TIPE2 is negatively correlated with tissue factor and thrombospondin-1 expression in patients with bronchial asthma

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Abstract. The interaction between inflammatory processes and a hypercoagulant state may aggravate the severity of asthma and stimulate the airway remodeling of asthma. The aim of the current study was to evaluate the association between the negative inflammatory regulator tumor necrosis factor α induced protein-8 like-2 (TIPE2) and the coagulating substances tissue factor (TF) and thrombospondin-1 (TSP-1) in patients with bronchial asthma. Compared with healthy controls, TIPE2 expression was significantly downregulated, whereas TF expression was upregulated in the peripheral blood mononuclear cells (PBMCs) of patients with bronchial asthma. In addition, levels of TF and TSP-1 in the sera were up-regulated in patients with asthma compared with healthy controls. TIPE2 expression was negatively correlated with TF in the PBMCs and sera and was negatively correlated with TSP-1 levels in the sera of patients with bronchial asthma. The results of the current study indicated that anti-inflammatory TIPE2 levels are associated with levels of the coagulation substances TF and TSP-1. However, further studies are required to determine whether TIPE2 participates in the pathogenesis of asthma by interacting with the coagulation substances TF and TSP-1.

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Abbreviations: TIPE2, tumor necrosis factor α induced protein-8 like-2; TF, tissue factor; TSP-1, thromobospondin-1; PBMCs, peripheral blood mononuclear cells; IgE, immunoglobulin E; IL, interleukin; TBST, Tris-buffered saline tween; IgG, immunoglobulin G; ELISA, Enzyme-linked immunosorbent assay; SLE, systemic lupus erythematosus

Key words: asthma, tumor necrosis factor α induced protein-8 like-2, tissue factor, thrombospondin-1

Introduction

Bronchial asthma is a chronic inflammatory disease of the airways affecting millions of people worldwide; there are currently ~300,000,000 patients with asthma (1,2). It has been demonstrated in a rat model of asthma that the interaction between inflammation and hypercoagulable state aggravates the severity of asthma and contributes to airway remodeling (3). In humans, lung inflammation in asthma is accompanied by a pulmonary procoagulant and antifibrinolytic environment (4). A number of cells, including eosinophilia, mastocytes, T lymphocytes, neutrophils and airway epithelium cells contribute to the development of chronic airway inflammation in asthma (2). Levels of coagulation parameters, platelets, fibrinogen, tissue factor, thromboxane A2, thrombin and activated protein C may reflect thrombosis formation during the pathophysiological process of asthma (5,6). Platelets activated by the up-regulation of cluster of differentiation 154 may release the platelet δ, α and λ granules, induce pulmonary inflammation and enhance the T helper cell 2 immune response, thus aggravating asthma severity (7). The extracellular signal-regulated kinase 1/2 signaling pathway serves an important role in the process of thrombus-promoting airway remodeling in ovalbumin allergic rats (8). This suggests that coagulation is closely associated with inflammation in asthma.

Tissue factors (TF) are a key connector of the coagulation and inflammation network and participate in thrombosis formation through the extrinsic pathway of blood coagulation. They may also contribute to the inflammation and remolding of the asthma airway (9,10). Mononuclear cells are the key cell type to express TF and are the primary source of blood-borne TF *in vitro and in vivo* (11). TF released by endothelial cells serves a major role in the initial stage, whereas blood-borne TF serves a key role in the amplification stage of thrombosis (11).

Thromobospondin-1 (TSP-1) is a mucoprotein involved in the formation of thrombosis (12). It is secreted by platelets, macrophages, mononuclear cells, vascular muscle cells, fibroblasts and endothelial cells following the onset of inflammation (13). Platelet activation is an important determinant of the severity of allergic asthma and TSP-1 is a marker of platelet activation that represents a higher level in severe asthma compared with non-severe asthma (14). In addition, TSP-1 may induce chemotaxis of the macrophagocytes and induce a pro-inflammatory

response (15). However, it remains unknown whether TIPE2 levels are correlated with TF or TSP-1 levels in asthma.

