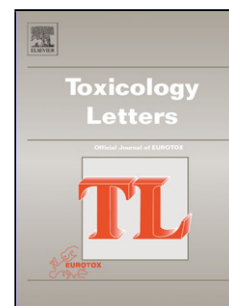


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RAGE mediates β -catenin stabilization via activation of the Src/p-Cav-1 axis in a chemical-induced asthma model

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Highlights

- RAGE plays an important role in TDI-induced airway inflammation and regulation of β -catenin signaling.
- Blockade of RAGE suppressed activation of Src/p-Cav-1 axis in TDI-induced asthma.
- Src/p-Cav-1 axis modulates β -catenin signaling via promoting the translocation of β -catenin from cytomembrane to cytoplasm.

Abstract

We previously demonstrated receptor for advanced glycation end products (RAGE) was required for β -catenin stabilization in a toluene diisocyanate (TDI)-induced asthma model, suggesting it plays an important role in TDI-induced airway inflammation. The aim of this study was to examine whether RAGE mediates β -catenin stabilization via activation of the Src/p-Cav-1 axis in TDI-induced asthma model. To generate a chemical-induced asthma model, male BALB/c mice were sensitized and challenged with TDI. Before each challenge, FPS-ZM1 (RAGE inhibitor) and PP2 (Src inhibitor) was given via intraperitoneal injection. In the TDI-exposed mice, airway reactivity, airway inflammation, goblet cell metaplasia, and the release of Th2 cytokines and IgE increased significantly. The level of membrane β -catenin decreased but was increased in the cytoplasm. Increased expression of RAGE, p-Src, and p-Cav-1 was also detected in TDI-exposed lungs. However, all these changes were inhibited by FPS-ZM1 and PP2. In TDI-HSA stimulated human airway epithelial (16HBE) cells, the expression of p-Src and p-Cav-1, and the abnormal distribution of β -catenin were significantly increased, and then inhibited in RAGE knockdown cells. Similarly, PP2 or non-phosphorylatable Cav-1 mutant (Y14F-Cav-1) treated 16HBE cells had the same effect on the

distribution of β -catenin. In addition, blockage of RAGE signaling and phosphorylation of Cav-1 eliminated the translocation of β -catenin from cytomembrane to cytoplasm. Our results showed that RAGE modulates β -catenin aberrant distribution via activation of Src/p-Cav-1 in a chemical-induced asthma model.

Keywords: asthma, toluene diisocyanate, β -catenin, RAGE, Src/p-Cav-1 axis

Introduction

Receptor for advanced glycation end products (RAGE) is a member of the immunoglobulin superfamily of cell surface receptors. It has emerged as a key pattern recognition receptor involved in the host response to tissue injury, infection, and inflammation (Oczypok et al., 2017). Many studies have identified RAGE and its ligands are associated with allergic airway sensitization and airway inflammation in asthma (Ullah et al., 2014; Kang et al., 2015). This is important in the early innate and adaptive immune responses to allergens (Ullah et al., 2014; Akirav et al., 2014). Two genome-wide association studies have suggested that RAGE is important in asthma pathogenesis in humans (Repapi et al., 2010; Hancock et al., 2010). In a house dust mite (HDM) extractiva and ovalbumin (OVA) asthma model, RAGE knockout (KO) mice were protected against airway eosinophilia, goblet cell hyperplasia, and impaired pulmonary function after methacholine challenge. In addition, RAGE has been shown to be important for the production of IL-5 and IL-13 via accumulation of group 2 innate lymphoid cells (ILC2s) in the lung (Milutinovic et al., 2012; Oczypok et al., 2015). Using a TDI-induced asthma model, we previously found the upregulation of RAGE and its ligands (HMGB1, S100A12, S100B, HSP70) in the airway (Yao et al., 2016), and that blocking

RAGE signaling alleviated airway inflammation, indicating that RAGE may play an important role in the pathological process of asthma.

β -catenin as an important component of the adherent junction, and its stabilization is a fundamental guarantee of barrier integrity. In addition, β -catenin is also a key nuclear effector of canonical Wnt signaling in the nucleus (Nelson et al., 2004). WNT-dependent activation of β -catenin signaling in asthma has been shown (Cohen et al., 2009; Moheimani et al., 2015). We previously demonstrated that β -catenin is abnormally distributed in the airway epithelium in a TDI-induced murine asthma model (Yao et al., 2015). More interesting, we found that the RAGE specific inhibitor (FPS-ZM1) decreased the abnormal distribution of β -catenin, and also reduced the expression of targeted gene expression (MMP2, MMP9, VEGF) (Yao et al., 2016). Furthermore, we found inhibitors of β -catenin signaling (XAV-939 and ICG-001) reduced the expression of many kinds of inflammatory factors, e.g., VEGF, HMGB1, TGF- β , MMP9 (Yao et al., 2017), suggesting that RAGE might act as a key link to promote lung inflammation through β -catenin signaling in TDI-induced asthma. However, the mechanism by which RAGE mediates β -catenin signaling remains unclear.

Caveolin-1(Cav-1), the major structural and functional protein of caveolae, has been regarded as a potential regulatory protein in the pathogenesis of asthma (Royce et al., 2014). Cav-1 co-localizes with adherent junction proteins E-cadherin, β -catenin, and γ -catenin in MDCK (Madin–Darby canine kidney) epithelial cells (Galbiati et al., 2000). Evidence suggests that the expression of Cav-1 is significantly lower in airway epithelia from patients with asthma, and downregulation of Cav-1 can break stabilization of the β -catenin/E-catenin complex (Hackett et al., 2013). Moreover, it has been confirmed that Src tyrosine kinase-

dependent phosphorylation of Cav-1 at Tyr14 in endothelial cells participates in the abnormal distribution of β -catenin (Sun et al., 2009). In vascular smooth muscle cells, S100B can increase the activation of Src kinase and tyrosine phosphorylation of Cav-1 by means of RAGE signaling (Hackett et al., 2013). Hence, we hypothesized RAGE can regulate β -catenin signaling via the p-src/p-Cav-1 axis in TDI-induced asthma.

Materials and Methods

Reagents

TDI (toluene-2, 4-diisocyanate, $\geq 98.0\%$), methacholine, and acetone were purchased from Sigma-Aldrich (Shanghai, China). The vehicle (AOO) used to dissolve TDI consisted of a mixture of 2 volumes of acetone and 3 volumes of olive oil for dermal sensitization, and 1 volume of acetone and 4 volumes of olive oil for airway challenge. Src tyrosine kinase inhibitor (PP2) and FPS-ZM1 were purchased from Selleck (SelleckChem, Shanghai, China). The ELISA kit for IgE, and kits for detecting IL-4, IL-5, IL-13 were purchased from eBioscience (San Diego, USA).

Animals and *in vivo* treatment with RAGE and Src inhibitors

Specific-pathogen-free male BALB/c mice, 6-8 weeks old, were purchased from Southern Medical University. The mice were housed in a SPF house with a 12 hour dark/light cycle (lighting: 7:00-19:00) at a temperature of $23 \pm 2^\circ\text{C}$ and humidity range of 40-70%. Mice were fed with sterile water and irradiated food ad libitum. All studies were conducted in accordance with the committee of Southern Medical University on the use and care of

animals. The protocols were approved by the Animal Subjects Committee of Nanfang Hospital.

A total of 40 mice were used (n=10 for each group). Mice were randomized to the following groups: (1) AOO-sensitized, AOO-challenged, and DMSO-treated (AOO/AOO group); (2) TDI-sensitized, TDI-challenged, and DMSO-treated (TDI/TDI group); (3) TDI-sensitized, TDI-challenged, and FPS-ZM1-treated (TDI/TDI+FPS-ZM1 group); (4) TDI-sensitized, TDI-challenged, and PP2-treated (TDI/TDI+PP2 group). Animal models were generated as described previously (Yao et al., 2015; Yao et al., 2016; Yao et al., 2017). Briefly, male BALB/c mice were dermally sensitized with 0.3% TDI on the dorsum of both ears (20 μ l per ear) on days 1 and 8. On days 15, 18, and 21 the mice were individually placed in a horizontal cylindrical chamber and challenged through the airway with 3% TDI dissolved in acetone/olive oil (1:4) by means of compressed air nebulization (NE-C28; Omron, Tokyo, Japan) for 3 h at each time. As a control, mice were sensitized and challenged by the same procedures with the same amount of AOO. FPS-ZM1 (1.5 mg/kg, i.p.), PP2 (5mg/kg, i.p.) dissolved in DMSO and diluted with PBS (pH=7.4) were respectively given to the mice immediately before each airway challenge. Sham mice received the same volume of vehicle by comparison.

