermore, thrombin upregulated Col-1, TGF- $\beta$ 1, and PAR-1 expression as well as ERK1/2 and Smad3 phosphorylation in PQ-stimulated rats. However, hirudin produced the opposite results. Additionally, the role of thrombin in promoting PF, increasing Col-1, TGF- $\beta$ 1,  $\alpha$ -SMA, PAR-1, T $\beta$ RI, and T $\beta$ RII expression and ERK1/2 and Smad3 phosphorylation in PQ-stimulated rats was reversed by SCH79797, while the inhibitory effects of SCH79797 were counteracted by SRI-011381.

**Conclusions.** Thrombin may promote PQ-induced PF by activating PAR-1-mediated TGF- $\beta$ 1, suggesting that PAR-1-mediated TGF- $\beta$ 1 is a potential target for preventing PQ-induced PF.

**Key words:** Thrombin, Paraquat, Pulmonary fibrosis, Protease-activated receptor-1, Transforming growth factor- $\beta$ 1

## Introduction

Paraquat (PQ) is a highly effective herbicide that is frequently used in agriculture (Inthama et al., 2021). However, it should be noted that PQ is highly toxic to the human body, and that PQ poisoning remains a significant public health concern due to its high lethality rates and lack of specific antidotes (Buckley et al., 2021). Pulmonary fibrosis (PF) is the main cause of death associated with PQ poisoning. PQ has similar chemical properties to polyamines, which are enriched in Clara cells and types I/II alveolar cells (Liu et al., 2022). These compounds are actively transported via the polyamine system and are prone to building up in lung tissues, where PQ can impair mitochondrial function, lead to excessive free radical production, trigger inflammation, promote apoptosis, and ultimately result in irreversible PF (Subbiah and Tiwari, 2021). Recently,

Corresponding Author: Zike Zhang, Department of Laboratory Medicine, The First Affiliated Hospital, Zhejiang University School of Medicine, No. 79, Qingchun Road, Shangcheng District, Hangzhou City, Zhejiang Province, 310003, China. e-mail: 1515112@zju.edu.cn  
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the mortality rate of PQ-induced PF has increased, but current medicines have no apparent therapeutic effects. Therefore, there is an urgent need to explore new molecular targets to prevent PQ-induced PF.

Thrombin is a serine proteinase that plays an important role in hemostasis and can cleave fibrinogen to fibrin. It has been well documented that thrombin can promote fibrosis (Herum et al., 2020), and an elevated plasma prothrombin time is associated with a poor prognosis in patients with PQ poisoning (Hu et al., 2018). Moreover, inhibiting thrombin pharmacologically has been demonstrated to protect against the development of fibrosis in animals (Saifi et al., 2021; Lin et al., 2022). Protease-activated receptor-1 (PAR-1), a thrombin receptor, has been identified as a significant contributor to fibrosis in multiple organs, including the lungs (Xia et al., 2024). There is increasing evidence that has suggested that PAR-1 contributes to fibroblast migration, proliferation, and extracellular matrix (ECM) generation, while PAR-1 deficiency mitigates fibrosis development in the kidney (Lok et al., 2020), liver (Poole et al., 2020), and skin (Duitman et al., 2014). TGF- $\beta$  is recognized as a profibrotic cytokine, and studies have reported a strong connection between PAR-1 and transforming growth factor- $\beta$  (TGF- $\beta$ ) (Song et al., 2024). Mechanistically, activated PAR-1 triggers rearrangement of the intracellular cytoskeleton (Jenkins et al., 2006; Shea et al., 2017). In epithelial cells, the cytoskeletal rearrangements lead to a conformational change in the cell surface  $\alpha\text{v}\beta_6$  integrin, which binds to the latent TGF- $\beta$  presented in the ECM. The conformational change of  $\alpha\text{v}\beta_6$  facilitates the release of TGF- $\beta$  from its latent complex and contributes to fibrosis (Munger et al., 1999; Jenkins et al., 2006). Furthermore, numerous studies have proved that thrombin can induce TGF- $\beta$  (Li et al., 2013; Chang et al., 2017). However, it is unclear whether thrombin promotes PQ-induced PF by activating PAR-1-mediated TGF- $\beta$ .

Thus, in this study, the rats were given PQ by gavage to establish PQ-induced PF animal models. Then, the rats were treated with thrombin, hirudin (a thrombin inhibitor), SCH79797 (a PAR-1 inhibitor), and SRI-011381 (a TGF- $\beta$  agonist) to further explore the mechanism of thrombin in PQ-induced PF. The findings of the study offered valuable targets for the prevention of PQ-induced PF.

## Materials and methods

### Experimental animals

The specific pathogen-free male Sprague Dawley rats (six weeks old and 200 g $\pm$ 20 g) used in this study were sourced from Shanghai SLAC Laboratory Animal Co., Ltd. (SCXK (hu) 2022-0004). All rats were maintained under standard conditions (18°C–24°C temperature, 50–60% humidity, and 12h/12h light/dark cycles). The animal experiments conducted in this study

were approved by the Laboratory Animal Management and Ethics Committee of Hangzhou Hunter Testing Biotechnology Co., Ltd., and the animals were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee.

### Animal grouping and treatments

After acclimatization for one week, the rats were randomized into six groups: the control, model, thrombin, hirudin, thrombin+SCH79797, and thrombin+SCH79797+SRI-011381 (n=9 per group). To establish PQ-induced PF models, all groups, except the control group, were administered 50 mg/kg/day of PQ (dissolved in normal saline to a concentration of 5 mg/mL; 36541, Sigma) via gavage for 14 days, while the control group received the same volume of normal saline via gavage (Liu et al., 2020). Meanwhile, the rats in the thrombin, thrombin+SCH79797, and thrombin+SCH79797+SRI-011381 groups were exposed to aerosolized thrombin for 14 days before each PQ administration. Specifically, thrombin (S10117) was dissolved in normal saline to a concentration of 2000 U/L, and an ultrasonic nebulizer was applied to generate the aerosol. During the exposure, the rats were placed in an inhalation chamber for 30 min, with filtered air drawn through the chamber at a flow rate of 17 L/min (Zhu et al., 2013; Bi et al., 2015). The control and model rats were exposed to aerosolized normal saline for the same period, while the rats in the hirudin group were exposed to aerosolized normal saline and also received a subcutaneous injection of 1 mg/kg of hirudin (a thrombin inhibitor, S31625) for 14 days before each PQ administration (Wei et al., 2024). In addition, after each PQ treatment, the rats in the thrombin+SCH79797 and thrombin+SCH79797+SRI-011381 groups received daily intratracheal instillations of 70  $\mu$ M SCH79797 (a PAR-1 inhibitor; T97487) for 14 days (Gupta et al., 2018), and rats in the thrombin+SCH79797+SRI-011381 group were further intraperitoneally injected with 30 mg/kg/day of SRI-011381 (a TGF- $\beta$  agonist; S88452) (Ren et al., 2023). In this study, thrombin, hirudin, SCH79797, and SRI-011381 were supplied by Shanghai Yuanye Biotechnology Co., Ltd. During the study, three rats were randomly selected from the control, model, thrombin, and hirudin groups and euthanized with an overdose of CO<sub>2</sub> on day 7; and the remaining rats were euthanized upon finalization of the experiment.

