

RESEARCH ARTICLE

Thrombin promotes airway remodeling via protease-activated receptor-1 and transforming growth factor- β 1 in ovalbumin-allergic rats

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Abstract

Background: Protease-activated receptor-1 (PAR-1) is widely distributed in platelets and involved in coagulation cascade activated by thrombin. In this study, we intend to explore the role of PAR-1 in the process of thrombin-inducing transforming growth factor- β 1 (TGF- β 1) to promote airway remodeling in ovalbumin (OVA)-allergic rats.

Materials and methods: A rat model of chronic asthma was set up by systemic sensitization and repeated challenge to OVA. The doses of thrombin, recombinant hirudin, PAR-1 inhibitor ER-112780-06 varied for different groups. We evaluated the bronchoalveolar lavage fluid (BALF) concentration of thrombin in these groups. The protein and gene expression of PAR-1 was assessed and the expression of TGF- β 1 was also detected.

Results: The PAR-1 mRNA level and the protein level were higher in the airway of asthmatic rats than those of normal rats, and were significantly increased by thrombin treatment but decreased by thrombin-inhibitor treatment. Airway remodeling was strengthened by thrombin but weakened by thrombin inhibitor and PAR-1 antagonist. Expression of TGF- β 1 protein in asthmatic rats was significantly increased by thrombin treatment and decreased by thrombin-inhibitor treatment and PAR-1 antagonist treatment.

Conclusion: The expression of PAR-1 is regulated by thrombin that induces the expression of TGF- β 1 to promote airway remodeling via PAR-1 in OVA-allergic rats.

Keywords

Airway remodeling, asthma, PAR-1, rat, TGF- β 1, thrombin

History

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Introduction

Asthma is a chronic inflammatory disease of the airway which is characterized by infiltration of inflammatory cells and structural changes. Structural changes are referred to as “airway remodeling”. Airway remodeling constitutes sub-epithelial fibrosis, increased deposition of extracellular matrix protein, goblet cell hyperplasia, mucus gland hypertrophy, smooth muscle hypertrophy and hyperplasia, and epithelial damage (Camateros et al., 2007; Chen et al., 2009; Takayama et al., 2011). Thrombin is a kind of serine protease. Except for the role in hemostasis, thrombin is recognized as a new candidate mediating airway remodeling. *In vitro* observations showed thrombin-induced airway smooth muscle (ASM) cell proliferation (Svensson Holm et al., 2011), and may contribute to ASM cell growth, contractile protein expression, and airway remodeling in asthma (Goncharova et al., 2010). Increase in vascular endothelial growth factor (VEGF) level leads to up-regulation of thrombin activity in asthmatic airways and elevated thrombin activity induces elevation of basic fibroblast growth factor level to promote airway

remodeling (Kanazawa & Yoshikawa, 2007). Extravascular fibrinogen and thrombin have been identified in the sputum of patients with asthma (Pizzichini et al., 1996). Thrombin activity in bronchoalveolar lavage fluid (BALF) was increased by segmental antigen provocation in patients with asthma, and BALF from these patients was able to induce proliferation of fibroblasts (Gabazza et al., 1999). These results suggest that there is a connection between thrombin and asthmatic airway remodeling.

PARs are a family of G-protein-coupled seven-transmembrane-domain receptors, including four members, PARs 1–4 (Ishihara et al., 1997; Kahn et al., 1998; Xu et al., 1998). PAR activation has been linked to a number of responses including platelet aggregation, vasodilation, vasoconstriction, increased vascular permeability, increased intestinal permeability, granulocyte chemotaxis and intestinal epithelial apoptosis (Buresi et al., 2001; Chin et al., 2003; Dery et al., 1998). Thrombin is an endogenous agonist for PAR-1, PAR-3 and PAR-4. PAR-1 is widely distributed in platelets, endothelial cells and smooth muscle cells. Thrombin, the main effector protease of the coagulation cascade, activates platelets by proteolytic activation via PAR-1 exists on cell surface (Chackalamannil et al., 2008). In the vessel wall biology, thrombin is known to be mitogenic in endothelial and smooth muscle cells via PAR-1 mechanism (Patterson et al., 2001).

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In respiratory system, PAR-1 is expressed in both the epithelial and smooth muscle cells (Hauck et al., 1999; Ossovskaya & Bunnett, 2004). Therefore, thrombin exerts a number of proinflammatory and profibrotic cellular effects *in vitro* that are critically important in tissue repair processes. These cellular effects are predominantly mediated via proteolytic activation of the major thrombin receptor PAR-1. PAR-1 activation may be a mechanism by which thrombin promotes airway remodeling in asthma.

Transforming growth factor-beta 1 (TGF- β 1), as a fibrogenic cytokine, promotes structural changes in airway remodeling (Chen et al., 2011; Goulet et al., 2007). TGF- β 1 stimulates fibroblast proliferation and promotes the synthesis and secretion of collagen from fibroblasts. TGF- β 1 is known as the strongest one of fibrosing factors so far (Broekelmann et al., 1991) and one of the most important factors involved in airway remodeling in asthma. Silva's research demonstrated that thrombin is a potent inducer of TGF- β 1 (Silva et al., 2012).

These data led us to hypothesize that the downstream cellular effects of thrombin, which are initiated following receptor activation, may be important in promoting tissue fibrosis *in vivo*. In this study, we focused on the reaction of thrombin and PAR-1, and the effect of this reaction. We hypothesized that thrombin promote the production of TGF- β 1 in the airway of asthmatic rats. To address this hypothesis, we assessed the effect of thrombin on TGF- β 1 expression *in vivo*. Our data show that for the first time, thrombin increases TGF- β 1 protein expression in asthma via PAR-1. The results also demonstrate that as a PAR-1 agonist, thrombin up-regulates both gene and protein expression of PAR-1. Moreover, this study can provide insight into a new class of potentially important therapeutic approaches for airway remodeling in asthma.