Assessment of airway hyper-responsiveness (AHR)

Airway reactivity to methacholine was assessed on day 22 according to a previously described method (Liang et al., 2015). Briefly, mice were placed in a barometric plethysmographic chamber (Buxco Electronics, Troy, NY) and challenged with vehicle

(sterile saline), followed by increasing concentrations of methacholine (6.25, 12.5, 25, and 50 mg/ml) by means of ultrasonic nebulization. Aerosols were generated with a matching ultrasonic nebulizer (Aeroneb Laboratory Nebulizer; Buxco Electronics, Inc., Troy, NY), and then nebulized into the main chamber through its inlet for 3 min. Pressure fluctuations caused by breathing of the mice were continuously monitored for 3 min after each nebulization, and subsequently these pressure fluctuations were quantified using the algorithm for enhanced pause (Penh, dimensionless parameter), which represents an accurate index of airway resistance (Lundblad et al., 2002). Penh is a dimensionless value that represents a function of the ratio of peak expiratory flow (PEF) to peak inspiratory flow (PIF), and a function of the timing of expiration (Pause) ($\text{Penh} = \text{PEF}/\text{PIF} \times \text{Pause}$) (Ye et al., 2012).

Quantification of total serum IgE

The mice were sacrificed as described previously (Liang et al., 2015). Blood samples collected were rested for 1 h at room temperature, then centrifuged ($3000 \times g$, 20 min) and supernatants were harvested and stored at -80°C . Total serum IgE was measured by ELISA (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Analysis of bronchoalveolar lavage (BAL) fluids

Recovered BAL fluids were pooled, and the total cells in the BAL fluids were counted, and the BAL fluids were centrifuged ($1000 \times g$, 10 min). The supernatant was frozen (-80°C) for further analysis of IL-4, IL-5, and IL-13 by ELISA (eBioscience) according to the manufacturer's instructions. For differential cell counts, a cytopsin sample (Shandon

Scientific, Runcorn, UK) was prepared and stained using hematoxylin and eosin (H&E) (Solarbio, Beijing, China). For each sample, a total of 200 cells were counted for the numbers of macrophages, eosinophils, neutrophils, and lymphocytes.

Pulmonary histopathological examination

Left lungs were dissected from mice, then fixed in 4% neutral formalin, dehydrated, and paraffin-embedded. Sections (4 μ m) were cut with a Leica microtome 2030 (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Lung tissues were stained with H&E and periodic acid-Schiff (PAS) for blinded histopathological assessment. Lung inflammation and airway goblet cell metaplasia were semiquantified as previously described (Tang et al., 2014). Scoring was performed at a magnification of 200 $\times$ by examining at least 40 image fields of 20 slices from 8 mice per group. Sections were assigned a random code to blind the examiner to the identity of each specimen.

Immunohistochemistry and western blotting

For immunohistochemistry analysis of β -catenin and RAGE, lung sections were deparaffinized, then subject to antigen retrieval. Samples were treated with H₂O₂ for 15 min to block endogenous peroxidase, and then incubated overnight at 4 $^{\circ}$ C in recommended dilutions of anti- β -catenin (Santa Cruz) and anti-RAGE (Abcam) antibodies. After washing with PBS, slices were incubated with a secondary antibody for 20 min at room temperature. Signals were visualized with DAB.

Pulmonary expression of β -catenin, RAGE, Src, p-Src(Tyr418), Cav-1, and p-Cav-1(Y14)

was detected by Western blotting. Whole lung extracts mixed with 5× SDS loading buffer were separated by 10% SDS-polyacrylamide gel and transferred onto PVDF membranes. Membranes were then probed with anti-β-catenin, anti-RAGE, anti-Src, anti-p-Src(Tyr418), anti-Cav-1, and anti-p-Cav-1(Y14) antibodies with the indicated dilutions. After incubation with aIRDye® 680WC-conjugated secondary antibody (LI-COR Biosciences), immunoreactive bands were exposed to an Odyssey® CLx Imager for image capture. Data analysis was done with Odyssey Software. Quantitative image analysis was performed with Image J software.

Preparation of TDI-HSA conjugates

TDI-HSA (both TDI and HSA were from Sigma-Aldrich) conjugates were prepared by a modification of Son's method (Son et al., 1998) previously described (Zhao et al., 2009). The molar ratio of TDI to HSA was 5.73. Each concentration of TDI-HSA used had no detectable effect on cell viability (data on file).

Cell culture and treatment

Human bronchial epithelial cell line 16HBE14o-(16HBE) cells (Bio-Rad Laboratories (Shanghai)Co, Ltd, ATCC, Portland, Oregon) were cultured in RPMI-1640 medium (Hyclone) containing 10% fetal calf serum (Hyclone) at 37°C and 5% CO₂. Medium was changed every other day, and cells passaged to five to eight generations were used for the following experiments. When reaching 90% confluence, the cells were passaged and seeded to proper culture plates at a density of 10⁴–10⁵ cells/cm². After 24 h incubation, the culture

medium was changed to serum free RPMI-1640. Different concentrations of TDI-HSA conjugates (0-100 $\mu\text{g/ml}$) were then added to the culture medium for indicated times with or without pretreatment with FPS-ZM1 (1 $\mu\text{g/ml}$, 1 h) or PP2 (20 μM , 1 h). The concentrations of TDI-HSA used in this experiment were partly based on previous studies (Song et al., 2013). We compared the effects of different concentrations of HSA control reagent (0-100 $\mu\text{g/ml}$) on p-Src and p-Cav-1 protein expression to the blank control. There were no statistical differences (data on file). Hence, we used the blank control as the control group for the following experiments. Non-phosphorylatable caveolin-1 mutant (Y14F-Cav-1) 16HBE cells were generated as described previously (Li et al., 1996). The lentiviral systems for knocking down RAGE were developed by Applied Biological Materials Inc. (Nanjing, China). The expression of p-Cav-1(Y14) and RAGE determined by Western blotting.

Western blotting

To evaluate the protein expression of β -catenin, RAGE, Src, p-Src(Tyr418), Cav-1, and p-Cav-1(Y14) *in vitro*, cells protein extracts were subjected to 10% or 12% SDS-PAGE, transferred to PVDF membranes (Millipore), and then probed with the antibodies anti- β -catenin, anti-RAGE, anti-Src, anti-p-Src(Tyr418), anti-Cav-1, and anti-p-Cav-1(Y14) and then incubated with an IRDye® 680WC-conjugated secondary antibody (LI-COR Biosciences).

Immunoreactive bands were exposed to an Odyssey® CLx Imager for image capture. Data analysis was done with Odyssey Software. Quantitative image analysis was performed with Image J software.

Immunofluorescence microscopy

Immunofluorescence microscopy was used to examine the localization of β -catenin after TDI-HSA stimulation. 16HBE cells (2×10^5 cells/well) were cultured in RPMI-1640 containing 10% (v/v) FBS on Glass Bottom Cell Culture Dishes (NEST, China), and were treated with TDI-HSA (60 μ g/mL) for 6 h at 37 °C with or without FPS-ZM1 (1 μ g/mL, 1 h) or PP2 (20 μ M, 1 h) pretreatment. Then, 16 HBE cells were fixed with 4% (v/v) formaldehyde in PBS for 10 min, treated with 0.5% (v/v) Triton X100 for 5 min, blocked with 5% (w/v) BSA for 2 h at room temperature, and then incubated with mouse monoclonal anti- β -catenin antibody overnight at 4°C. The cells were incubated with Alexa 488-labeled goat anti-rabbit IgG antibody for 1 h in the dark at room temperature. The cells nuclei were stained with 4', 6-Diamidino-2-phenylindole (DAPI) dihydrochloride (Beyotime Biotechnology, China) for 10 min. A laser-scanning confocal microscope (Olympus, Tokyo, Japan) was used to examine the distribution of β -catenin.