### Detection of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX)

Following euthanasia, lung tissue samples were collected from the rats. Following the manufacturer's guidelines, the lung tissues were homogenized, and the supernatants were collected by centrifugation. The corresponding assay kits from Nanjing Jiancheng Bioengineering Institute were then utilized to assess MDA (A003-1), SOD (A001-1), and GSH-PX (A005-1)

levels in the supernatants.

#### *Hematoxylin and eosin (H&E) and Masson staining*

The lung tissues were fixed using 4% para-formaldehyde, dehydrated through a series of ethanol concentrations, embedded in paraffin, and cut into 4- $\mu$ m sections. H&E and Masson staining were then separately conducted.

For H&E staining, the samples were dewaxed, stained with hematoxylin (H3136, Sigma) and eosin (E4009, Sigma), dehydrated in a series of ethanol concentrations, penetrated with xylene, and finally mounted with neutral resin. The morphological alterations in the lung tissues were viewed and captured using a microscope. According to the severity of the lesion, the scores were allocated as follows: no lesion or very mild lesion was 0 points; mild lesion was 1 point; moderate lesion was 2 points; severe lesion was 3 points; and extremely severe lesion was 5 points.

For Masson staining, the samples were first hydrated, then stained with hematoxylin, and immersed in a Lichun red magenta solution (71033761, Sinopharm Chemical Reagent Co., Ltd.). After treatment with acetic acid (10000208, Sinopharm Chemical Reagent Co., Ltd.) and phosphomolybdic acid (XW514297441, Beijing Wokai Biotechnology Co., Ltd.), the tissues were stained with aniline blue staining solution (71003644, Sinopharm Chemical Reagent Co., Ltd.), dehydrated with alcohol, penetrated with xylene, and mounted using neutral resin. The extent of pulmonary fibrosis was assessed and captured using a microscope.

#### *Measurement of collagen levels*

Following euthanasia, bronchoalveolar lavage fluid (BALF) was collected from the rats by cannulating the trachea. Specifically, 3 mL of sterile saline was slowly infused into the lungs of the rats, and 2 mL of BALF was collected. Then, the BALF was centrifuged to obtain the supernatant. After that, soluble collagen assay kits (SIRC2, Biocolor) were applied to detect collagen levels in the BALF, following the operating instructions.

#### *Determination of hydroxyproline (Hyp)*

In short, the collected lung tissues were cut into small pieces using scissors and then homogenized in phosphate-buffered saline. Subsequently, the homogenates were centrifuged to collect the

supernatants. After that, the Hyp content in the tissues was measured using enzyme-linked immunosorbent assay kits (CB10702-Ra, COIBO BIO Inc.).

#### *Quantitative polymerase chain reaction (qPCR)*

qPCR was employed to assess mRNA expression levels in lung tissues. Total RNA was extracted using an EZ-10 Total RNA Mini-Preps kit (B618583-0250, BBI) and subsequently converted to complementary DNA with a TRUEScript RT MasterMix kit (PC7002, Aidlab Biotechnologies Co., Ltd.). The qPCR analysis was performed using a 2 x Dual SYBR Green qPCR Mix kit (PC6202, Aidlab Biotechnologies Co., Ltd.), and RNA expression levels were determined by the  $2^{-\Delta\Delta C_q}$  method. The primer sequences of the genes used in the qPCR analysis are presented in Table 1.

#### *Western blot assay*

Total proteins were extracted from the collected lung tissues using radioimmunoprecipitation assay lysis buffer, and the total protein concentrations were then determined using bicinchoninic acid protein quantification kits (P0012, Beyotime Biotech). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was employed to separate proteins of different molecular weights. Then, the proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked for 2h with 5% skim milk and then incubated overnight with the corresponding primary antibodies. The following day, after washing, the membranes were treated with secondary antibodies (1:6000, 7074, CST) for 2h and washed three times. Then, enhanced chemiluminescence kits were used to visualize the proteins, and ImageJ software was applied to quantify the western blots. The details of the primary antibodies are presented in Table 2.

#### *Immunohistochemical analysis*

Initially, the paraffin-embedded tissues were deparaffinized and incubated in hydrogen peroxide to eliminate endogenous peroxidase activity. Antigen retrieval was then performed by placing the sections in an antigen retrieval solution. After blocking with 5% bovine serum albumin, the sections were incubated with primary antibodies against PAR-1 (1:200, AF0263, Affinity), TGF- $\beta$ 1 (1:200, AF1027, Affinity), type I collagen (Col-1) (1:200, bs-10423R, Bioss), and alpha-

**Table 1.** Primer sequences of the genes for qPCR analysis.

| Gene                                | Forward Primer        | Reverse Primer       |
|-------------------------------------|-----------------------|----------------------|
| Rat <i>Col-1</i>                    | CCAGCCGCAAAGAGTCTACA  | ATCAGCACCAGGGAAACCAC |
| Rat <i>TGF-<math>\beta</math>1</i>  | TACCTTTCCTTGGGAGACCCC | CAGCCACTCAGGCGTATCAG |
| Rat <i><math>\beta</math>-actin</i> | GGGAAATCGTGCGTGAC     | AGGCTGGAAAAGAGCCT    |



smooth muscle actin ( $\alpha$ -SMA) (1:200, BF9212, Affinity) at 4°C overnight. Afterward, the sections were treated with horseradish peroxidase-conjugated secondary antibodies (1:5000, ab97080, Abcam) and incubated with 3,3-diaminobenzidine. This was followed by counterstaining with hematoxylin and dehydrating in a series of ethanol concentrations. Finally, the protein levels of PAR-1, TGF- $\beta$ 1, Col-1, and  $\alpha$ -SMA in lung tissues were examined using a microscope.

Statistical analysis

The results were analyzed using SPSS 20.0, and the values are displayed as mean $\pm$ standard deviation. For comparisons involving multiple groups, one-way analysis of variance followed by Tukey tests was performed. Furthermore, the Kruskal-Wallis H test was utilized when the data did not meet the criteria for

normal distribution, and the Dunnett T3 test was employed for data with unequal variances.  $p<0.05$  was considered statistically significant.

Table 2. Information on the primary antibody for Western blot analysis.

| Antibody                    | Source      | Cat No.    | Dilutions |
|-----------------------------|-------------|------------|-----------|
| PAR-1                       | Affinity    | AF0263     | 1:1000    |
| TGF- $\beta$ 1              | Affinity    | BF8012     | 1:1000    |
| T $\beta$ RI                | Affinity    | AF5347     | 1:1000    |
| T $\beta$ RII               | Affinity    | AF5449     | 1:1000    |
| ERK1/2                      | CST         | 4695s      | 1:1000    |
| p-ERK1/2                    | CST         | 4370T      | 1:1000    |
| Smad3                       | Affinity    | AF6362     | 1:1000    |
| p-Smad3                     | Affinity    | AF3362     | 1:1000    |
| Anti-rabbit IgG, HRP-linked | CST         | 7074       | 1:6000    |
| $\beta$ -actin              | Proteintech | 81115-1-RR | 1:10000   |