Materials and methods

Reagents

Chicken egg ovalbumin (OVA, grade V, >98% pure) and aluminum hydroxide were from Sigma (St Louis, MO). Hirudin was purchased from Knoll AG-BASF Pharma (Ludwigshafen, Germany). The PAR-1 inhibitor, ER-112780-06 was from Eisai Pharmaceuticals (Tokyo, Japan). Bovine thrombin was purchased from Haematologic Technologies Inc. (Essex Junction, VT). Heat-killed *Bordetella pertussis* bacteria (6×10^9 per ml) were obtained from National Institute for Food and Drug Control (Beijing, China). Rabbit monoclonal anti-PAR-1 and anti-TGF β 1 antibody (Abcam, Cambridge, MA). Anti-rabbit secondary antibody and diaminobenzidine (DAB) reagent were from Beijing Biosynthesis Biotechnology Co., Ltd (Beijing, China).

Animals and sensitization

Female Wistar rats, weighing 150–160 g, were purchased from the Experimental Animal Center of Shandong University. All experimental protocols involving animals were approved by the ShanDong University Animal Care Committee and complied with the guidelines of the China Council on Animal Care. Rats were sensitized by subcutaneous injection of 1 mg OVA and 200 mg/ml aluminum hydroxide in 1 ml saline

solution and intraperitoneal (i.p.) injection of 1 ml heat-killed *Bordetella pertussis* bacteria on days 0 and 7 (Zhou et al., 2008). Control (non-sensitized) animals received i.p. injection of normal saline on days 0 and 7.

Inhalational exposure

OVA was dissolved in normal saline at a concentration of 2% (20 mg/ml). Sensitized rats were exposed to aerosolized 2% OVA for 30 min twice a day from day 14 (3 d/week) for 6 weeks (OVA group). An AZWELL UN-511 ultrasonic nebulizer (Osaka, Japan) was used for aerosol generation. During the exposure, the animals were placed in a plastic inhalation chamber, and the filtered air was drawn through the chamber at a flow rate of 17 L/min. The volume of the chamber was about 17 L maintained under normoxic and normocapnic conditions. Control (non-sensitized) animals were exposed to aerosolized normal saline for 30 min/d twice a day from day 14 (3 d/week) for 6 weeks.

Inhalation of thrombin and administration of hirudin

Thrombin was dissolved in normal saline at a concentration of 2000 U/L (Moffatt et al., 2004). The animals were treated with aerosolized thrombin for 30 min from day 14 (3 d/week) for 6 weeks and immediately before each inhalational exposure to OVA using a chamber and an ultrasonic nebulizer as described in Section "Inhalational exposure". Correspondingly, hirudin (1 mg/kg) was administrated subcutaneously before each inhalational exposure to OVA (Marty et al., 2001). To evaluate the effect of thrombin on airway remodeling, the rats were divided into the following treatment groups: (1) OVA-sensitized rats treated with inhaled normal saline before exposure to aerosolized OVA (OVA + SAL group; $n = 6$), (2) OVA-sensitized animals treated with inhaled thrombin before exposure to aerosolized OVA (thrombin + OVA group; $n = 6$), (3) OVA-sensitized animals treated with hirudin subcutaneous injection before exposure to aerosolized OVA (hirudin + OVA group; $n = 6$) and (4) non-sensitized rats that received i.p. injection of saline and inhaled normal saline (SAL group; $n = 6$).

Inhalation of PAR-1 inhibitor

To assess the role of PAR-1 on thrombin activity, in a separate experiment, OVA-sensitized rats were treated with 20 μ M inhaled form of the PAR-1 inhibitor, ER-112780-06 (OVA + thrombin + PAR-1-inh group; $n = 6$) or normal saline (OVA + thrombin + SAL group; $n = 6$) prior to treatment with inhaled thrombin and before exposure to aerosolized OVA.

Bronchoalveolar lavage sampling

At the end of each experimental protocol, the animals were killed by i.p. overdoses of pentobarbital to take samples for biochemical and histologic examinations. Sampling of BALF was performed as previously described (Yasui et al., 2001). BALF was obtained by cannulating the trachea with a 20-gauge needle and infusing the lungs four times with 1 ml of saline. The recovery of BALF ranged between 2.0 and 3.5 ml, with no significant differences in the volume recovered between control and treated rats. The recovered

Table 1. Primers used in the experiments.

Gene and type	Sequence	Position	Size (bp)
PAR-1 (NM_012950.2)			
Forward	GGAGGCAATAACACCTGCACATC	2257–2401	145
Reverse	GTCAGTCAGTCTACGGCCCCACTATC		
β -Actin			
Forward	GGAGATTACTGCCCTGGCTCCTA	1026–1175	150
Reverse	GACTCATCGTACTCCTGCTTGCTG		

fluid was filtered through a single layer of gauze to remove mucus. The BALF was centrifuged (1000 g, 10 min, 4 °C) and the cell-free supernatant was stored immediately at –80 °C until use for biochemical analysis.

Measurement of thrombin in BALF

Thrombin activity was measured by the enzymatic assay, using the chromogenic substrate, H-D-hexahydrotyrosol-alanyl-arginine-*para*-nitroanilide diacetate (American Diagnostica, Greenwich, CT).

Goblet cell hyperplasia, airway wall thickness, collagen deposition and mucus production

Left lungs were obtained at 24 h after the final OVA administration, fixed with periodate-lysine-paraformaldehyde, embedded in paraffin and sectioned at a thickness of 4 μ m. Slides were stained with periodic acid-Schiff or Masson's trichrome staining.

Under light microscopy, four representative cross-sections of the trachea which were completely cartilage-free were randomly selected. MIAS-2000 image measurement and analysis software was used for measurement and analysis; measurement parameters include lumen perimeter (Pi), the area enclosed by the outer wall of the airway (WAo), the area enclosed by the walls of the airways (Wai) and the total wall area (WA) = WAo – Wai. WA/Pi (m^2/m) represents the bronchial wall thickness.

Immunohistochemistry

Tissue sections were prepared as mentioned earlier. To eliminate endogenous peroxidase activity, tissue sections were treated with 3% H_2O_2 in methanol and permeabilized with 0.25% Triton X in PBS for 20 min at room temperature. The sections were then incubated with rabbit monoclonal anti-PAR-1 (1:200; Abcam) or anti-TGF β 1 antibody (1:400; Abcam). The sites of primary antibody binding were visualized with anti-rabbit secondary antibody (1:500) and DAB reagent. The specimens were also counterstained with hematoxylin (Merck, Darmstadt, Germany).