Statistical analysis

Statistical analysis was performed using SPSS version 16.0 software. Data were expressed as mean $\pm$ standard error (SE), and comparisons among groups were analyzed by one-way analysis of variance (ANOVA) accompanied by Bonferroni post hoc testing for multiple comparisons. Values of $P < 0.05$ were considered statistically significant.

Results

RAGE inhibition suppressed activation of the Src/p-Cav-1 axis and decreased airway

hyper-reactivity as well as airway inflammation in TDI-induced asthma

As can be seen in Figure 3B, RAGE, p-Src(Tyr418), and p-Cav-1(Y14) were upregulated after TDI exposure. Immunohistochemistry analysis also showed that the airway epithelia exhibited more RAGE immunoreactivity as compared with AOO/AOO after TDI exposure (Fig. 3A), indicating RAGE and the Src/p-Cav-1 axis were activated. As expected, these changes were interrupted by FPS-ZM1 (Fig. 3A, B). At the same time, consistent with previous study, the asthmatic mice had increased airway reactivity (Fig. 1A), and higher serum IgE concentration (Fig. 1B). The numbers of total and differential inflammatory cells were counted in BAL fluid. Higher numbers amounts of neutrophils and eosinophils were found after sensitization and challenge with TDI (Fig. 1E, F), as well as elevated levels of IL-4, IL-5, and IL-13 (Fig. 1C). In addition, numerous neutrophils and eosinophils infiltrating into the airway, pronounced bronchial epithelial hyperplasia, and goblet cell metaplasia was noted (Fig. 2A). Treatment with FPS-ZM1 eliminated these responses, further proving a central role of RAGE in the pathogenesis of TDI-induced asthma.

Both RAGE and Src/p-Cav-1 axis inhibition ameliorated the redistribution of β -catenin in TDI-induced asthma

As expected, β -catenin was present at the lateral side and apicolateral border in the airway epithelium of control mice (Fig. 3A), and significantly decreased at the epithelial cell-cell contact and diffused in the cytoplasm and nucleus of the airway epithelium in TDI group. RAGE inhibition ameliorated the redistribution of β -catenin in TDI-induced asthma. In addition, we found the blockade of Src/p-Cav-1 by PP2 can maintain β -catenin anchoring in the cytomembrane. Of note, PP2 reduced airway hyper-reactivity and inflammation in TDI-

induced asthma.

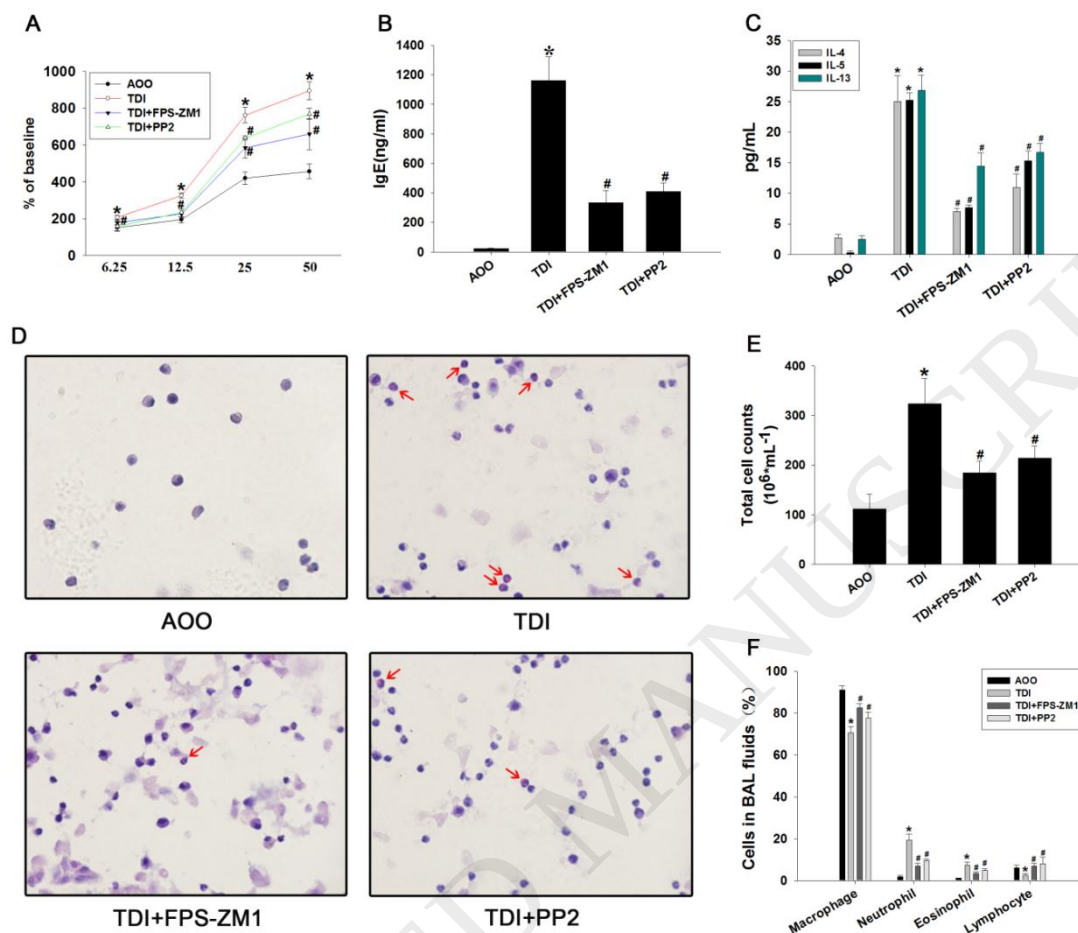


Fig.1. Effects of RAGE inhibitor (FPS-ZM1) and Src inhibitor (PP2) on TDI-induced asthmatic responses. (A) Airway hyper-responsiveness was measured by lung resistance (RL). Results were shown as percentage of baseline value (n=6).

(B) IgE in the serum. n=6. **(C)** IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid (BALF) (n=6). **(E)** Total and differential inflammatory cells **(D, F)** in BALF in the AOO-treated group and TDI-treated group (n=8). Percentages of different inflammatory cells were calculated by counting a total of 200 cells in H&E stained cytospin samples. *P < 0.05 compared with AOO/AOO group; #P < 0.05 compared with TDI/TDI group.

Blockade of RAGE suppressed Src/p-Cav-1 axis activation in TDI-HSA-treated 16HBE cells

Consistent with the animal experiments, the Src/p-Cav-1 axis was activated after stimulation by TDI-HSA in 16HBE cells (Fig. 4A, B). Western blotting showed that Src(Tyr 418) and Cav-1(Y14) phosphorylation were increased, and were diminished by PP2 (Fig. 4C).

Intriguingly, FPS-ZM1 or silence of RAGE also suppressed the phosphorylation of Src and Cav-1 (Fig. 4D, E), revealing that RAGE participates in the regulation of the Src/p-Cav-1 axis in TDI-exposed 16HBE cells.