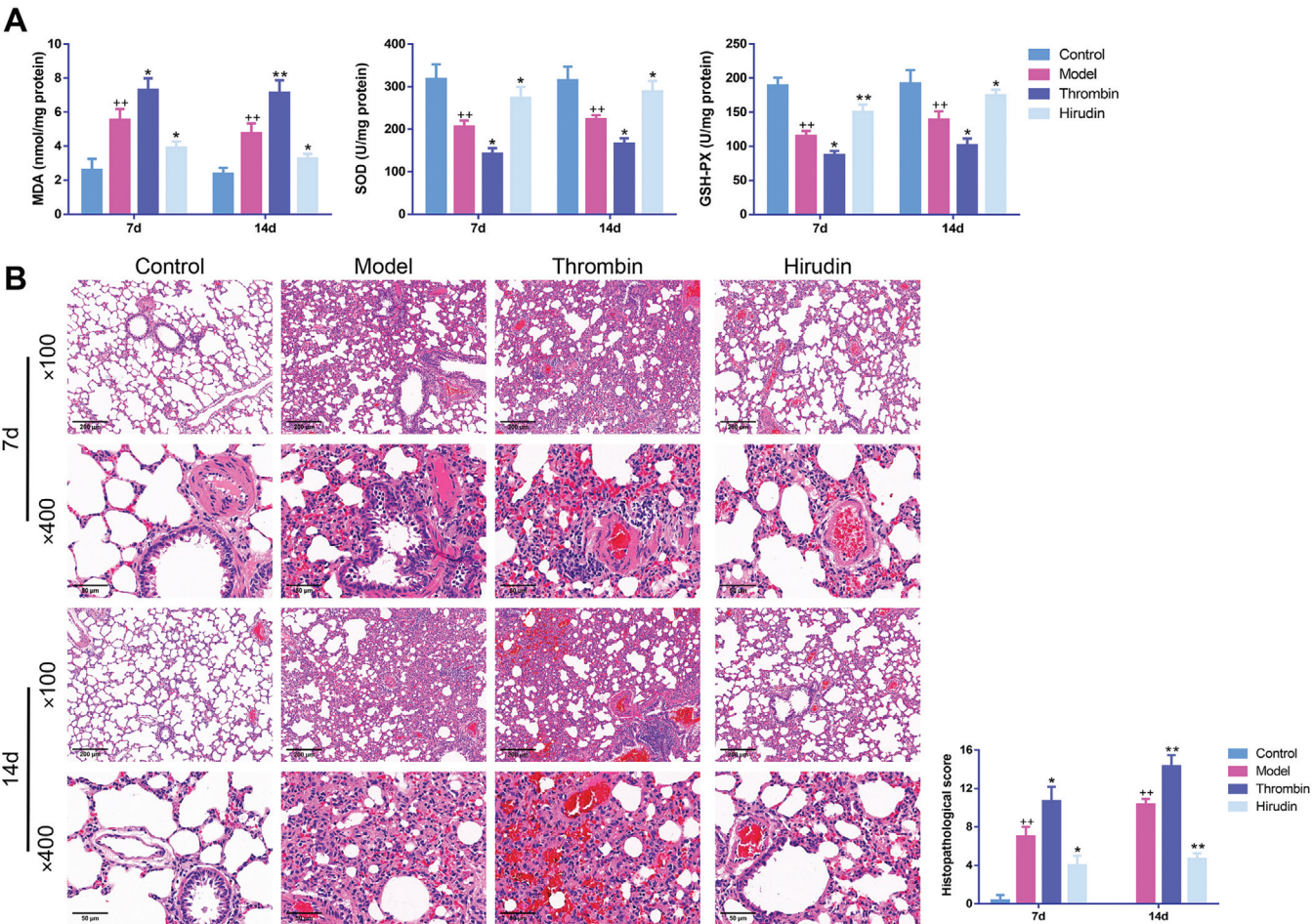


Fig. 1. Thrombin induced oxidative stress and exacerbated lung histopathological damage in paraquat (PQ)-stimulated rats. **A.** After treatment with thrombin or hirudin (a thrombin inhibitor), the changes in malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) levels in the lung tissues of PQ-stimulated rats were examined. **B.** The histopathological damage in the lung tissues of the rats in each group was determined by hematoxylin and eosin staining and histopathological score. Magnification:  $\times 100$ , scale bar: 200  $\mu$ m; Magnification:  $\times 400$ , scale bar: 50  $\mu$ m.  $n=3$ .  $^{+}p<0.05$  and  $^{++}p<0.01$  vs. Control;  $^{*}p<0.05$  and  $^{**}p<0.01$  vs. Model. Results are presented as mean $\pm$ standard deviation (SD).

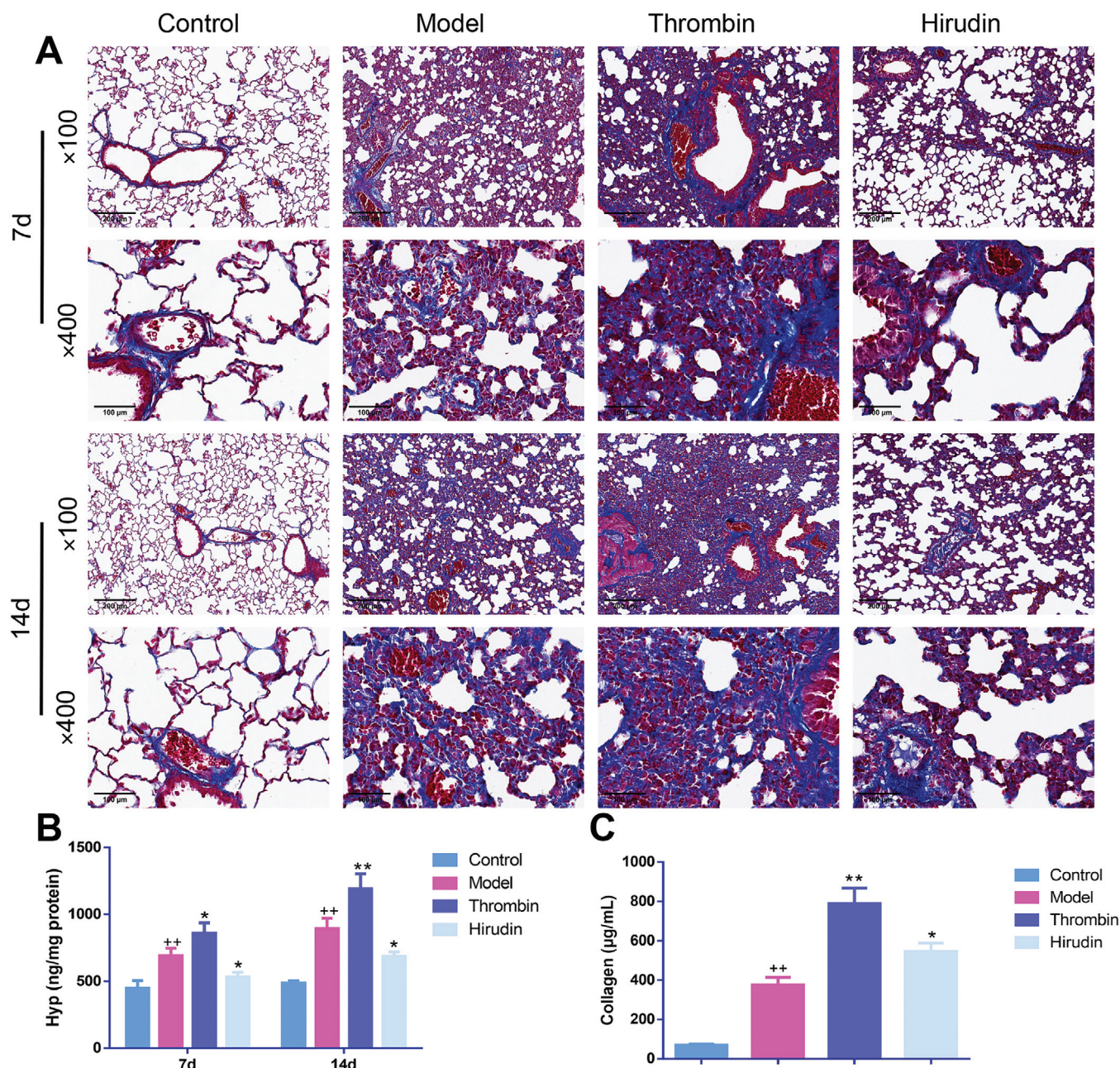


## Results

*Thrombin triggered oxidative stress (OS) and increased histopathological damage to the lungs of PQ-stimulated rats*