RT-PCR

For RT-PCR, total RNA from the middle lobe of right lung was isolated with Trizol LS reagent (Takara, Dalian, China) according to the manufacturer's instructions. To exclude any genomic DNA contamination, all RNA samples were pre-treated with DNA-free DNase treatment and removal reagents (Takara). Total RNA were reverse-transcribed with random primer according to the manufacture's protocol using High-Capacity cDNA Reverse Transcription kit (Applied

Biosystems, Foster City, CA). Real-time PCR was performed using Fast SYBR Green Master Mix (Applied Biosystems) with the Applied Biosystems 7500 Fast Real-Time PCR system. The reactions were amplified at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C for 60 s. The relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ as described previously (Jiang et al., 2008). Each sample was run in triplicate. The primers used for these reactions are shown in Table 1.

Western blotting

PCR data of PAR-1 expression were also confirmed at the protein level by Western blotting. TGF- β expression was determined by Western blotting. The upper lobe of right lung tissues were rapidly collected and frozen after the last OVA inhalation. Each sample was homogenized in an ice-cold tissue extraction buffer (Invitrogen, Carlsbad, CA) containing 1% protease inhibitor cocktail. The homogenates were centrifuged at 11 000g for 30 min at 4 °C. The bicinchoninic acid protein assay kit (Pierce, UK) was used to assay the total protein of each sample. Following denaturing, samples, with equal protein concentrations, were loaded onto an 8% SDS-PAGE. After electrophoresis, proteins were transferred to a poly(vinylidene fluoride) membrane in transfer buffer using a blotting apparatus (Biometra, Göttingen, Germany). Membranes were blocked in 5% milk in TBST (Tris buffer solution + tween) for an hour, and then incubated in the primary antibody (β -actin, 1:10 000; or PAR-1, 1:500; or TGF- β , 1:1000) overnight at 4 °C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature and then processed with DAB Western blotting detection reagents (ZSJQ Corp., Beijing, China). The results were documented and analyzed with image J (National Institute of Health, Bethesda, MD). The ratio of band intensity in Western blots in comparison with the internal control was calculated.

Statistics

All results were expressed as the mean $\pm$ SD. A two-way ANOVA with multiple comparison tests to assess differences between groups was performed. All data analysis was performed using SPSS 18.0 software. *p* Values less than 0.05 were considered statistically significant.

Results

BALF concentration of thrombin is increased in asthmatic rat

Thrombin activity in BALF was significantly higher in OVA-allergic rats than in normal control group:

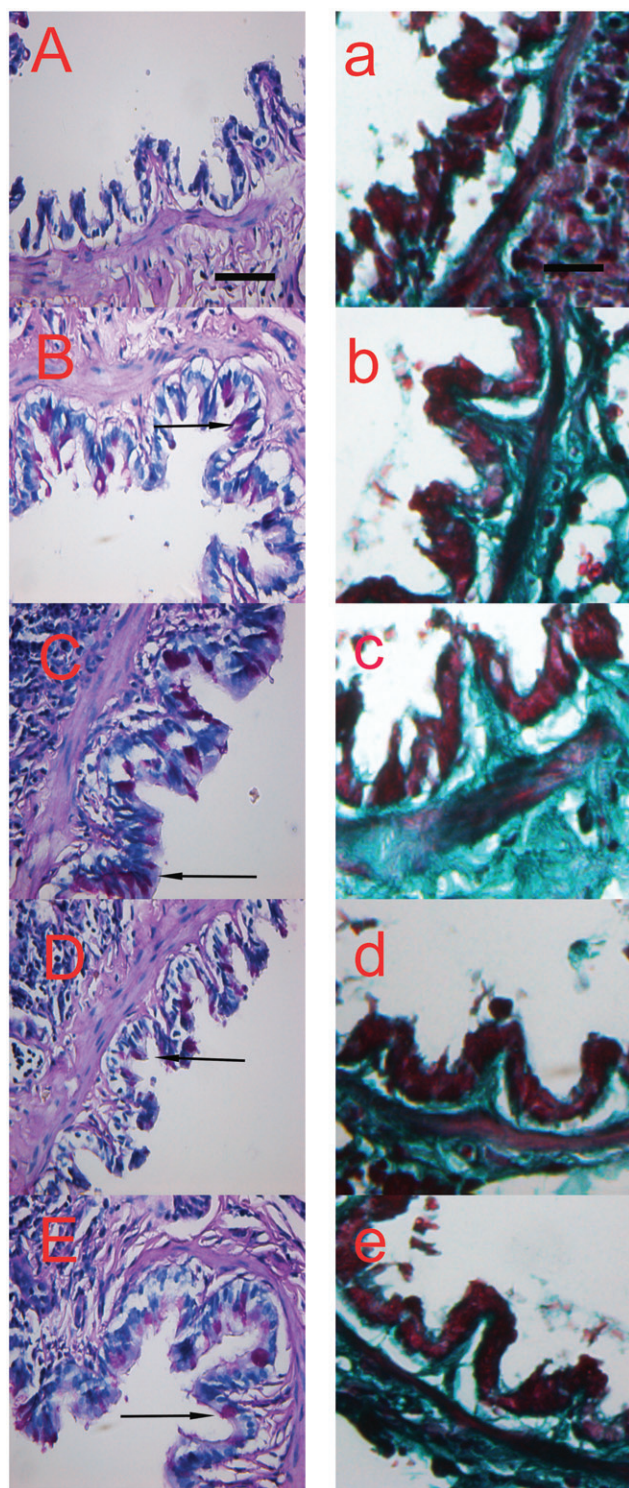


Figure 1. Periodic acid-Schiff (A–E) and Masson trichrome (a–e) staining of whole lung sections from rats with different treatment. The photomicrographs are representative of lungs removed from SAL group (A, a, $n = 6$), OVA + SAL group (B, b, $n = 6$), OVA + thrombin group (C, c, $n = 6$), OVA + hirudin group (D, d, $n = 6$) and OVA + thrombin + PAR-1-inh group (E, e, $n = 6$). The images included are representative of all animals within each treatment group. Goblet cell hyperplasia in OVA-challenged rats were observed around the airways (arrow in B), goblet cell hyperplasia was increased more evident in rats treated with thrombin and OVA (arrow in C), but goblet cell hyperplasia was decreased in rats treated with hirudin and OVA (arrow in D), and rats treated with OVA, thrombin and PAR-1-inh (arrow in E). More enhanced collagen deposition were observed in thrombin + OVA group (c, dark-green areas) relative to OVA + SAL group, hirudin + OVA group and OVA + thrombin + PAR-1-inh group (b, d and e, respectively). Original magnification: $\times 400$. Bar = 25 μm .