Tumor necrosis factor-α-induced protein-8 like-2 (TIPE2) is a gene that was initially identified in a mouse model of autoimmune encephalomyelitis (16). As a type of tumor necrosis factor-α-induced protein-8, TIPE2 is primarily expressed by the myeloid and lymphoid immune cells, particularly by T lymphocytes, mononuclear cells and macrophages (17,18). TIPE2 is a negative regulator of inflammation in certain diseases including chronic hepatitis B (19), chronic hepatitis C (20), systemic lupus erythematosus (SLE) (21), diabetic nephropathy (22) and abdominal aortic aneurysm (23), suggesting that it serves an important role in the pathogenesis of inflammatory diseases.

It has been demonstrated that TIPE2 is down-regulated in the peripheral blood mononuclear cells (PBMCs) of children with bronchial asthma and that TIPE2 expression is negatively correlated with immunoglobulin (Ig)E, interleukin (IL)-4 and eosinophil counts (24). However, the association between the inflammatory regulator TIPE2 and the coagulation substances TF and TSP-1 in asthma remains unclear. In the current study, the relative expression of TIPE2 and TF in PBMCs, as well as the levels of TF and TSP-1 in the sera, were measured in patients with bronchial asthma and healthy controls. The association between TIPE2, and TF and TSP-1 in patients with asthma was subsequently analyzed.

Patients and methods

Patients. A total of 65 patients (male:female, 35:30) aged 38-88 years, diagnosed with acute asthma were recruited from the First Affiliated Hospital of Zhengzhou University (Henan, China) between November 2015 and May 2016. Asthma diagnoses were based on the criteria established by Global Initiative for asthma (25). None of the patients had other diseases, including pulmonary embolism, chronic bronchitis, pulmonary tuberculosis, chronic obstructive pulmonary disease and hematological tumors. Furthermore, patients had not received oral corticosteroids and did not experience any upper respiratory infections for 2 months prior to enrollment. A total of 40 healthy individuals (male:female, 20:20) aged 34-85 years old were also recruited from the First Affiliated Hospital of Zhengzhou University (Henan, China). There were no statistical differences in sex and age between the two groups. All participants gave their written informed consent for inclusion in the current study and ethical approval was granted by the local Ethics Committee of the First Affiliated Hospital of Zhengzhou University.

Western blotting. Levels of TIPE2 and TF in the PBMCs were assessed as previously described (24). Briefly, PBMCs were respectively separated from 1 ml EDTA-K2 anticoagulation peripheral blood from 65 asthmatic patients and 40 healthy controls using density gradient centrifugation at 600 x g for 30 min at 25˚C with Human lymphocyte separation fluid (Dakewe Biotech Co., Ltd, Shenzhen, China) according to the manufacturer's instructions. Proteins of PBMCs were extracted using a western and IP lysate solution (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentration was determined using a Bradford kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (10 μg) were separated on 12% SDS-polyacrylamide gel and transferred onto 0.22 μm PVDF membranes (Merck KGaA, Darmstadt, Germany) by electrotransfer. Following blocking with 5% skimmed powder milk in TBST for 2 h at room temperature, the PVDF membrane was incubated overnight at 4˚C with a 1:500 dilution of rabbit anti-human TF monoclonal antibodies (cat no. AB151748; Abcam, Cambridge, UK) or 1:1,000 dilution of mouse anti-human TIPE2 polyclonal antibodies (cat no. 15940-1-AP; ProteinTech Group, Inc., Chicago, IL, USA). A 1:1,000 dilution of mouse anti-human β-actin monoclonal antibodies (cat no. 66,009-1-Ig; ProteinTech Group, Inc.) was used as internal reference. The membrane was then washed four times with TBST and incubated with a 1:1,000 dilution of goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies, respectively, for 1 h at room temperature. Following four washes with TBST, the membrane was visualized using an ECL western blot detection kit (Beyotime Institute of Biotechnology). Images were analyzed using a Gel analyzing Program analyzer version 4.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Enzyme-linked immunosorbent assay (ELISA). Sera were separated from the peripheral blood of asthmatic patients and healthy controls at 1,000 x g for 5 min at 25˚C. To assess levels of TF and TSP-1 in the sera of 65 patients with asthma and 40 healthy controls, sandwich ELISA kits (cat nos. E-EL-H0040c and E-EL-H1589c; Elabscience Biotechnology, Houston, TX, USA) were used according to the manufacturers' protocols. Each sera sample was diluted with sample dilution buffer from the kit and then added to plates pre-coated with the TF or TSP-1 antibodies, which were also part of the kit. Plates were incubated for 90 min at 37˚C. Following three washes, 1:100 diluted biotinylated TF or TSP-1 detection antibodies were added to the plates and incubated at 37˚C for 60 min. Plates were washed a further three times, then horseradish peroxidase-conjugated streptavidin was added and incubated at 37˚C for 30 min. Subsequently, etramethylbenzidine substrate solution was added to the plates. Following 10 min incubation in the dark at room temperature, the reaction was halted by the addition of the stop solution and measured at 450 nm. The concentration of samples was calculated according to the standard curve, which was produced by different dilutions of reference standard.