On both the 7th and 14th day, relative to the control

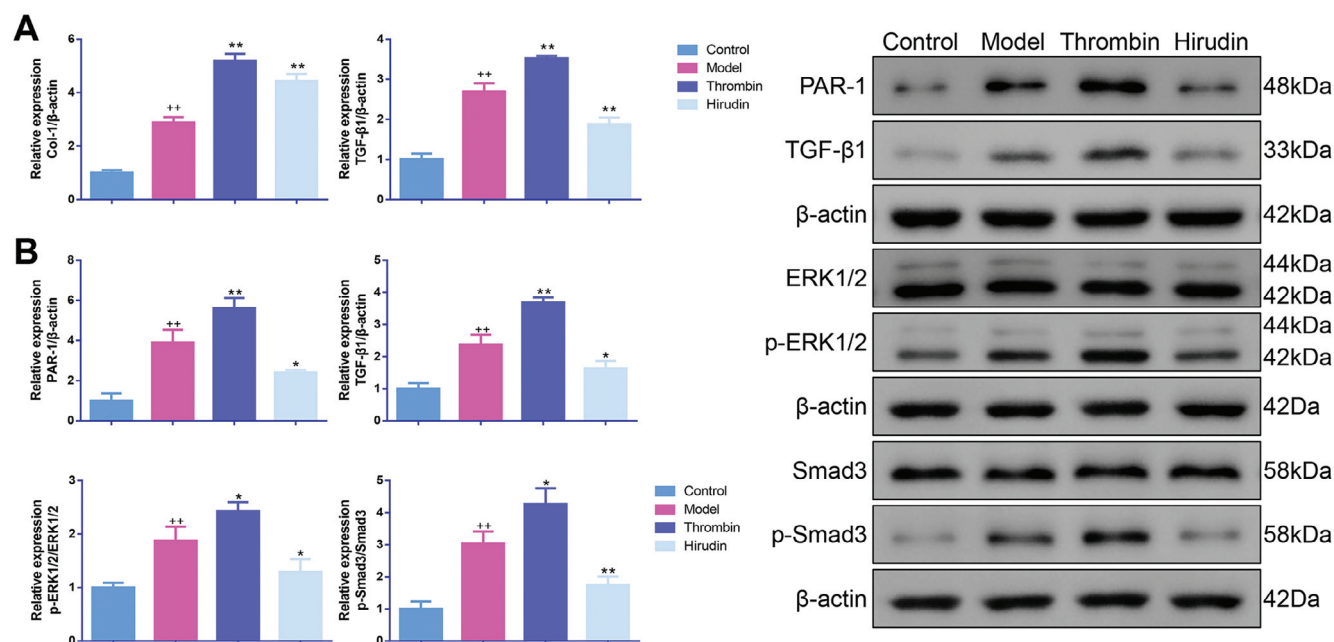
group, model rats exhibited dramatic increases in MDA, SOD and GSH-PX levels in lung tissues. Following thrombin treatment, MDA, SOD, and GSH-PX levels in the model rats were further elevated, but treatment with hirudin (a thrombin inhibitor) produced the opposite results (Fig. 1A). Additionally, Fig. 1B revealed that, on the 7th day, the model rats exhibited notable alveolar



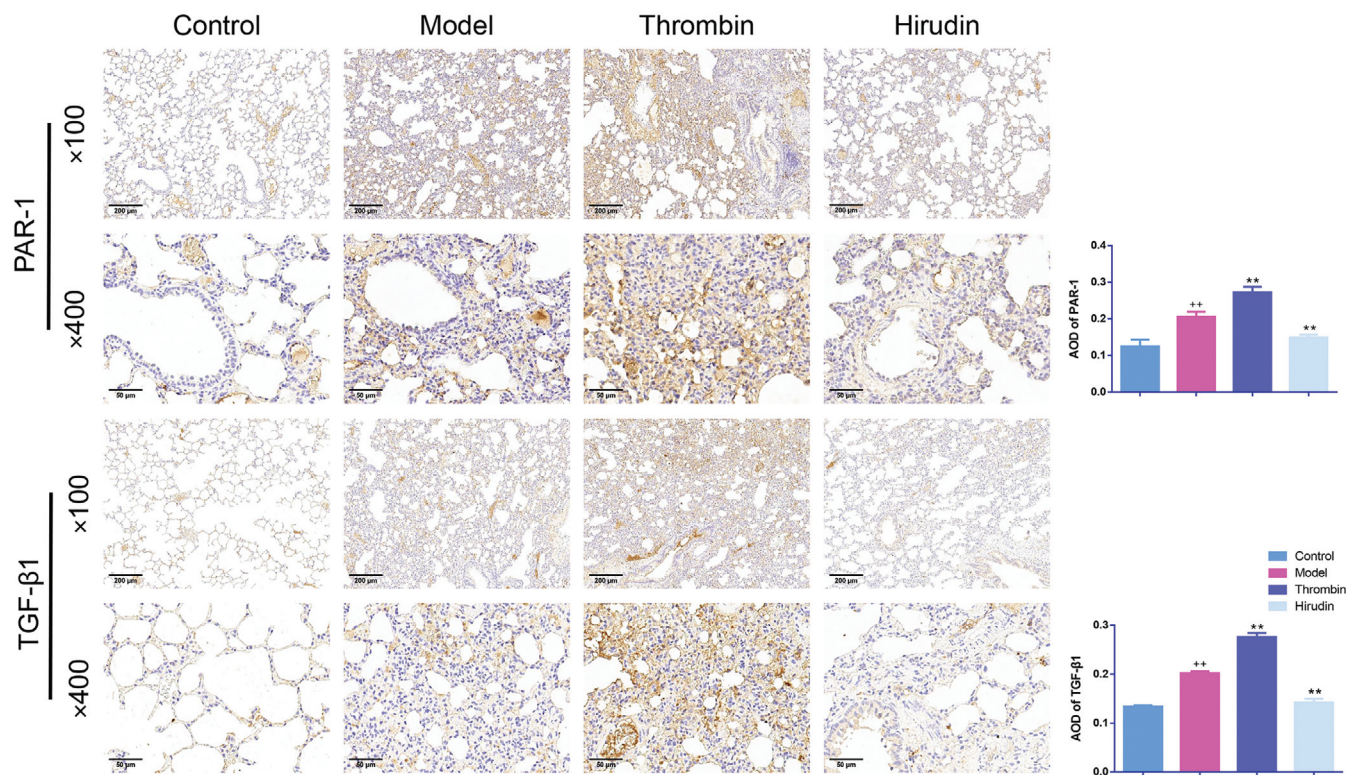
**Fig. 2.** Thrombin facilitated pulmonary fibrosis (PF) in PQ-stimulated rats. **A.** The degree of PF in rats from each group was evaluated by Masson staining. Magnification:  $\times 100$ , scale bar: 200  $\mu\text{m}$ ; Magnification: 400, scale bar: 50  $\mu\text{m}$ . **B.** The levels of hydroxyproline in the lung tissues were tested to determine PF levels in the rats. **C.** Collagen levels in the bronchoalveolar lavage fluid (BALF) of the rats were detected to evaluate PF levels.  $n=3$ . <sup>\*</sup> $p<0.05$  and <sup>++</sup> $p<0.01$  vs. Control; <sup>\*</sup> $p<0.05$  and <sup>\*\*</sup> $p<0.01$  vs. Model. Results are presented as mean $\pm$ SD.