$237.33 \pm 34.93 \text{ ng/ml}$ versus $104.8 \pm 8.35 \text{ ng/ml}$ ($p < 0.01$). In thrombin + OVA group, thrombin activity was increased compared with OVA + SAL group: $450 \pm 43.20 \text{ ng/ml}$ versus $237.33 \pm 34.93 \text{ ng/ml}$ ($p < 0.01$).

Effect of thrombin on goblet cell hyperplasia, collagen deposition and mucus production

Periodic acid-Schiff staining (goblet cells) and mucus secretion were increased in lung tissue from OVA + thrombin group (Figure 1C) compared with OVA + SAL group (Figure 1B) and OVA + hirudin group (Figure 1D). In addition, collagen deposition and smooth muscle thickness were increased in OVA + thrombin group (Figure 1c) compared with OVA + SAL group (Figure 1b) and OVA + hirudin group (Figure 1d). The bronchial wall thickness was increased significantly in OVA + thrombin group in comparison with OVA + SAL group (Figure 2) and decreased significantly in OVA + hirudin group in comparison with OVA + SAL group (Figure 2).

These results suggest that thrombin promotes the remodeling of airway in OVA-sensitized rats.

Role of PAR-1 inhibitor on thrombin activity on goblet cell hyperplasia, airway wall thickness, collagen deposition and mucus production

Goblet cells, mucus secretion, collagen deposition and smooth muscle thickness were decreased in lung tissue from OVA + thrombin + PAR-1-inh group (Figure 1E, e) compared with OVA + thrombin + SAL group (Figure 1C, c). The effect of PAR-1-inh on the bronchial wall thickness is showed in Figure 2.

These results suggest that inhalation of PAR-1 inhibitor diminishes the role of thrombin on airway remodeling in OVA-sensitized rats.

Effect of thrombin on the expression of TGF- $\beta 1$

The ability of thrombin to promote airway remodeling was evaluated by the expression of TGF- $\beta 1$. Positive staining showed TGF- $\beta 1$ expression in the epithelium, macrophage leukocyte and smooth muscle (Figure 3). The immunostaining areas and intensity of TGF- $\beta 1$ in the OVA + thrombin group (Figure 3C) were markedly greater than those in the OVA + SAL group (Figure 3B). In contrast, positive staining of TGF- $\beta 1$ expression was decreased in OVA + hirudin group (Figure 3D) compared with OVA + SAL group (Figure 3B).

To confirm these results, expression of TGF- $\beta 1$ was determined by Western blotting (Figure 4). The result showed that the protein expression of TGF- $\beta 1$ was increased in OVA + thrombin group, whereas decreased in OVA + hirudin group compared with OVA + SAL group.

Effect of PAR-1 inhibitor on thrombin activity on the expression of TGF- $\beta 1$

Both immunohistochemistry (Figure 3) and Western blotting results (Figure 4) demonstrated that the protein expression of TGF- $\beta 1$ was decreased in OVA + thrombin + PAR-1-inh group (Figure 3E) compared with OVA + thrombin + SAL

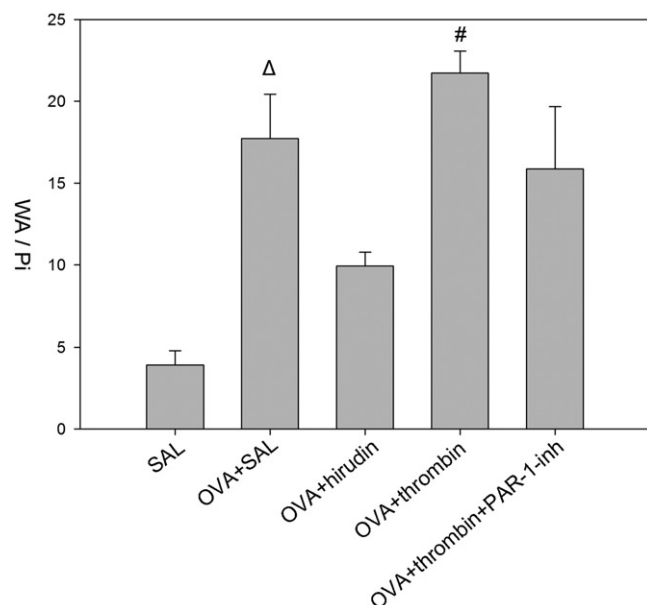
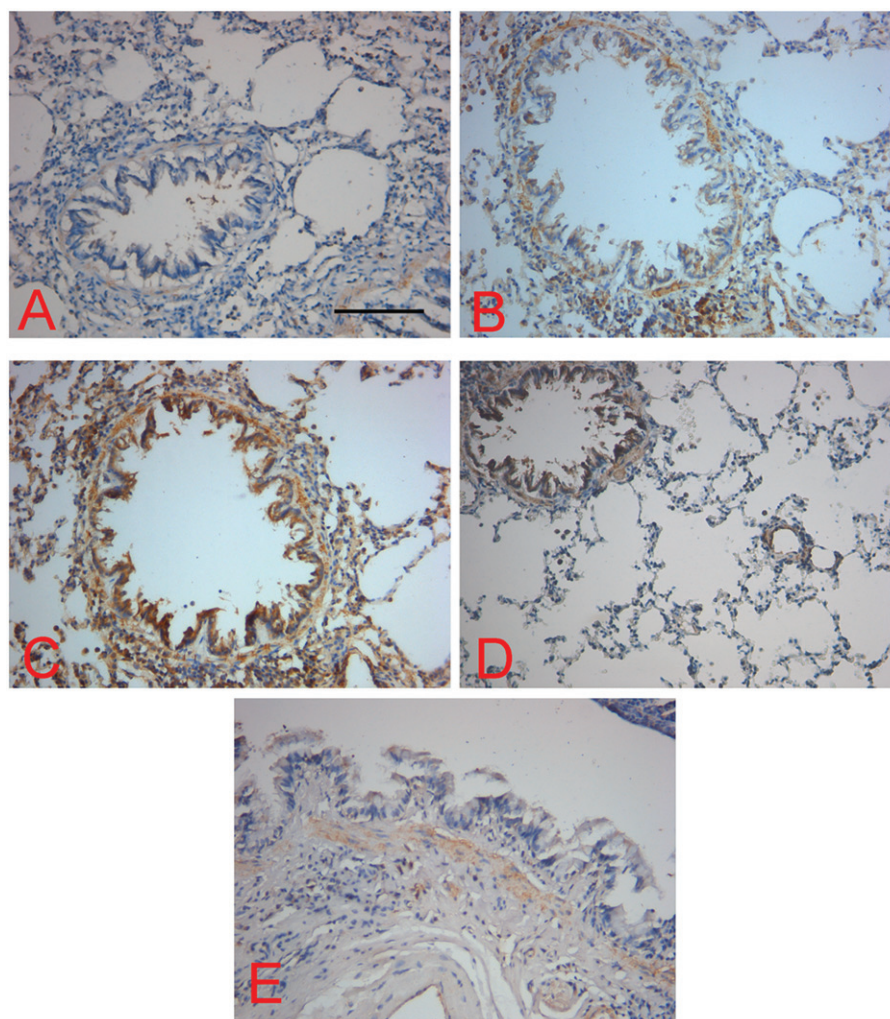