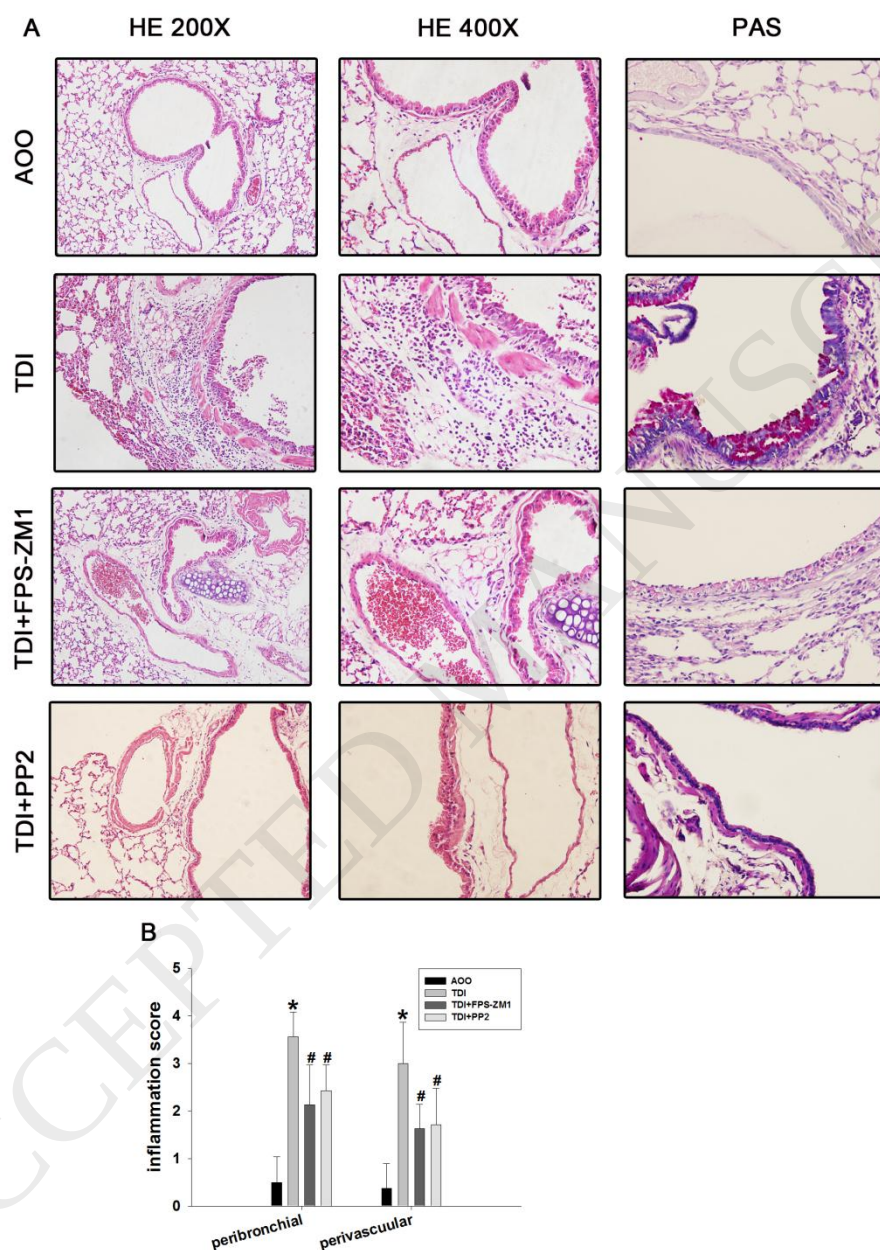


Fig. 2. Effects of FPS-ZM1 and PP2 on TDI-induced asthmatic responses. (A) Representative H&E stained lung sections of different groups at 200× (left panel) and 400× (middle panel) original magnification, and periodic acid-Schiff base (PAS) stained sections at 400× original magnification. **(B)** Semiquantification of airway inflammation was performed in the AOO-treated group (n=10) and TDI-treated groups (n=10). *P < 0.05 compared with the AOO/AOO group; #P < 0.05 compared with the TDI/TDI group.

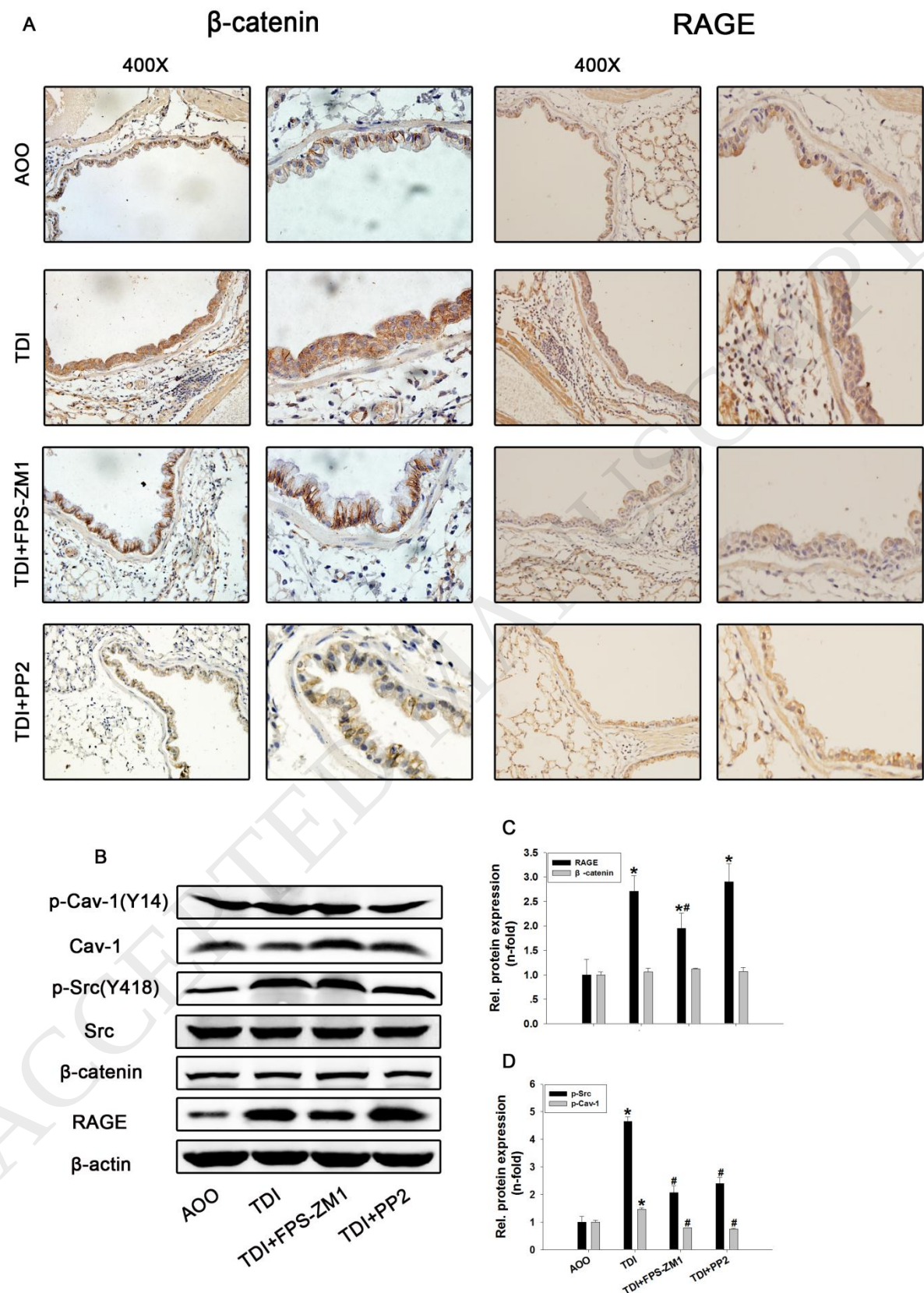
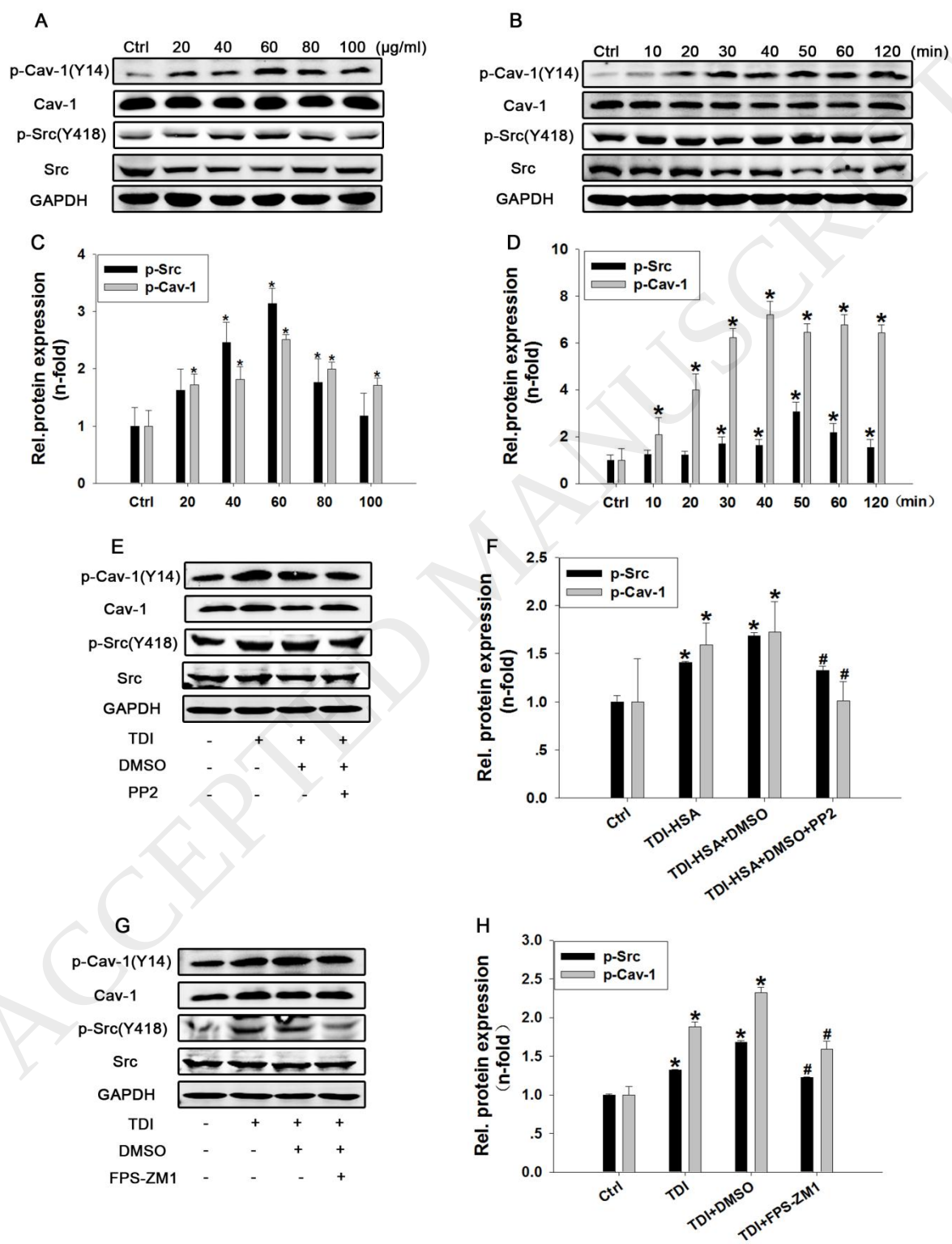