# Thrombin contributes to paraquat-induced PF



**Fig. 3.** Thrombin activated the protease-activated receptor-1 (PAR-1) and transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) pathway in PQ-stimulated rats. **A.** The expression of type I collagen (Col-1) and TGF- $\beta$ 1 mRNA in the lung tissues of the rats was evaluated. **B.** PAR-1 and TGF- $\beta$ 1 protein expression, as well as extracellular signal-regulated kinase 1/2 (ERK1/2) and mothers against decapentaplegic homolog 3 (Smad3) phosphorylation in the lung tissues of the rats, were detected.  $n=3$ .  $^{+}p<0.05$  and  $^{++}p<0.01$  vs. Control;  $^{*}p<0.05$  and  $^{**}p<0.01$  vs. Model. Results are presented as mean $\pm$ SD.



**Fig. 4.** Thrombin increased the expression of PAR-1 and TGF- $\beta$ 1 proteins in the lung tissues of PQ-stimulated rats. After receiving treatments, the expression of PAR-1 and TGF- $\beta$ 1 proteins in the lung tissues of the rats was detected by immunohistochemical analysis. Magnification:  $\times 100$ , scale bar: 200  $\mu$ m; Magnification:  $\times 400$ , scale bar: 50  $\mu$ m.  $n=3$ .  $^{+}p<0.05$  and  $^{++}p<0.01$  vs. Control;  $^{*}p<0.05$  and  $^{**}p<0.01$  vs. Model. Results are presented as mean $\pm$ SD.



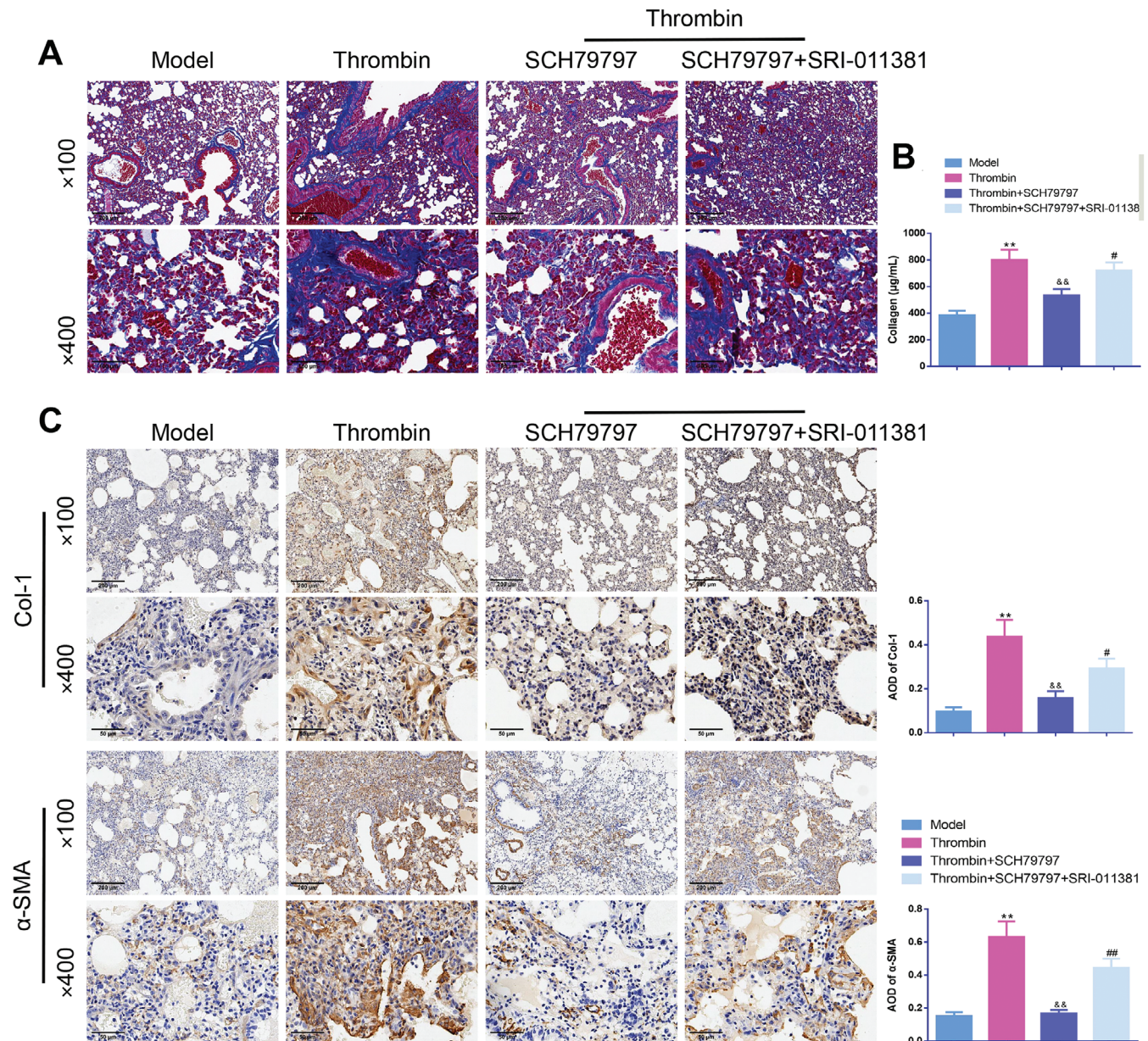
# Thrombin contributes to paraquat-induced PF

structural deterioration, interstitial hemorrhage, evident thickening of the alveolar septa, and substantial inflammatory cell infiltration. In comparison with the model group, the thrombin group showed more severe histopathological damage. However, hirudin treatment effectively alleviated the histopathological damage to the lung tissues in PQ-stimulated rats. The H&E staining results observed on the 14th day were similar to those observed on the 7th day, i.e., model rats exhibited serious histopathological damage in the lungs, while

thrombin treatment exacerbated this damage, and hirudin treatment alleviated the damage.