Figure 2. Effects of thrombin and PAR-1-inh on airway wall thickness in bronchi in rats challenged with OVA. Data represent the mean $\pm$ SEM ($n=6$) for each group. $\Delta p < 0.05$ versus SAL-treated rats, $\#p < 0.05$ versus OVA+SAL-treated rats, $\#p < 0.05$ versus OVA+hirudin-treated rats and $\#p < 0.05$ versus OVA + thrombin + PAR-1-inh-treated rats.

Figure 3. Effect of thrombin on expression of transforming growth factor- β 1 (TGF- β 1) protein in the lung tissue of asthmatic rats. The photomicrographs are representative of lungs removed from SAL group (A, $n=6$), OVA + SAL group (B, $n=6$), OVA + thrombin group (C, $n=6$), OVA + hirudin group (D, $n=6$) and OVA + thrombin + PAR-1-inh group (E, $n=6$). The images included are representative of all animals within each treatment group. Expression of TGF- β 1 in lung tissue was determined by immunohistochemical staining. Positive staining is depicted in brown. Positive staining showed TGF- β 1 expression in the epithelium, macrophage leukocyte and smooth muscle cells. Administration of thrombin to rats challenged with ovalbumin (C) significantly increased the immunostaining area of TGF- β 1. Administration of hirudin (D) and PAR-1-inh (E) to rats challenged with ovalbumin markedly reduced the immunostaining area of TGF- β 1 (magnification $\times 200$). Bar = 50 μ m.



group (Figure 3C). Immunohistochemistry result showed TGF- β 1 expression in the epithelium, macrophage leukocyte and smooth muscle.

These results suggest inhalation of PAR-1 inhibitor diminishes the role of thrombin on the expression of TGF- β 1 in the airway of OVA-sensitized rats.

Thrombin induces the expression of PAR-1

As the receptor of thrombin, the data showed that PAR-1 could be regulated by thrombin. PAR-1 was detected by real-time PCR, Western blotting and immunohistochemistry.

As shown in Figure 5, the gene expression of PAR-1 was increased in OVA + thrombin group compared with OVA + SAL group, and decreased in OVA + hirudin group compared with OVA + SAL group.

This observation could be confirmed by immunohistochemistry (Figure 6) and Western blotting (Figure 7); the protein expression of PAR-1 had the same trend with gene expression. In detail, immunostaining with the monoclonal antibody to PAR1 revealed a patchy distribution over the ASM cells and a high level of staining among the cells of the epithelium.

These results suggest that thrombin could up-regulate the expression of PAR-1 in airway of OVA-sensitized rats.

Figure 4. Western blotting result of TGF- β 1 (A). Quantification of the band intensity (B) showed that activated TGF- β 1 level was increased in thrombin-treated, OVA-challenged rats and PAR-1-inh treatment significantly reduced activation of TGF- β 1. Quantitative densitometry analysis of Western blot analysis for TGF- β 1. Data represent the mean $\pm$ SEM ($n=6$) for each group. β -actin is used as the control. # $p<0.01$ compared with the SAL group, $\Delta p<0.01$ compared with the OVA + SAL group, $\Delta p<0.01$ compared with the OVA + hirudin group and $\Delta p<0.01$ compared with OVA + thrombin + PAR-1-inh group.