Fig. 3. Pulmonary β -catenin distribution and expressions of RAGE. (A) Representative immunohistochemistry analysis of β -catenin (left panel) and RAGE(right panel) and the amplified view of them in the lung (panel 2 and 4) (n=6). (B) Western blot analysis of RAGE, β -catenin, p-Src, and p-Cav-1 in the whole lung, and (C) densitometric analysis of the blots (n=6). *P < 0.05 compared with the AOO/ AOO group; #P < 0.05 compared with TDI/ TDI group.



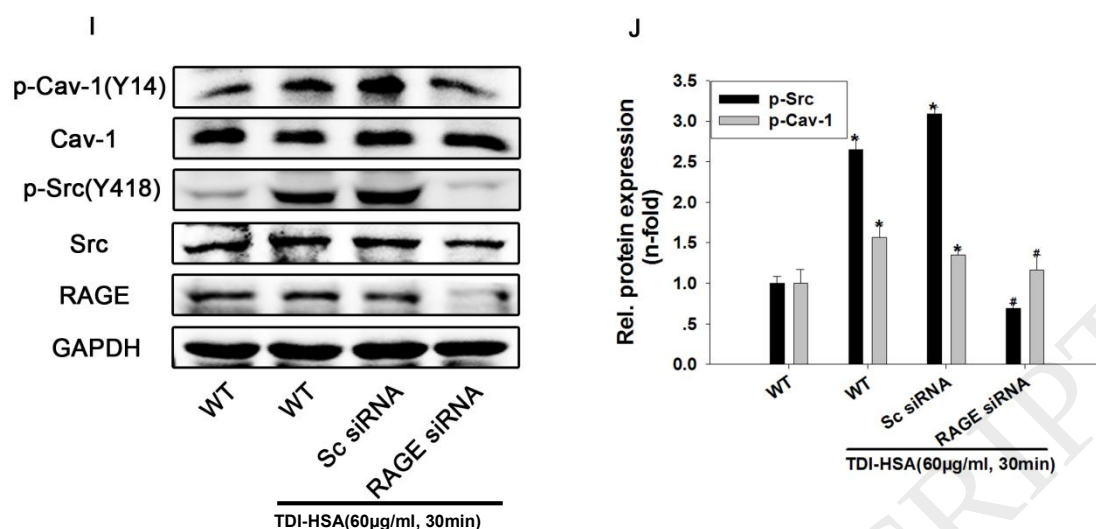
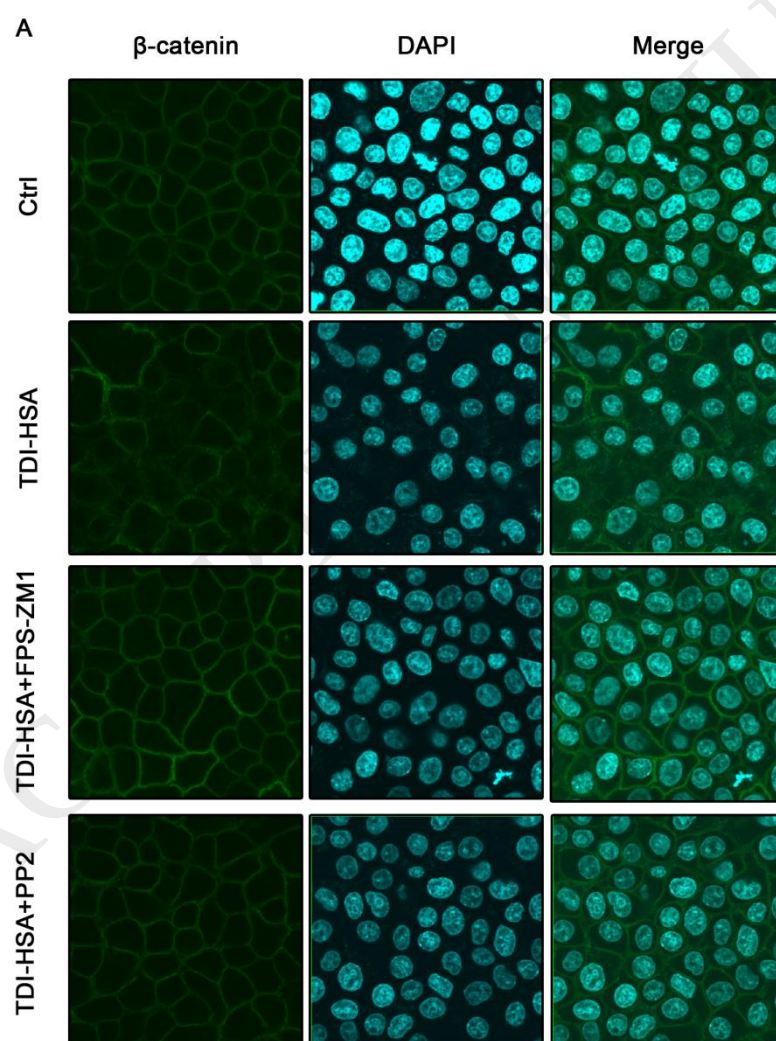


Fig. 4. TDI-HSA-induced activation of the Src/p-Cav-1 axis via RAGE in 16HBE cells. (A) TDI-HSA induced the activation of the Src/p-Cav-1 axis. 16HBE cells were exposed to different concentration of TDI-HSA (20 to 100 mg/ml) for 30 min. (C) Time course of TDI-HSA-induced Src/p-Cav-1 axis activation. Cells were stimulated with 60 µg/ml TDI-HSA for the indicated time. (E) Effect of PP2 on Src/p-Cav-1 axis activation induced by TDI-HSA. Cells were stimulated with 60 µg/ml TDI-HSA for 30 min after treatment with PP2 (20 µM) or vehicle for 1 h. (G) Effect of FPS-ZM1 on Src/p-Cav-1 axis activation induced by TDI-HSA. Cells were pretreated with FPS-ZM1 (1 µg/ml) or vehicle for 1 h and then stimulated with 60 µg/ml TDI-HSA for 30 min. (I) Effect of RAGE on activation of the Src/p-Cav-1 axis induced by TDI-HSA. RAGE knockdown 16HBE cells were exposed to 60 µg/ml TDI-HSA. (C, D, F, J) Desitometric analysis of the blots. Data are mean $\pm$ standard deviation of three independent experiments. *P < 0.05 compared with control group; #P < 0.05 compared with TDI-HSA group.