## Thrombin promoted PF in PQ-stimulated rats

On both the 7th and 14th day, compared with control rats, the extent of PF was increased after PQ administration. Then, thrombin intervention further worsened the extent of PF, but hirudin treatment improved it in model rats (Fig. 2A). As the Hyp level in



**Fig. 5.** Thrombin promoted PF in PQ-stimulated rats by upregulating PAR-1-mediated TGF- $\beta$ 1. **A.** After receiving treatments, the degree of PF in all rats was tested by Masson staining. Magnification:  $\times 100$ , scale bar: 200  $\mu$ m; Magnification:  $\times 400$ , scale bar: 50  $\mu$ m. **B.** Collagen levels in the BALF of the rats were detected in each group. **C.** The expression of Col-1 and alpha-smooth muscle actin ( $\alpha$ -SMA) in the lung tissues of the rats was measured by immunohistochemical analysis. Magnification:  $\times 100$ , scale bar: 200  $\mu$ m; Magnification:  $\times 400$ , scale bar: 50  $\mu$ m.  $n=3$ . \* $p<0.05$  and \*\* $p<0.01$  vs. Model; & $p<0.05$  and && $p<0.01$  vs. Thrombin; # $p<0.05$  and ## $p<0.01$  vs. Thrombin+SCH79797. Results are presented as mean $\pm$ SD.



lung tissue can reflect the extent of PF, Hyp levels were detected in the lung tissues of all groups. Similarly, on both the 7th and 14th day, Hyp levels in the lung tissues were elevated after PQ administration; thrombin intervention further upregulated Hyp levels, while hirudin treatment decreased them in model rats (Fig. 2B). Moreover, it was noted that collagen levels in the BALF of model rats were higher than those of control rats. Consistently, thrombin intervention further increased collagen levels, while hirudin treatment decreased collagen levels in the BALF of model rats (Fig. 2C).

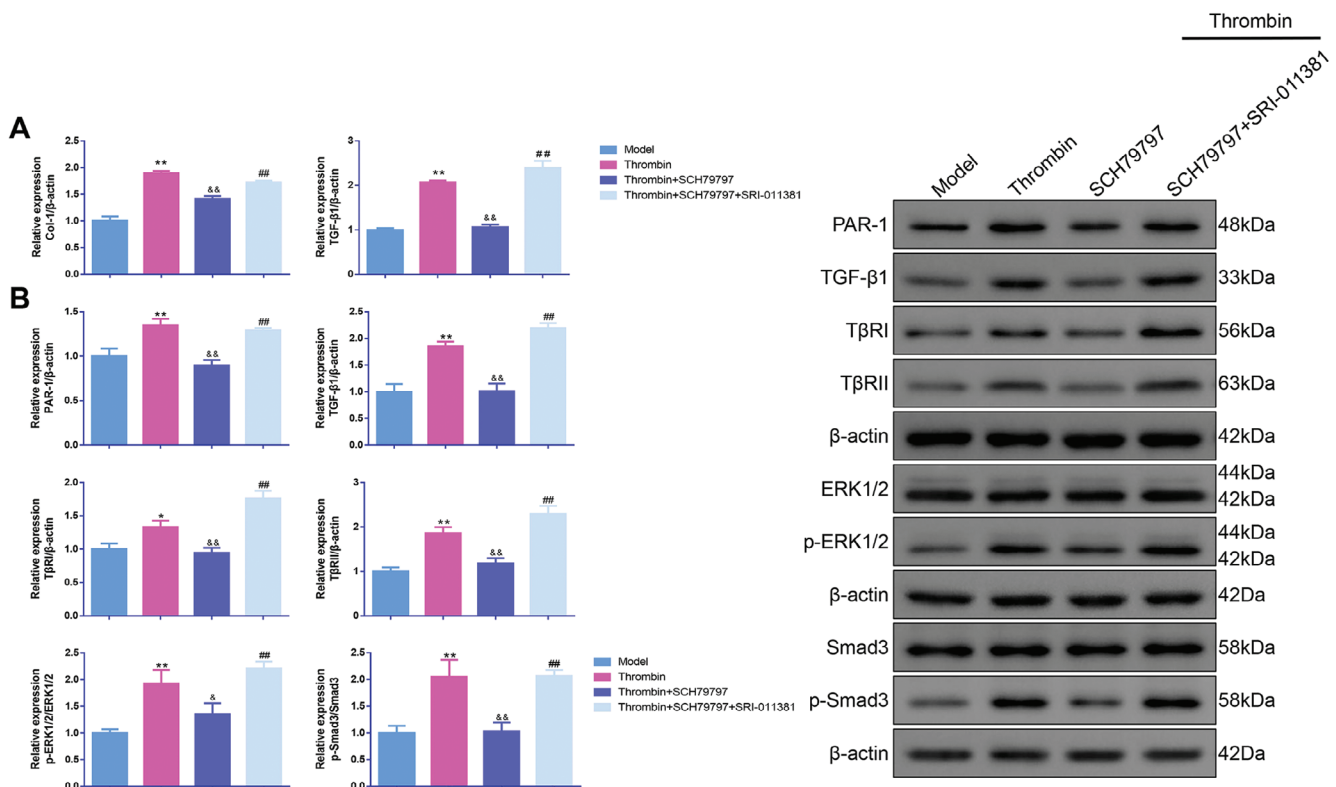
*Thrombin boosted PAR-1 and TGF- $\beta$ 1 protein expressions as well as extracellular signal-regulated kinase 1/2 (ERK1/2) and mothers against decapentaplegic homolog 3 (Smad3) phosphorylation for PQ-stimulated rats*

As exhibited in Fig. 3A, exposure to PQ for 14 days increased the mRNA expression of Col-1 and TGF- $\beta$ 1 in lung tissues. Following thrombin treatment, Col-1 and TGF- $\beta$ 1 mRNA expression was further enhanced in rats exposed to PQ, while hirudin intervention led to opposite results. Furthermore, it can be observed from

Fig. 3B that PAR-1 and TGF- $\beta$ 1 protein expression, as well as ERK1/2 and Smad3 phosphorylation, in rat lung tissues, were enhanced after stimulation with PQ for 14 days. As anticipated, treatment with thrombin further augmented PAR-1 and TGF- $\beta$ 1 protein expression, as well as ERK1/2 and Smad3 phosphorylation in PQ-stimulated rats, while hirudin intervention diminished these effects. The results of the immunohistochemical analysis for PAR-1 and TGF- $\beta$ 1 protein expression in rat lung tissues were similar to those observed in the western blot analysis (Fig. 4).

*Thrombin worsened PF in PQ-stimulated rats by activating PAR-1-mediated TGF- $\beta$ 1*

Next, to investigate the mechanism of thrombin in PQ-stimulated PF, PQ-stimulated rats were further treated with SCH79797 (a PAR-1 inhibitor), either alone or in combination with SRI-011381 (a TGF- $\beta$  agonist). Fig. 5A shows that thrombin promoted the degree of PF in model rats; however, this condition was mitigated by SCH79797, while SRI-011381 further counteracted the protective effect of SCH79797 on PF in rats. Furthermore, thrombin increased collagen levels in the BALF of model rats; however, this increase was partially



**Fig. 6.** Thrombin activated the PAR-1-mediated TGF- $\beta$ 1 pathway in PQ-stimulated rats. **A.** The quantitative polymerase chain reaction was applied to detect the expression of Col-1 and TGF- $\beta$ 1 mRNA in the lung tissues of the rats. **B.** Western blot assays were conducted to measure the protein expression of PAR-1, TGF- $\beta$ 1, TGF $\beta$  receptor type I (T $\beta$ RI), and type II (T $\beta$ RII), as well as the phosphorylation of ERK1/2 and Smad3 in the lung tissues of the rats.  $n=3$ . \* $p<0.05$  and \*\* $p<0.01$  vs. Model; & $p<0.05$  and && $p<0.01$  vs. Thrombin; # $p<0.05$  and ## $p<0.01$  vs. Thrombin+SCH79797. Results are presented as mean $\pm$ SD.

diminished by SCH79797, while SRI-011381 reversed the effect of SCH79797 (Fig. 5B). In addition, immunohistochemical analysis was conducted to evaluate the expression levels of fibrosis markers (Col-1 and  $\alpha$ -SMA) in the lung tissues of the rats. The results in Fig. 5C show that thrombin elevated Col-1 and  $\alpha$ -SMA levels in the lung tissues of model rats; however, relative to the thrombin group, Col-1 and  $\alpha$ -SMA levels in the thrombin+SCH79797 group were significantly decreased, while SRI-011381 reversed the effect of SCH79797 on the rats.