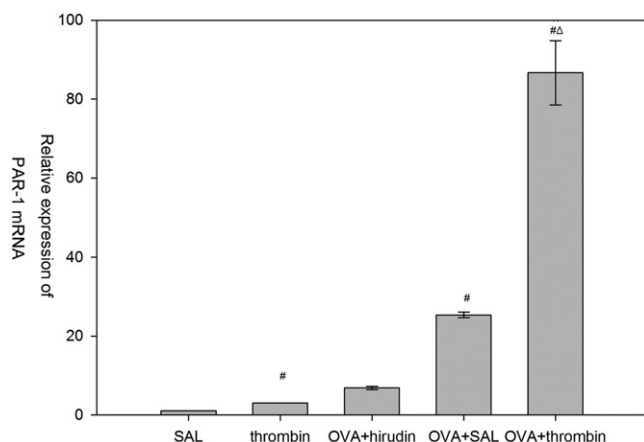
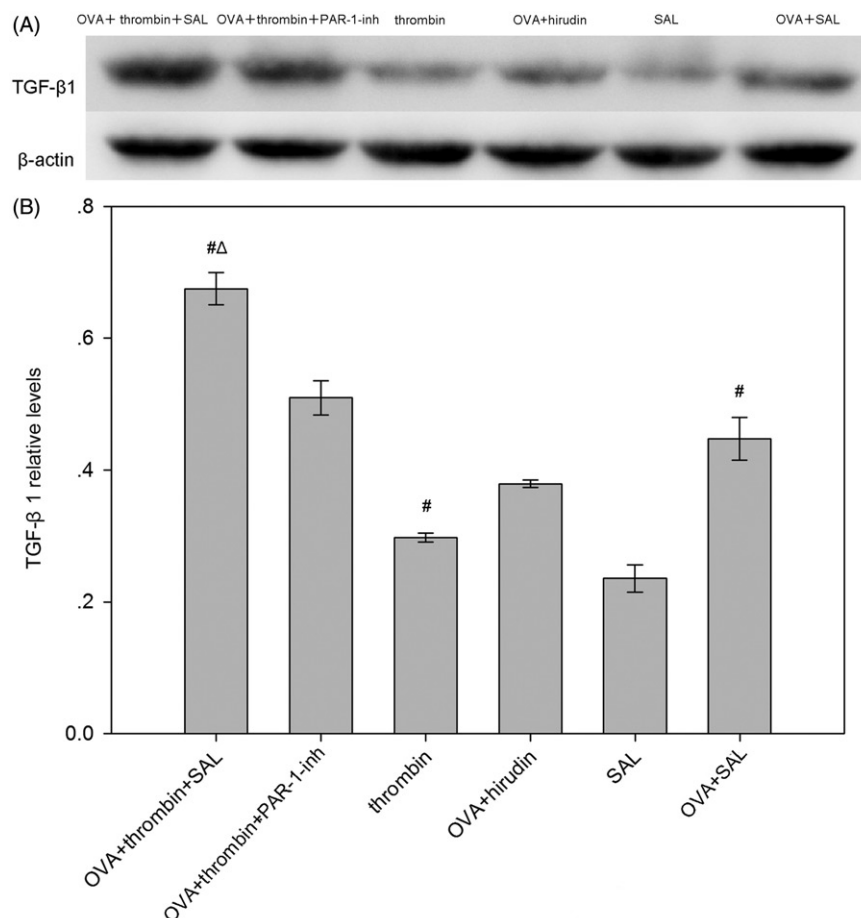


Figure 5. Thrombin induces the expression of PAR-1. Total RNA was isolated and assessed in triplicate for the expression of PAR-1 by means of real-time PCR. Expression levels were normalized to the housekeeping gene β -actin and calculated as a mean level of induction in comparison with lung tissue from control untreated rats. Results show mean $\pm$ SE of three separate experiments, each in triplicate (# $p<0.05$ compared with SAL group, $\Delta p<0.05$ compared with the OVA + SAL group and $\Delta p<0.05$ compared with the OVA + hirudin group).

Discussion

The prime function of the coagulation cascade is to generate a stable, insoluble, cross-linked fibrin blood clot to promote hemostasis at the vascular injury sites. The formation of the provisional clot is generated after the stepwise activation of coagulation proteases via two highly regulated, independent

pathways: the extrinsic and intrinsic systems. The two pathways merge to trigger a set of reactions in the common pathway. Factor Xa binds to a cell-surface receptor called effector protease receptor-1 and forms the prothrombinase complex, which is responsible for the conversion of prothrombin into thrombin. Thrombin cleaves soluble fibrinogen into insoluble fibrin and also activates Factor XIII which cross-links the fibrin clot and also promotes platelet aggregation, resulting in the formation of stable haemostatic plugs.

A number of investigators have shown that activation of the coagulation cascade is a common feature of asthma. Levels of thrombin are increased in BALF from asthmatic patients and in animal models of this disorder. For example, significant activation of thrombin was found in the asthmatic model from previous study (Yuda et al., 2004). Compared with normal individuals, thrombin activity in sputum and BALF of patients with asthma increased (Gabazza et al., 1999; Naureckas et al., 1999). The experimental data indicated that BALF concentration of thrombin in OVA-allergic rats was significantly increased (see Figure 1). Although Kanazawa & Yoshikawa (2007) have reported increase in VEGF level leads to up-regulation of thrombin activity in asthmatic airways, the mechanism of high thrombin activity in the airways is not well understood. Possibly, it is relative to tissue factor. As the main initiator of coagulation activation, tissue factor binds to activated factor VII and activates the extrinsic pathway of coagulation system, leading to the generation of thrombin, which cleaves fibrinogen to fibrin (Gabazza et al., 1999, 2004). In the asthmatic airway,

Figure 6. Effect of thrombin on the expression of PAR-1 protein in the lung tissue of asthmatic rats. The photomicrographs are representative of lungs removed from SAL group (A, $n = 6$), OVA + SAL group (B, $n = 6$), OVA + thrombin group (C, $n = 6$) and OVA + hirudin group (D, $n = 6$). Expression of PAR-1 in lung tissue was determined by immunohistochemical staining. Positive staining is depicted in brown. Positive staining showed PAR-1 expression in the epithelium, macrophage leukocyte and smooth muscle. Administration of thrombin to rats challenged with ovalbumin markedly increased the immunostaining area of PAR-1 (C). Administration of hirudin to rats challenged with ovalbumin markedly reduced the immunostaining area of PAR-1 (D) (magnification $\times 400$). Bar = 25 μ m.