Inhibition of the RAGE-Src/p-Cav-1 pathway ameliorated TDI-HSA-induced redistribution of β -catenin in 16HBE cells

In order to further elucidate RAGE-Src/p-Cav-1 pathway involvement in the redistribution of β -catenin, we observed the distribution of β -catenin via immunofluorescence microscopy (Fig. 5A). Confocal imaging showed wide spread gap formation, increased β -catenin translocation into the cytosol, and a loss of β -catenin staining in membranes at sites of cell-cell contact in the TDI-HSA group, whereas β -catenin localization was unaffected and gaps in the monolayer did not form with blockade of RAGE and Src/p-Cav-1 signaling by FPS-ZM1 or siRNA of RAGE and PP2, respectively. To identify the effect of the RAGE-Src/p-Cav-1

pathway on different pools of β -catenin, we analyzed subcellular fractions isolated from 16HBE cells stimulated by TDI-HSA. First, we verified the purity of the subcellular extracts by immunoblotting (Fig. 5C). Similarly, TDI-HSA stimulation resulted in decreased membrane expression of β -catenin (Fig. 5D), along with increased amounts in the cytoplasm, indicating β -catenin translocation from the membrane to cytosolic compartments. In line with the results of immunohistochemistry analysis, blockade of RAGE and Src/p-Cav-1 signaling notably impaired the cytosolic accumulation of β -catenin, indicating the RAGE-Src/p-Cav-1 pathway may play a critical role in translocation of β -catenin.



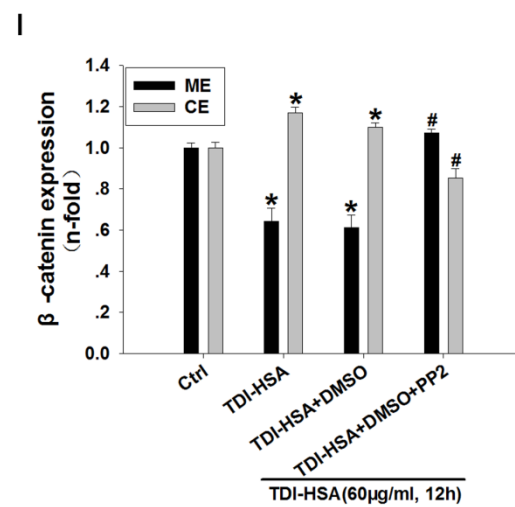
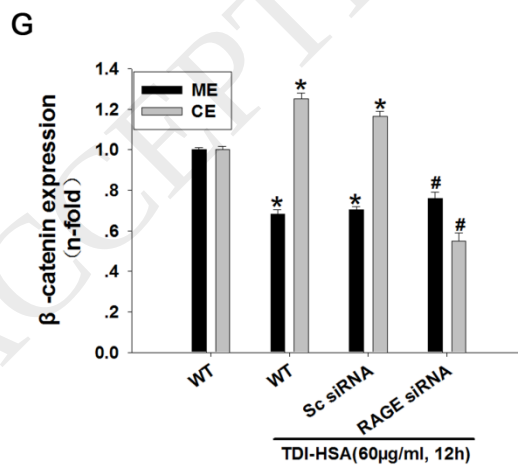
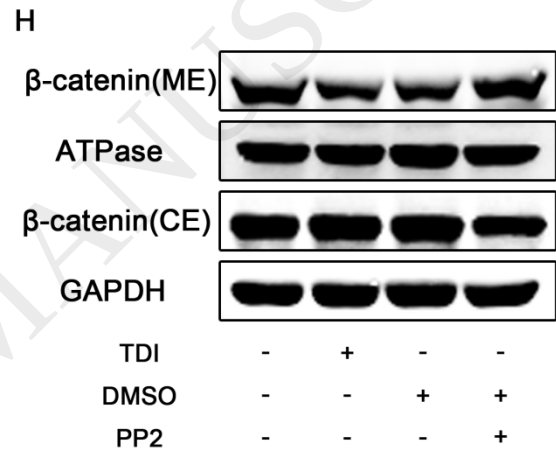
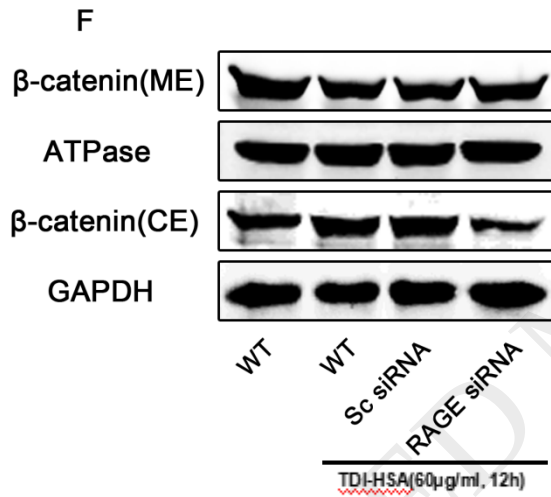
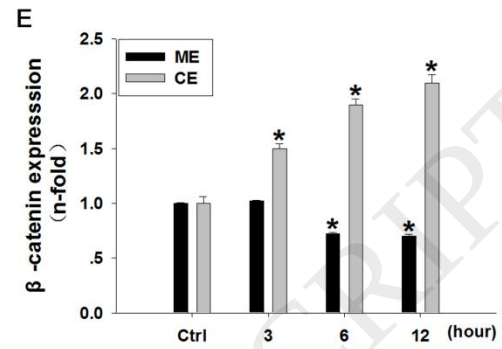
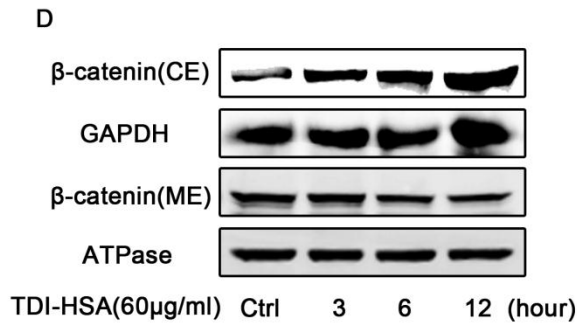
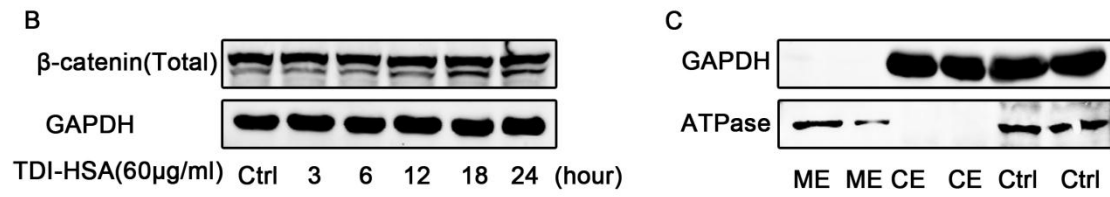


Fig. 5. TDI-HSA induced redistribution of β -catenin and effects of RAGE. (A) FPS-ZM1 and PP2 restrained redistribution of β -catenin analyzed immunofluorescent examination. Cells were stimulated with TDI-HSA (60 μ g/ml) for 12 h with or without pre-treatment with FPS-ZM1 (1 μ g/ml, 1 h) or PP2(20 μ M, 1 h), and then β -catenin was stained with rabbit polyclonal antibody and Alexa 488-labeled goat anti-rabbit IgG antibody. The cell nuclei were stained with DAPI. (B) TDI-HSA did not influence expression of total- β -catenin. Cells were exposed to TDI-HSA (60 μ g/ml) for the indicated times (3 h to 24 h). (C) The purity of membrane extracts (ME) and cytoplasmic extracts (CE) was verified by Western blotting with antibodies against Na/K ATPase and GAPDH. (D) TDI-HSA induced redistribution of β -catenin. Cells were stimulated with TDI-HSA (60 μ g/ml) for different times, and lysates were separated into cytosolic and membrane fractions followed by Western blotting with β -catenin antibody to determine β -catenin localization. (E) RAGE is required for TDI-HSA induced β -catenin redistribution. RAGE knockdown 16HBE cells were exposed to TDI-HSA (60 μ g/ml) for 6 h, and lysates separated into cytosolic and membrane fractions followed by Western blotting. (H) PP2 restrained redistribution of β -catenin as examined with Western blotting. (E, G, I) Desitometric analysis of the blots. Data are mean $\pm$ standard deviation of three independent experiments. *P < 0.05 compared with control group; #P < 0.05 compared with TDI-HSA group or wild-type 16HBE cells.