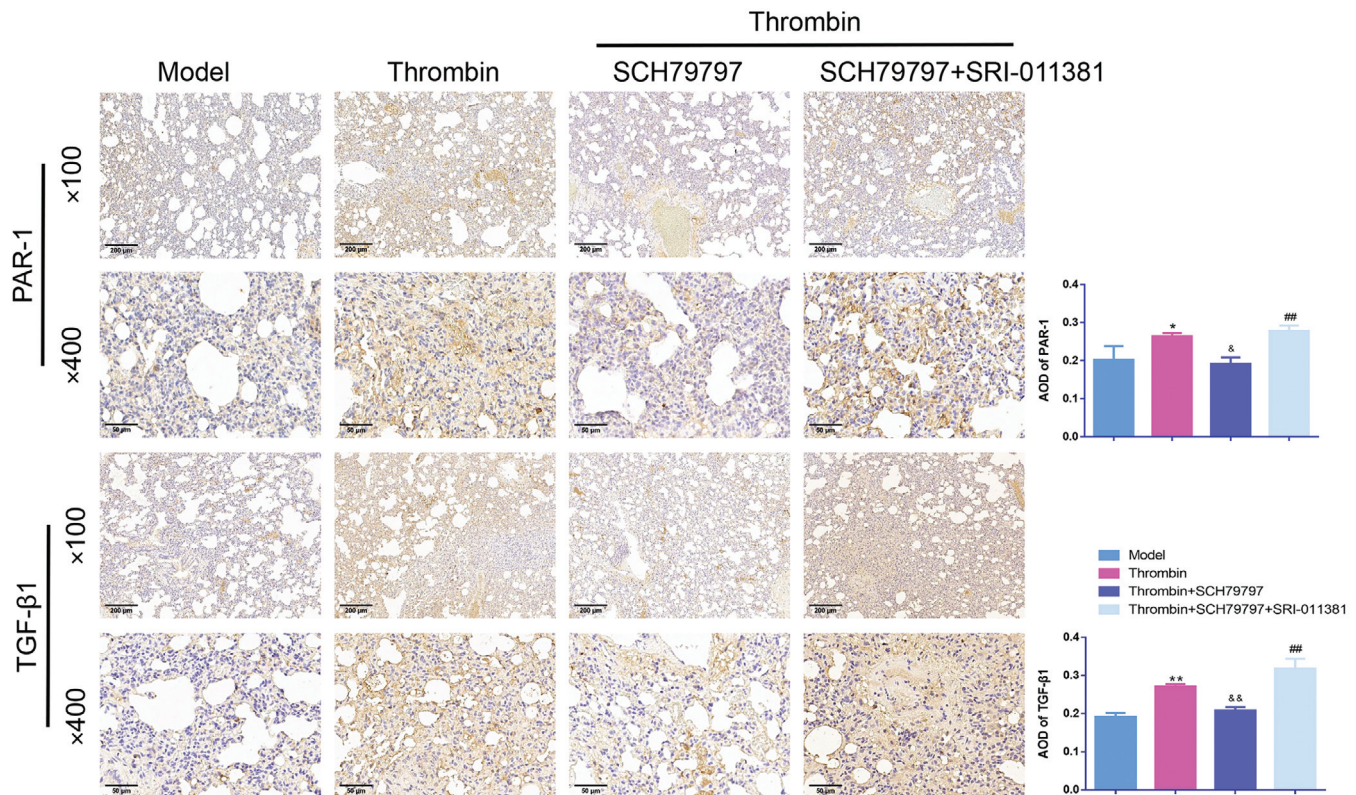
The qPCR results presented in Fig. 6A were similar to those observed in the immunohistochemical analysis. Thrombin increased Col-1 and TGF- $\beta$ 1 mRNA levels in the lung tissues of model rats; however, compared with the thrombin group, these levels were effectively reduced in the thrombin+SCH79797 group, whereas SRI-011381 reversed the effect of SCH79797 on the rats. Furthermore, Fig. 6B illustrates that the upregulated levels of PAR-1, TGF- $\beta$ 1, TGF $\beta$  receptor type I (T $\beta$ RI), and type II (T $\beta$ RII) proteins, as well as ERK1/2 and Smad3 phosphorylation, observed in the thrombin group were all reversed by SCH79797; however, the inhibitory effects of SCH79797 on these proteins were counteracted by SRI-011381. The immunohistochemical

results for PAR-1 and TGF- $\beta$ 1 protein expression in rat lung tissues were similar to those observed in the Western blot analysis (Fig. 7).

## Discussion

PQ is widely used globally as a fast-acting herbicide. However, the highly toxic effects on humans have recently been reported. The poisoning rate associated with PQ, whether intentional suicide attempts or accidental ingestion, has recently shown a notable upward trend (Kumar et al., 2021). Currently, many findings have suggested that PF is a leading cause of mortality after PQ ingestion (Li et al., 2015). Although immunosuppressive therapy has emerged as the most efficient approach to treating PF, its application remains limited due to insufficient supporting evidence (Agarwal et al., 2007; Parimon et al., 2021). Therefore, elucidating the relevant molecular mechanisms in PQ-induced PF is crucial to facilitate the development of effective clinical interventions for PF. In this study, we discovered that thrombin contributes to PQ-induced PF by activating PAR-1-mediated TGF- $\beta$ 1 (Fig. 8).

Thrombin is a multifunctional serine proteinase that plays a pivotal role in activating platelets and facilitating



**Fig. 7.** Thrombin upregulated PAR-1-mediated TGF- $\beta$ 1 expressions in PQ-stimulated rats. Following treatments, immunohistochemical analysis was used to assess the expression of PAR-1 and TGF- $\beta$ 1 proteins in the rat lung tissues. Magnification:  $\times 100$ , scale bar: 200  $\mu$ m; Magnification:  $\times 400$ , scale bar: 50  $\mu$ m.  $n=3$ . \* $p<0.05$  and \*\* $p<0.01$  vs. Model;  $^{\&}$  $p<0.05$  and  $^{\&\&}$  $p<0.01$  vs. Thrombin;  $^{\#}$  $p<0.05$  and  $^{\#\#}$  $p<0.01$  vs. Thrombin+SCH79797. Results are presented as mean $\pm$ SD.