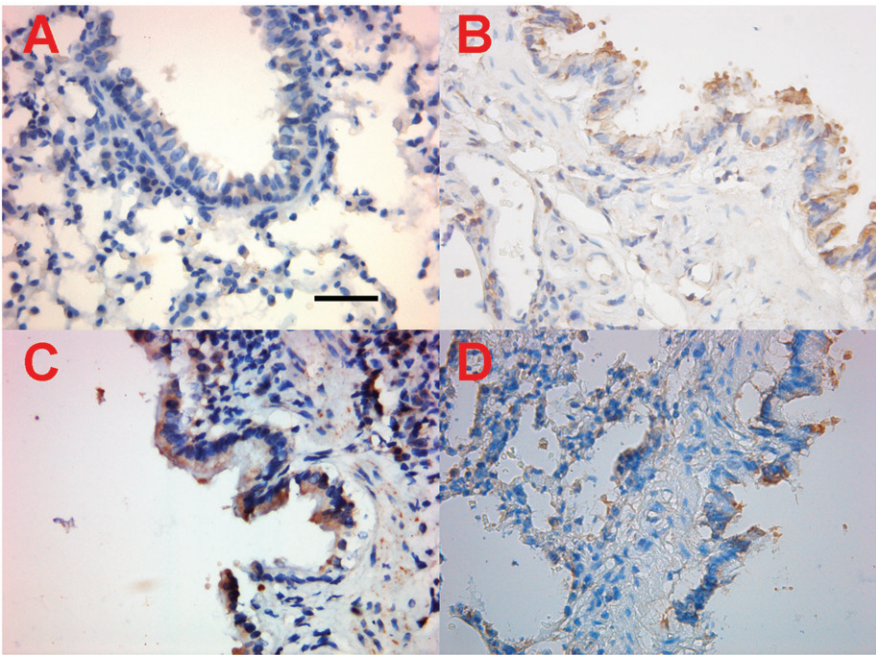
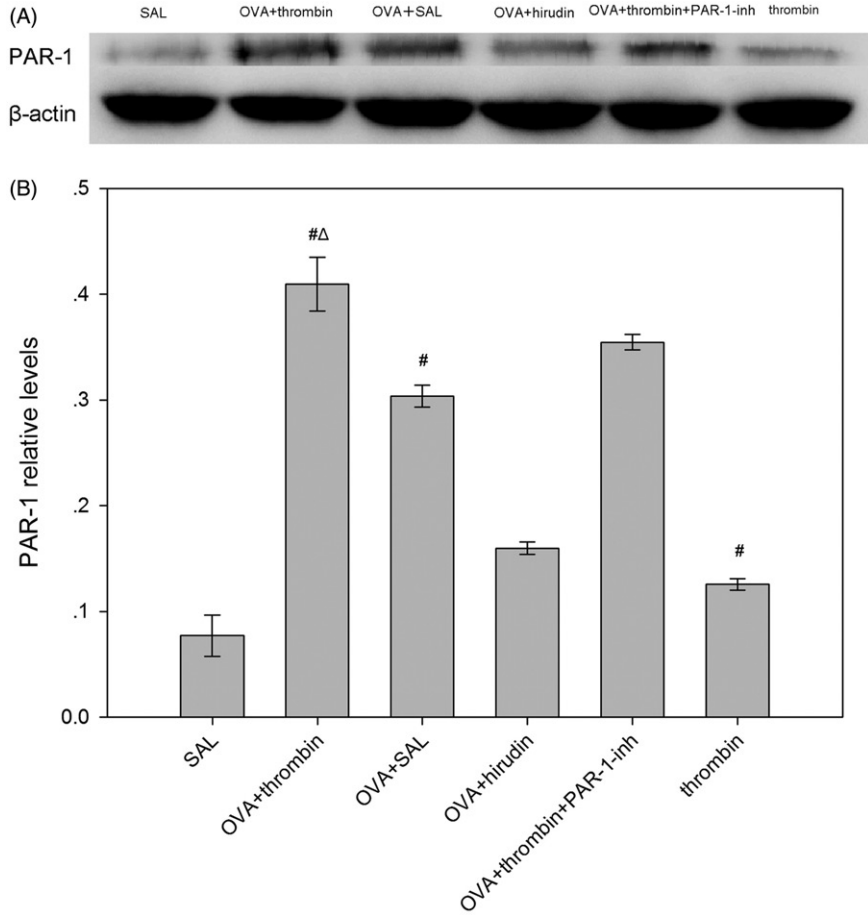


Figure 7. Western blotting result of PAR-1 (A). Quantification of the band intensity (B) showed that activated PAR-1 level was increased in thrombin-treated, OVA-challenged rats and hirudin treatment significantly reduced the activation of PAR-1. Quantitative densitometry analysis of Western blot analysis for PAR-1. Data represent the mean $\pm$ SEM ($n = 6$) for each group. $\#p < 0.01$ compared with the SAL group, $\Delta p < 0.01$ compared with the OVA + SAL group, $\Delta p < 0.01$ compared with the OVA + hirudin group and $\Delta p < 0.01$ compared with OVA + thrombin + PAR-1-inh group.



increased concentration of inflammatory cytokines can be detected (Kay et al., 2004). Inflammatory cytokines can stimulate a number of cells including monocytes/macrophages, endothelial and epithelial cells which express tissue factors (Kanazawa & Yoshikawa, 2007). Therefore, cytokine-mediated tissue factor induces thrombin generation in the lung during allergic responses.

Research performed over the last 10 years has shown that thrombin mediates a number of cellular responses that may play critical roles in tissue repair responses. Thrombin plays an important role in mediating biological responses, it participates in inflammation and tissue repair responses following injury. Allergic inflammation in bronchial asthma is characterized by activation and airway infiltration of

inflammatory cells. As an important source of inflammatory mediators, infiltrating cells contribute to tissue injury (Thorburn & Hansbro, 2010). Airway remodeling is a manifestation of repair of tissue injury, and experimental animal models of asthma have also shown that thrombin is associated with tissue remodeling following injury (Yuda et al., 2004; Ma et al., 2009; Shinagawa et al., 2007; Shimizu et al., 2008). Moreover, previous observations of Gabazza and Naureckas and their colleagues have shown that sputum (Gabazza et al., 1999) and BALF (Naureckas et al., 1999) obtained from patients with asthma induced proliferation of ASM cells. Terada showed that the fibroblast mitogenic activity of BALF obtained from asthmatic patients was inhibited by the thrombin-specific inhibitor hirudin (Terada et al., 2004).