Statistical analysis. When the data were normally distributed, an unpaired student's t-test was used to compare the difference between patients with bronchial asthma and healthy controls. If the data were not normally distributed, the Mann-Whitney U-test was used. Spearman's rank correlation coefficient was used to determine whether there was a correlation between TIPE2 and either TF or TSP-1. All statistical analyses were performed using Graphpad 5.0 software (Graphpad Software, Inc., La Jolla, CA, USA) and $P<0.05$ was considered to indicate a statistically significant difference.

Results

The expression of TIPE2 and TF proteins in PBMCs from patients with bronchial asthma compared with healthy controls. Relative protein levels of TIPE2 and TF in PBMCs

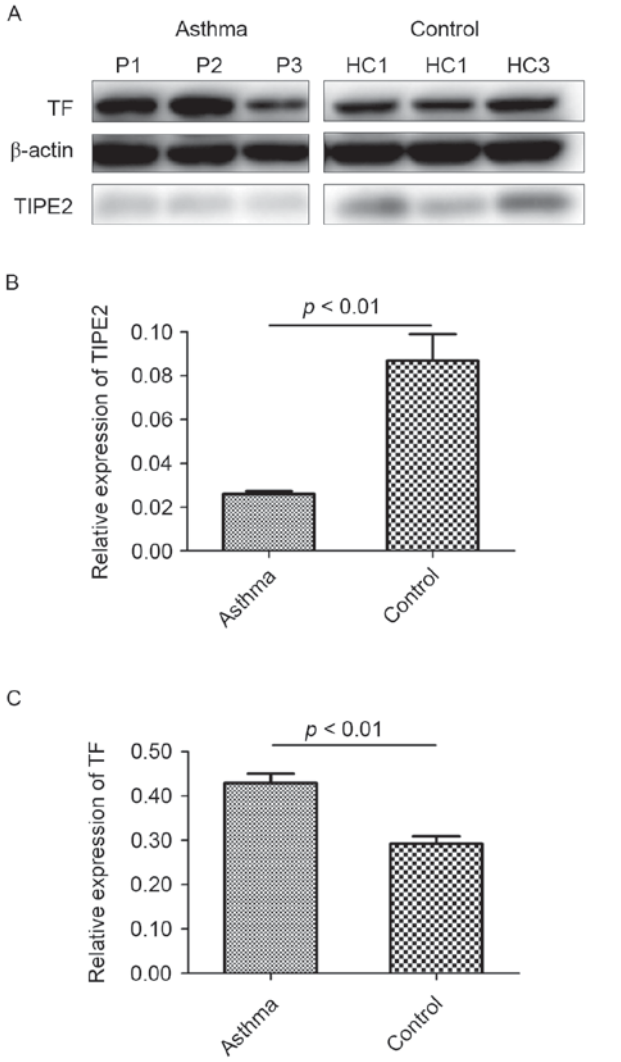

Figure 1. Levels of TIPE2 and TF in the PBMCs of patients with asthma compared with healthy controls. The expression of TIPE2 and TF in the PBMCs of 65 patients with asthma and 40 healthy controls were determined by western blotting. β-actin was used as an internal reference to ensure that equal amounts of protein were loaded in all lanes. (A) Expression of TIPE2 and TF in asthmatic patients and healthy controls. (B) Relative expression of TIPE2 in PBMCs of asthmatic patients (0.026±0.001) and healthy controls (0.087±0.012). (C) Relative expression of TF in PBMCs of asthmatic patients (0.429±0.021) and healthy controls (0.292±0.016). Data are presented as the mean ± standard error of the mean. TIPE2, tumor necrosis factor α induced protein-8 like-2; TF, tissue factor; PBMCs, peripheral blood mononuclear cells; P, patient; HC, healthy control.