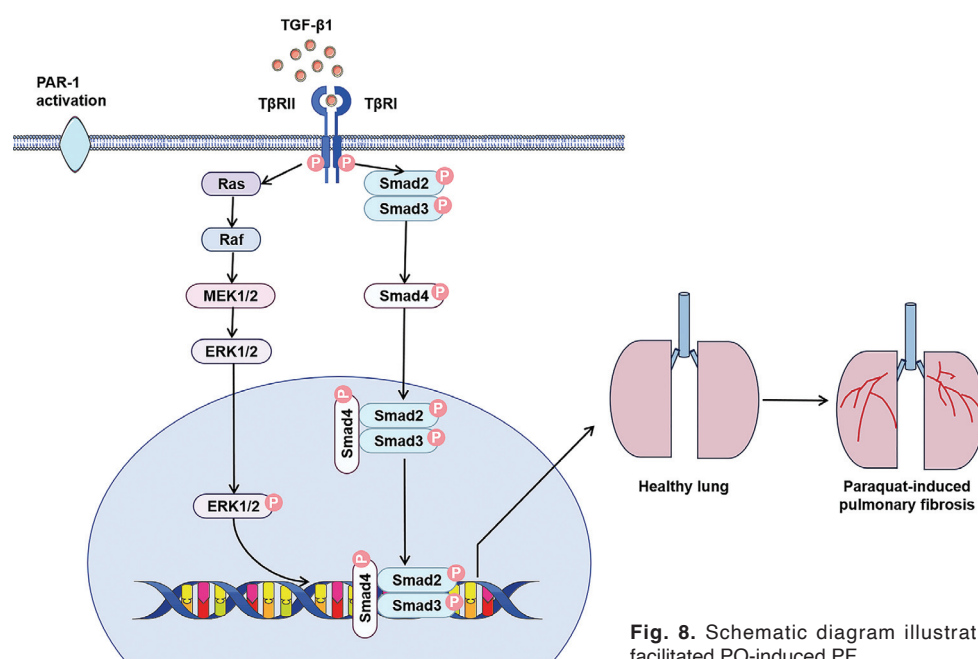


blood coagulation. In addition to its primary function in maintaining homeostasis, thrombin can catalyze the cleavage of fibrinogen to fibrin, thereby facilitating the formation of blood clots after tissue injury. At present, numerous studies have explored the role and mechanism of thrombin in central nervous system diseases (Shlobin et al., 2021), cardiovascular diseases (Ferreira et al., 2022), and cancers (Alexander and Gilmour, 2022). Since Kubo et al. reported that anticoagulant therapy has a beneficial effect on survival in patients with idiopathic pulmonary fibrosis (IPF), more attention has been paid to the relationship between thrombin and lung diseases (Kubo et al., 2005). For example, clinical research has found that thrombin exacerbates extravascular lung water and the outcomes of septic patients with acute respiratory distress syndrome (Chung et al., 2023). Huang et al. have found that administering thrombin inhibitors can effectively treat lung inflammation by modulating type 2 innate lymphoid cells through interleukin-33 maturation (Huang et al., 2022). It has been indicated that OS significantly contributes to the onset and progression of PF (Sul et al., 2022). OS has been shown to facilitate myofibroblast formation by stimulating the release of various cytokines. The increase in myofibroblasts resulting from OS can increase ECM, thereby further promoting fibrosis progression (Zeng et al., 2022). It has been well documented that PF can be effectively alleviated through the antioxidant N-acetylcysteine (Mata et al., 2003), and inhibiting thrombin can decrease OS (Johnson et al., 2020).

In line with previous studies, this study found that, in addition to triggering OS in the lung tissues of PQ-stimulated rats, thrombin also aggravated histo-

pathological damage to lung tissues and promoted PF. To further explore the role and mechanism of thrombin in PQ-induced PF, we used a thrombin inhibitor. Notably, although warfarin is a direct thrombin inhibitor, it does not exhibit a benefit in the treatment of patients with progressive IPF, and treatment with warfarin is associated with an increased risk of mortality in the IPF population who lack other indications for anti-coagulation (Noth et al., 2012). Therefore, hirudin (another thrombin inhibitor) was selected for this study. The results of the study revealed that treatment with hirudin led to opposite results to thrombin, indicating that thrombin may promote PF development in PQ-stimulated rats.

PAR-1, a receptor for thrombin, is considered a promising target in the treatment of fibrotic diseases (Xiao et al., 2023). In patients with IPF, PAR-1 expression is notably elevated in fibroproliferative and inflammatory foci, and PAR-1 activation regulates angiogenesis and mitogenesis, changes pulmonary vascular permeability, contributes to fibroblast proliferation, migration, and ECM synthesis, and increases inflammation in the pulmonary epithelium (Lin et al., 2014). Additionally, TGF- $\beta$  also plays a crucial role in the regulation of PF (Inui et al., 2021). Several potential candidates, such as Biochanin-A (Andugulapati et al., 2020), Yupingfeng (Chen et al., 2024), and Sinomenine (Yao et al., 2024), have been demonstrated to improve PF by inhibiting TGF- $\beta$ . On the other hand, it has been reported that TGF- $\beta$ 1 and OS can cooperate to drive fibrosis (Louzada et al., 2021). More importantly, research has demonstrated that regulating PAR-1 influences TGF- $\beta$  expression. For example, enhancing PAR-1 activity significantly increases the gene



**Fig. 8.** Schematic diagram illustrating the mechanism by which thrombin facilitated PQ-induced PF.

expressions of fibronectin, TGF- $\beta$ , and Col-1 *in vitro* (Seo et al., 2021), and agonists of PAR-1 can activate TGF- $\beta$  in an  $\alpha$ v $\beta$ 6 integrin-specific manner (Jenkins et al., 2006). In addition, research by Zhu et al. demonstrated that thrombin promotes airway remodeling via PAR-1 and TGF- $\beta$  in ovalbumin-stimulated animals (Zhu et al., 2013). Conversely, treatment with hirudin has been reported to decrease PAR-1 and TGF- $\beta$ 1 protein expressions in rats with unilateral ureteral obstruction (Yang et al., 2020) and in mice with renal interstitial fibrosis (Lin et al., 2022).

Consistently, in this study, we observed that PAR-1 and TGF- $\beta$ 1 expression as well as ERK1/2 and Smad3 phosphorylation (TGF- $\beta$  downstream proteins) (Li et al., 2020; Wu et al., 2023) were elevated in PQ-stimulated rats after thrombin treatment; however, hirudin treatment led to opposite results. In addition, the effect of thrombin on PF, PAR-1, and TGF- $\beta$ 1 expression, and ERK1/2 and Smad3 phosphorylation for PQ-stimulated rats was reversed by SCH79797 (a PAR-1 inhibitor), while SRI-011381 (a TGF- $\beta$  agonist) further counteracted the effect of SCH79797. Based on these results, it can be speculated that thrombin contributes to PQ-induced PF by activating PAR-1-mediated TGF- $\beta$ 1.

Nevertheless, it should be noted that the study has some limitations. The primary limitation is the small sample size. In addition, inflammation is important during PF development; therefore, the other limitation of the study is the lack of inflammatory cytokine detection. In the future, we will increase the sample size and detect the levels of interleukin-1 beta, interleukin-6, and tumor necrosis factor-alpha in rat lung tissues, thereby enhancing the reliability of the key findings in the study.

In conclusion, our findings imply that thrombin can promote PQ-induced PF, and the mechanisms by which thrombin contributes to PQ-induced PF may relate to the activation of PAR-1-mediated TGF- $\beta$ 1. We believe these discoveries pave the way for new strategies to prevent PQ-induced PF.

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**Conflicts of interest.** The authors declare that there is no conflict of interest regarding the publication of this article.

**Ethics approval and consent to participate.** The animal experiments conducted in this study received approval from the Laboratory Animal Management and Ethics Committee of Hangzhou Hunter Testing Biotechnology Co., Ltd., and the animals were cared for according to the guidelines of the Institutional Animal Care and Use Committee.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon reasonable request.

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