Airway remodeling in patients and animal models with asthma involves a wide array of pathophysiologic features, including shedding of the epithelium, increased smooth muscle mass, collagen deposition, goblet cells hyperplasia, etc. These pathophysiologic features were shown in our results, as shown in Figure 2. This result further supports the role of thrombin in airway remodeling in asthma and airway hyper-responsiveness to non-specific and allergen-specific triggers.

TGF- β 1 is a factor involved in tissue regeneration, remodeling and fibrosis. A large number of studies were carried out on the relationship between TGF- β 1 and airway remodeling. These studies demonstrated that TGF- β 1 is an important cytokine in airway remodeling. It can stimulate the production of different cytokines involved in airway remodeling (Murphy et al., 2008), induce goblet cell hyperplasia and proliferation of fibroblasts (Makinde et al., 2007). Cell research showed that TGF- β 1 could enhance ASM cell proliferation (Okona-Mensah et al., 1998; Black et al., 1996) and mediate connective tissue growth factor, elastin and fibronectin mRNA expression (Kucich et al., 1998, 2001). TGF- β 1 signaling transduction pathways include the activation of TGF- β 1 type I and II receptors, and the subsequent phosphorylation and translocation of the intracellular effectors Smad2 and Smad3 to the nucleus where they regulate gene transcription. In the airway of a mouse model of asthma, these signaling pathways have been demonstrated (Bartram & Speer, 2004; Chen et al., 2011; Yamaguchi et al., 2008). Triptolide could inhibit asthma airway wall remodeling, and the role of triptolide is achieved through mechanisms involving a decrease in the production of TGF- β 1 mRNA and TGF- β 1 as well as modulation of active TGF- β 1 signaling in the lung (Chen et al., 2011). TGF- β 1 signaling can possibly be a targeting therapy for asthma airway remodeling.

Therefore, TGF- β 1 plays an important role in promoting the structural changes of airway remodeling in asthma. Thrombin activity in BALF from patients with asthma correlated with TGF- β 1 (Terada et al., 2004). Our result demonstrated that thrombin could regulate the expression of TGF- β 1. This may be a manner for thrombin to participate in airway remodeling in asthma.

Accumulating evidences indicate that PARs are involved in respiratory disorders. Thrombin is considered to be a major activator of PAR-1, PAR-3 and PAR-4. PAR-1 is a G-coupled membrane protein, which is involved in physiological

and malignant invasion processes. Previous studies on the relationship of thrombin and PAR-1 have mainly focused on coagulation system (Ossovskaya & Bunnett, 2004) and have not resolved the effect in airways. In addition, no study has examined the effect of thrombin on PAR-1 expression. Studies have demonstrated that there is staining for immunoreactive PAR-1 in ciliated and basal epithelial cells and in smooth muscle cells of rat trachea and bronchi (Chow et al., 2000; Howell et al., 2002). Treatment of peritoneal mast cells with PAR-activating peptides up-regulated the expression of PAR-1 mRNA (Stenton et al., 2002). Our data confirm previous studies of the expression of PAR-1 in airway. Moreover, the gene and protein expression of PAR-1 is increased in asthmatic airway and thrombin could up-regulate PAR-1 expression. Thrombin inhibitor hirudin prevents the effect of thrombin on PAR-1 expression. The identification of PAR-1 expression in asthmatic airway brought questions about the role of PAR-1 in asthma.

PAR-1 has been shown to be the major receptor involved in mediating thrombin's mitogenic, pro-fibrotic and pro-inflammatory effects *in vitro* (Cunningham et al., 2000). Through PAR-1, thrombin may stimulate the migration and activation of monocytes and lymphocytes and the secretion of inflammatory mediators from several cell types (Coughlin, 2000). Thrombin may also stimulate contraction of human bronchial rings through PARs (Hauck et al., 1999). Hauck and coworkers demonstrated that cultured human ASM cells express a family of PARs that may mediate the effects of thrombin on cytosolic calcium mobilization, contraction and cell proliferation in human ASM cells (Bachhuber et al., 1997). Indeed, there is emerging evidence that thrombin contributes to airway fibrosis *in vivo*, and thrombin-mediated PAR-1 activation may play a key role in the fibrotic response (Howell et al., 2002). But no study has attempted to study *in vivo* the subsequent effect after reaction between thrombin and PAR-1 in asthma.

In this study, thrombin could induce the expression of TGF- β 1, but when PAR-1 inhibitor was given after thrombin inhalation, the expression of TGF- β 1 was decreased. Thus, the role of thrombin on up-regulation of TGF- β 1 is PAR-1 dependent. Previous *in vitro* studies have shown that PAR-1 agonists stimulate the release of TGF- β in vascular smooth muscle cells, kidney mesangial and epithelial cells and lung epithelial cells (Howell et al., 2005; Shirato et al., 2003; Tsunoda et al., 2001; Yamabe et al., 1997). Now, we use animal model to demonstrate this observation in the airway of asthma. This is a good evidence to show that the effect of thrombin on airway remodeling is mediated, at least in part, via promoting production and release of TGF- β 1 by the reaction with PAR-1. Thus, inappropriate thrombin generation in the airways stimulate cytokine after interaction with PAR-1 to promote airway remodeling in asthma. In our study, a PAR-1 inhibitor decreased the effect of thrombin on airway remodeling, indicating that thrombin promotes airway, which means that thrombin promote airway remodeling through PAR-1.

Conclusions

In this paper, we report that the coagulation cascade protease thrombin is a potent inducer of TGF- β 1 expression in the

airway of OVA-allergic rat and further confirms that this effect is mediated via activation of PAR-1. Previous studies have shown the role of thrombin and PAR-1 in hemostasis, fibrosis and smooth muscle cell proliferation. Most of these studies were based on cell experiments. Based on the animal research, we demonstrate that thrombin induces TGF- β 1 to promote airway remodeling in OVA-allergic rat through reaction with PAR-1. This observation supports a role for thrombin as a target in the treatment of asthmatic airway remodeling.

Declaration of interest

The authors report no declarations of interest.

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