from patients with bronchial asthma and healthy controls were detected and β-actin was used as an internal reference (Fig. 1A). The results demonstrated that TIPE2 expression in PBMCs is significantly lower in patients with asthma compared with healthy controls (P<0.01; Fig. 1B). However, TF expression in PBMCs was significantly higher in patients with asthma compared with healthy controls (P<0.01; Fig. 1C). Furthermore, it was determined that there was a negative correlation between the expression of TIPE2 and TF in the PBMCs of patients with asthma (r=-0.3828, P<0.01; Fig. 2).

TF and TSP-1 levels in the sera of patients with bronchial asthma compared with healthy controls. Bronchial asthma is a chronic inflammatory disease of the airways and previous

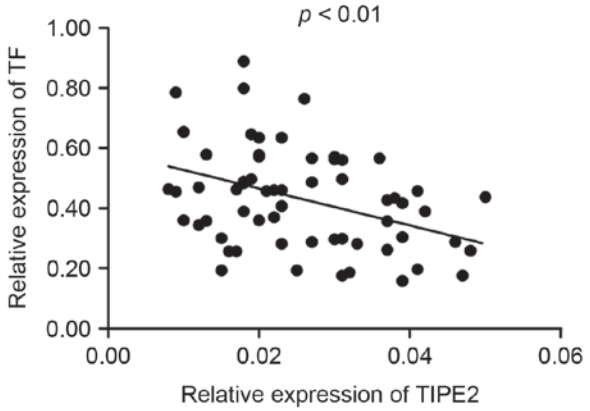

Figure 2. Correlation between TIPE2 and TF expression in the peripheral blood mononuclear cells of patients with asthma. There was a significantly negative correlation between TIPE2 and TF expression in patients with asthma (r=-0.3828, P<0.01). TIPE2, tumor necrosis factor α induced protein-8 like-2; TF, tissue factor.

studies have determined that TF and TSP-1 serve important roles in the inflammatory process (10,15). Therefore, in the current study, the levels of TF and TSP-1 in the sera of patients with bronchial asthma and healthy controls were measured using sandwich ELISA. The results demonstrated that TF levels in the sera of patients with bronchial asthma were significantly higher than those of healthy individuals (P<0.01; Fig. 3A). Furthermore, TSP-1 levels in the sera of patients with bronchial asthma were significantly higher than those of healthy controls (P<0.01; Fig. 3B).

Correlation of TIPE2 expression with TF and TSP-1. To further investigate the mechanism of TIPE2 in patients with bronchial asthma, the correlation between TIPE2 expression and sera TF and TSP-1 levels was determined. The relative expression of TIPE2 was negatively correlated with TF levels in the sera of patients with asthma (r=-0.5422, P<0.01; Fig. 4A). Furthermore, the relative expression of TIPE2 in the PBMCs was negatively correlated with TSP-1 levels in the sera of patients with asthma (r=-0.3013, P<0.05; Fig. 4B).

Discussion

Asthma is chronic inflammatory disease of the airways (2). It has been demonstrated that patients with bronchial asthma also develop hyper-coagulation (4). However, the mechanism between inflammation and coagulation in asthma is not fully understood. Therefore, the current study investigated the association between the negative inflammatory regulator TIPE2 and the coagulation substances TF and TSP-1 to provide innovative insights into the pathological mechanism and clinical diagnosis of asthma, as well as identify a potential novel method of treating asthma.