Cav-1 phosphorylation mediates TDI-HSA–induced redistribution of β -catenin

To assess the role of Cav-1 phosphorylation in the mechanism of TDI-HSA induced redistribution of β -catenin, non-phosphorylatable Cav-1 mutant (Y14F-Cav-1) 16HBE cells were established (Fig. 6B). As shown in Figure 6A, immunofluorescence staining revealed TDI-HSA led to the redistribution of β -catenin from the cytomembrane to cytoplasm, which was remitted by inhibition of Cav-1 phosphorylation. Moreover, the result was verified through analyzing subcellular extracts by immunoblotting (Fig. 6C). Therefore, Cav-1 phosphorylation plays critical role in TDI induced redistribution of β -catenin.

Discussion

In this study, for the first time, we demonstrated activation of the Src/p-Cav-1 axis in TDI-induced asthma. Blockade of RAGE signaling with specific antagonists not only attenuated TDI-induced airway inflammation, but also blocked the activation of the Src/p-Cav-1 axis and β -catenin signaling. In addition, inhibition of phosphorylation of Cav-1 reduced abnormal distribution of β -catenin and TDI-induced airway inflammation.

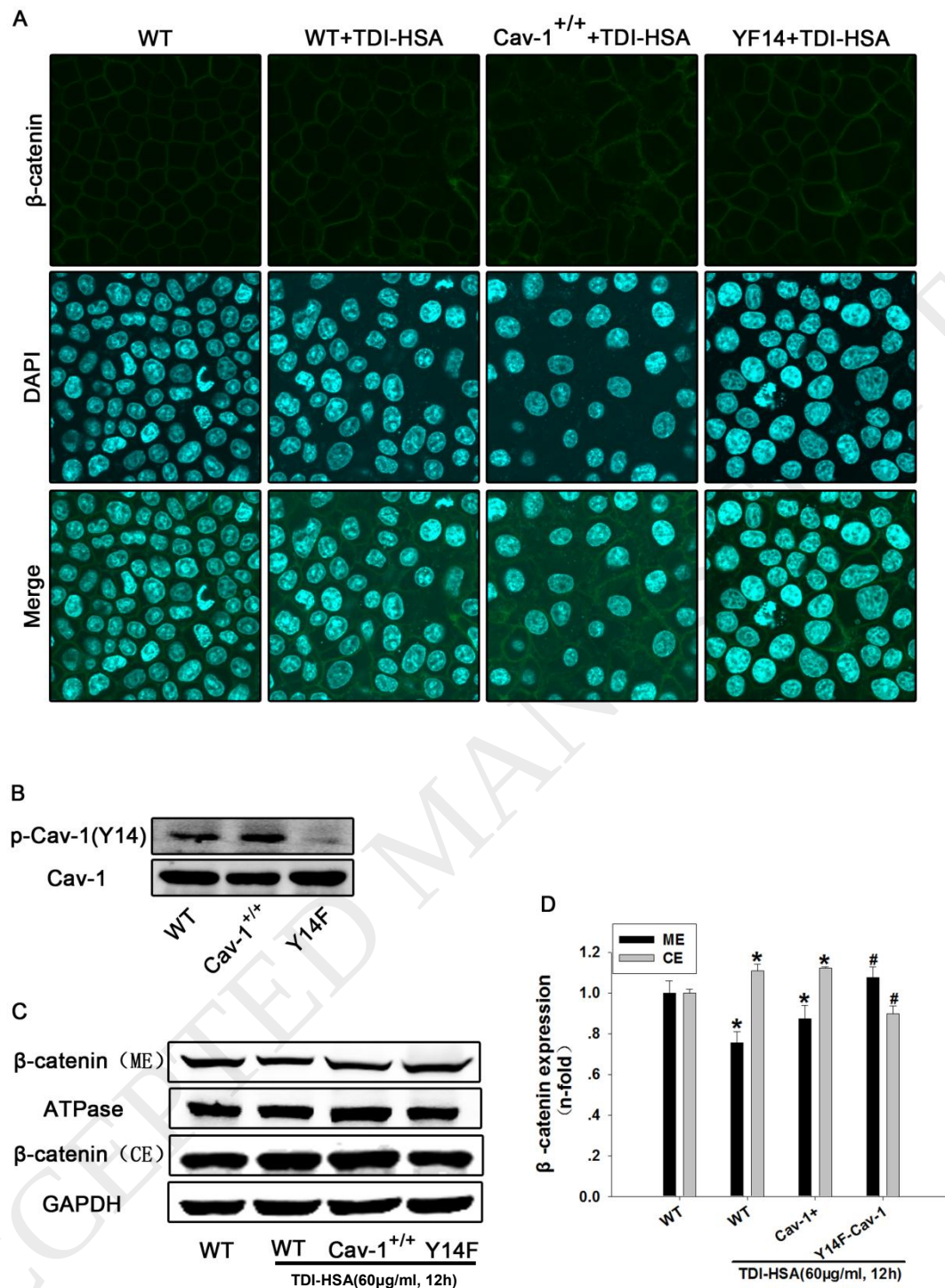


Fig. 6. Cav-1 phosphorylation is required for TDI-HSA-induced β-catenin redistribution. (A) Non-phosphorylated Cav-1 mutant (Y14F-Cav-1) 16HBE cells restrained redistribution of β-catenin as analyzed by immunofluorescent examination. Wild-type, Cav-1-overexpression, or Y14F-Cav-116HBE cells were stimulated with TDI-HSA (60μg/ml) for 12 h, and then β-catenin was stained with the rabbit polyclonal antibody and Alexa 488-labeled goat anti-rabbit IgG antibody. The cell nuclei were stained with DAPI. (B) Detection of Cav-1 phosphorylation Y14F-Cav-116HBE cells by Western blotting. (C) Western blotting analysis of β-catenin distribution in wild-type, Cav-1-overexpression, or Y14F-Cav-1 16HBE cells. (D) Desitometric analysis of the blots. Data are mean ± standard deviation of three independent experiments. *P < 0.05 compared with wild-type 16HBE cells control group; #P < 0.05 compared with wild-type 16HBE cells TDI-HSA group.

Consistent with a previous study, FPS-ZM1 or silencing RAGE through siRNA reduced delocalization of β -catenin from the cytomembrane in 16HBE cells as demonstrated by immunohistochemistry staining and Western blotting. Additionally, *in vivo* study further established that RAGE contributes to accumulation of β -catenin in the cytoplasm by facilitating β -catenin isolation from the cytomembrane. However, the mechanisms underlying RAGE-mediated β -catenin accumulation in the cytoplasm in TDI-induced asthma are still open to speculation.

The key factors of β -catenin signaling are its stabilization and accumulation in the cytoplasm (Valenta et al., 2012). The stabilization of β -catenin is attributed to a multiprotein complex known as the ‘destruction complex’, which consists of APC, Axin1/2, CK1 α , and GSK3 β , which phosphorylates β -catenin on N-terminal Ser/Thr residues (S45, S33, S37, T41), thus priming it for ubiquitination and proteasomal degradation (Monga et al., 2015). Activation of Wnt signaling causes phosphorylation of LRP6, which is the critical step that leads to rescuing of β -catenin from this destructive complex, and maintains its stabilization, resulting in its nuclear translocation (Valenta et al., 2012). We have also proved that RAGE could partly maintain the stabilization of β -catenin by the PI3K/Akt/GSK3 β pathway (Yao et al., 2016; Yao et al., 2017). This is consistent with the study that HMGB1-induced epithelial mesenchymal transition through RAGE and the PI3K/AKT/GSK3 β / β -catenin signaling pathway in human airway epithelial cells (Chen YC et al., 2016). Recently, our group further demonstrated RAGE modulates Lrp6 phosphorylation, contributing to β -catenin stabilization in a TDI-induced murine model (Xiong et al., 2018). All this studies reminder RAGE is crucial in β -catenin stabilization. However, it is worth noting that the expression of β -catenin

in cytomembrane is much higher than that of cytoplasm, so the inversion of β -catenin from the cytomembrane to the cytosol is also a crucial factor leading to β -catenin accumulation in the cytoplasm.