In the present study, the relative expression of TIPE2 and TF in the PBMCs of patients with bronchial asthma were measured and compared with those of healthy controls. In addition, levels of TF and TSP-1 in sera were assessed. It was determined that the expression of TIPE2 in the PBMCs of patients with bronchial asthma was significantly down regulated (Fig. 1B), which was consistent with the results of a study

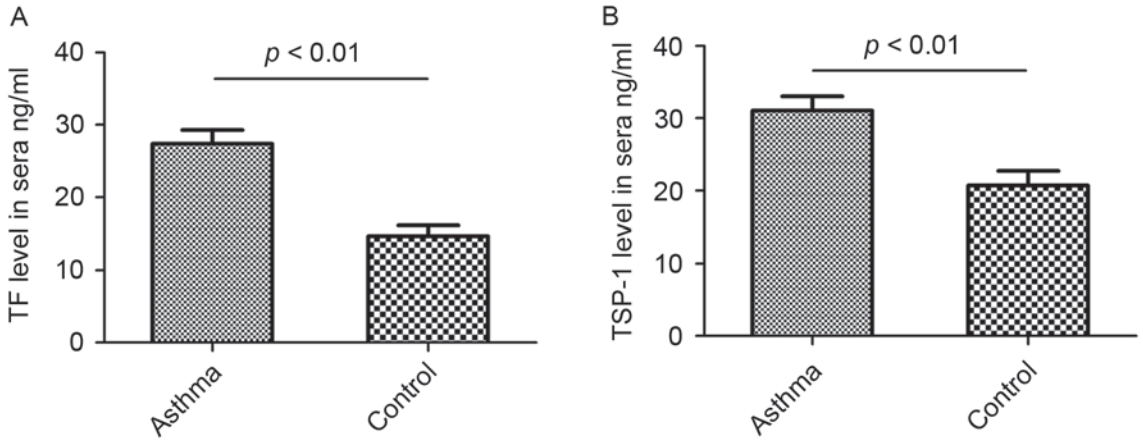

Figure 3. Sera TF and TSP-1 levels in the 65 patients with asthma and 40 healthy controls. (A) Sera TF levels in patients with asthma (27.385±1.898 ng/ml) were significantly higher than in healthy controls (14.695±1.415 ng/ml) (P<0.01). (B) Sera TSP-1 levels in patients with asthma (31.064±1.972 ng/ml) were significantly higher than in healthy controls (20.719±1.994 ng/ml) (P<0.01). Data are presented as the mean ± standard error of the mean. TIPE2, tumor necrosis factor α induced protein-8 like-2; TF, tissue factor.

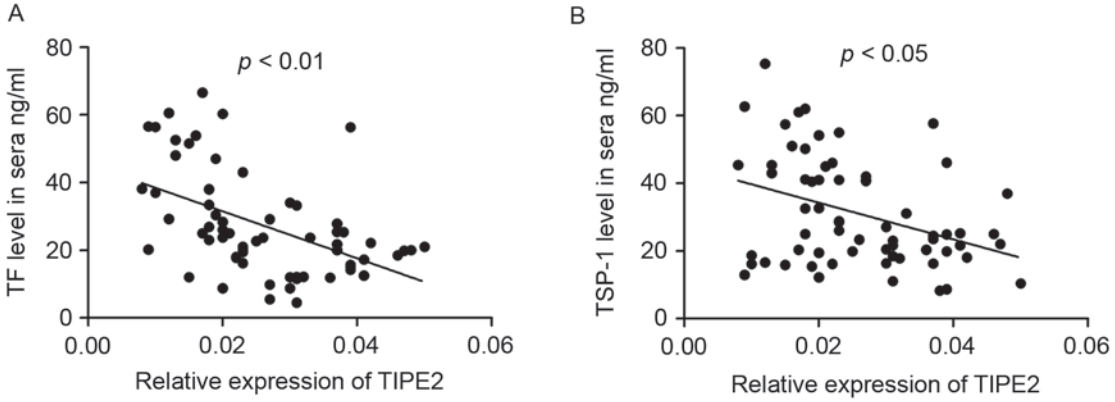

Figure 4. Correlations between PBMC TIPE2 expression and sera TF and TSP-1 levels in patients with asthma. (A) There was a significantly negative correlation between TIPE2 and TF expression in the sera (r=-0.5422, P<0.01). (B) There was a significantly negative correlation between PBMC TIPE2 expression and sera TSP-1 levels in patients with asthma (r=-0.3013, P<0.05). TIPE2, tumor necrosis factor α induced protein-8 like-2; TF, tissue factor; PBMCs, peripheral blood mononuclear cells.

by Ma *et al* (24). By contrast, levels of TF were significantly up regulated in the PBMCs of patients with bronchial asthma compared with healthy controls (Fig. 1C). Furthermore, significantly higher levels of circulating TF and TSP-1 were detected in the sera samples of patients with asthma compared with healthy controls (Fig. 3). A negative correlation between TIPE2 and TF in the PBMCs and sera was identified in patients with bronchial asthma (Figs. 2 and 4A) and there was a negative correlation between TIPE2 and levels of TSP-1 in the sera of patients with bronchial asthma (Fig. 4B). These results suggest that there is a link between the negative inflammatory regulator TIPE2 and activation of the coagulation cascade in the peripheral blood circulation.

Previous studies have indicated that TIPE2 serves an important role in the pathogenesis of inflammatory diseases including chronic hepatitis B (19), chronic hepatitis C (20) and SLE (21). TIPE2 may alleviate SLE by inducing macrophage polarization to an M2 phenotype and it has been demonstrated that TIPE2 overexpression significantly decreases the severity of SLE (21). In addition, TIPE2 may inhibit the synthesis of inducible nitric oxide, which inhibits inflammation (26). However, silencing of TIPE2 expression may counteract the reduced inflammation and myocardial injury in NOD2-/-ischemic mice (27). Furthermore, down-regulation of TIPE2 expression may increase the expression of pro-inflammatory factors including IL-1, IL-10, IL-12 and tumor necrosis factor-α, which in turn induce liver inflammation and the progression of liver disease in TIPE2$^{-/-}$ mice (16). In the present study, it was demonstrated that the expression of TIPE2 was reduced in the PBMCs of patients with asthma. This was in accordance with the results of a previous study, which identified the down-regulation of TIPE2 in PBMCs of patients with asthma (24). The results of the present demonstrated that there was a significant correlation between TIPE2 expression in PBMCs and TF expression in the PBMCs and sera, as well as with TSP-1 in sera samples from patients with asthma.

As a key connector of the coagulation and inflammation network, TF may contribute to the inflammation and remolding of asthmatic airways, as well as participate in the formulation of thrombosis via the extrinsic blood coagulation pathway (3,9,10). The serum pro-inflammatory cytokine IL-33, which is increased in asthmatic patients, amplifies the

coagulation function of human endothelial cells by increasing the production and activity of TF, which increases disease severity (28). The data collected in the current study indicated that the expression of TF was unregulated in the PBMCs and sera of patients with asthma. Furthermore, the results of the current study indicated that the expression of the negative inflammatory regulator TIPE2 was negatively correlated with TF. These results suggest that TIPE2 may participate in the pathogenesis of asthma by regulating the expression of TF.

TSP-1, which is known for its antiangiogenic function (29), has been extensively studied in cancer (30) and wound healing (31). However, TSP-1 is a multifunctional protein and serves a significant role in inflammation. It has been demonstrated that TSP-1 contributes to the development of vascular inflammation by regulating the cell motility of monocytes in a mouse model of abdominal aortic aneurysm (23). Furthermore, TSP-1 efficiently down-regulates TF-induced coagulation by binding to TFPI and locating to surfaces within the extravascular space following vascular injury (32). Platelet activation is an important determinant of the severity of allergic asthma. Levels of TSP-1, a marker of platelet activation, are generally higher in patients with severe asthma than those with less severe asthma (14). The results of the current study identified high levels of TSP-1 in the sera samples of patients with asthma compared with healthy controls. Furthermore, a negative correlation between TSP-1 and TIPE2 was identified in patients with asthma. Thus, TIPE2 may act via TSP-1 to regulate inflammation and coagulation in asthma. However, further studies are required to determine the specific mechanism by which TIPE2 regulates the pathogenesis of asthma through the coagulation substances TSP-1 and TF.

In conclusion, the results of the current study indicated that patients with asthma exhibit reduced TIPE2 expression in the PBMCs but higher levels of TF and TSP-1 in the sera compared with healthy controls. Furthermore, TIPE2 expression is negatively correlated with TF expression in PBMCs and sera, and negatively correlated with TSP-1 levels in the sera of patients with bronchial asthma. TIPE2 may participate in the pathological mechanism of asthma by interacting with the coagulating substances TF and TSP-1. However, the specific mechanism by which TIPE2 regulates the coagulation substances TF and TSP-1 remains unknown and requires further investigation. This may facilitate the development of novel methods to diagnose and treat bronchial asthma.

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