In this study, we found that TDI led to phosphorylation of Cav-1 *in vivo* and *in vitro* and inhibition of Cav-1 phosphorylation by PP2 or phosphorylation-defective Cav-1 mutant tools reduced the cytosolic accumulation of β -catenin. Usually, Cav-1 serves as the pivotal scaffolding protein of Caveolae, which is reduced in the airways of patients with asthma, leading to dysfunction of the epithelial barrier as well as increased Th2 inflammation (Hackett, et al., 2013). In addition, Cav-1 expression is also crucial for the maintenance of barrier function in brain microvascular endothelial cells (Song et al., 2007). In these studies, they explored the total expression levels of Cav-1, but not the p-Cav-1. Consistent with our study, Sun et al. showed that phosphorylation of caveolin-1 by p-Src following H₂O₂ exposure resulted in the dissociation of VE-cadherin/ β -catenin complexes in vascular endothelial cells, coupled with accumulation of β -catenin in cytoplasm (Sun et al., 2009). Therefore, phosphorylation of Cav-1 may play an important role in β -catenin translocation from cytomembrane to cytoplasm in TDI-induced asthma, however, the definite mechanism needs to be further explored.

In this study, we found that blockade of RAGE signaling could suppress activation of the Src/p-Cav-1 axis –induced by TDI *in vivo* and *in vitro*. Consistent with our results, Shang et al. reported that HMGB1 boosted endothelial albumin transcytosis via RAGE-dependent Src kinase activation and Cav-1 phosphorylation (Shang et al., 2012). In 2006, Reddy et al. also found that antibody specific to RAGE could block S100B-induced activation of Src

kinase and tyrosine phosphorylation of caveolin-1 in vascular smooth muscle cells (Reddy et al., 2006). Thus, RAGE may involve in regulating Src/p-Cav-1 axis mediating β -catenin translocation from cytomembrane to cytoplasm. Moreover, as blockade of RAGE, the inhibition of the Src/p-Cav-1 axis by PP2 potentially attenuates the TDI-induced elevation of serum IgE and IL-4, IL-5, IL-13 in BALF, coupled with diminishing airway eosinophilia, neutrophil infiltration, and airway hyper-reactivity. Therefore, we inferred that the Src/p-Cav-1 axis may participate in airway inflammation through RAGE/ β -catenin signaling.

Previous study indicates that Cav-1 increases GSK-3 β activity, which is an important element of β -catenin degradation (Bai et al., 2017). Furthermore, Cav-1 is necessary for Wnt-3a-dependent internalization of LRP6 and accumulation of beta-catenin. (Yamamoto et al., 2006). Our recent study also found RAGE regulates the phosphorylation of LRP6 in TDI asthma model (Xiong et al., 2018), these suggested Cav-1 may participate in β -catenin stabilization by inhibiting the activity of 'destruction complex'. However, how Cav-1 or p-Cav-1 involved in RAGE phosphorylating LRP6 and mediating abnormal distribution of β -catenin remained to be clarified..

In conclusion, our results demonstrated that RAGE modulates β -catenin aberrant distribution via activation of Src/p-Cav-1, which may play an important role in inflammation of the airway in a chemical-induced asthma model.

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Author contributions

Haijin Zhao and Shaoxi Cai conceived the study, and supervised the scientific work. Wenqu Zhao performed the experiments, and wrote the first draft of the manuscript. Yun Lin, Jing Xiong, Yanhong Wang, Guohua Huang, Qiuhua Deng, Lihong Yao, Yu Changhui, and Hangming Dong contributed to the research plan and design. All of the authors contributed to, and approved the final manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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Figure Legends

Fig.1. Effects of RAGE inhibitor (FPS-ZM1) and Src inhibitor (PP2) on TDI-induced asthmatic responses.

(A) Airway hyper-responsiveness was measured by lung resistance (RL). Results were shown as percentage of baseline value (n=6). (B) IgE in the serum. n=6. (C) IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid (BALF) (n=6). Total (E) and differential inflammatory cells (D, F) in BALF in the AOO-treated group and TDI-treated group (n=8). Percentages of different inflammatory cells were calculated by counting a total of 200 cells in H&E stained cytospin samples. *P < 0.05 compared with AOO/AOO group; #P < 0.05 compared with TDI/TDI group.

Fig. 2. Effects of FPS-ZM1 and PP2 on TDI-induced asthmatic responses. (A) Representative H&E stained lung sections of different groups at 200 $\times$ (left panel) and 400 $\times$ (middle panel) original magnification, and periodic acid-Schiff base (PAS) stained sections at 400 $\times$ original magnification. (B) Semiquantification of airway inflammation was performed in the AOO-

treated group (n=10) and TDI-treated groups (n=10). *P < 0.05 compared with the AOO/AOO group; #P < 0.05 compared with the TDI/TDI group.

Fig. 3. Pulmonary β -catenin distribution and expressions of RAGE. (A) Representative immunohistochemistry analysis of β -catenin (left panel) and RAGE(right panel) and the amplified view of them in the lung (panel 2 and 4) (n=6). (B) Western blot analysis of RAGE, β -catenin, p-Src, and p-Cav-1 in the whole lung, and (C) desitometric analysis of the blots (n=6). *P < 0.05 compared with the AOO/AOO group; #P < 0.05 compared with TDI/ TDI group.

Fig. 4. TDI-HSA-induced activation of the Src/p-Cav-1 axis via RAGE in 16HBE cells. (A) TDI-HSA induced the activation of the Src/p-Cav-1 axis. 16HBE cells were exposed to different concentration of TDI-HSA (20 to 100 mg/ml) for 30 min. (C) Time course of TDI-HSA-induced Src/p-Cav-1 axis activation. Cells were stimulated with 60 ug/ml TDI-HSA for the indicated time. (E) Effect of PP2 on Src/p-Cav-1 axis activation induced by TDI-HSA. Cells were stimulated with 60 ug/ml TDI-HSA for 30 min after treatment with PP2 (20 uM) or vehicle for 1 h. (G) Effect of FPS-ZM1 on Src/p-Cav-1 axis activation induced by TDI-HSA. Cells were pretreated with FPS-ZM1 (1ug/ml) or vehicle for 1 h and then stimulated with 60 ug/ml TDI-HSA for 30 min. (I) Effect of RAGE on activation of the Src/p-Cav-1 axis induced by TDI-HSA. RAGE knockdown 16HBE cells were exposed to 60 ug/ml TDI-HSA. (C, D, F, J) Desitometric analysis of the blots. Data are mean $\pm$ standard deviation of three independent experiments.

*P < 0.05 compared with control group; #P 0 < 0.05 compared with TDI-HSA group.

Fig. 5. TDI-HSA induced redistribution of β -catenin and effects of RAGE. (A) FPS-ZM1 and PP2 restrained redistribution of β -catenin analyzed immunofluorescent examination. Cells were stimulated with TDI-HSA (60 ug/ml) for 12 h with or without pre-treatment with FPS-ZM1 (1ug/ml, 1 h) or PP2(20 uM,1 h), and then β -catenin was stained with rabbit polyclonal antibody and Alexa 488-labeled goat anti-rabbit

IgG antibody. The cell nuclei were stained with DAPI. (B) TDI-HSA did not influence expression of total- β -catenin. Cells were exposed to TDI-HSA (60 μ g/ml) for the indicated times (3 h to 24 h). (C) The purity of membrane extracts (ME) and cytoplasmic extracts (CE) was verified by Western blotting with antibodies against Na/K ATPase and GAPDH. (D) TDI-HSA induced redistribution of β -catenin. Cells were stimulated with TDI-HSA (60 μ g/ml) for different times, and lysates were separated into cytosolic and membrane fractions followed by Western blotting with β -catenin antibody to determine β -catenin localization. (F) RAGE is required for TDI-HSA induced β -catenin redistribution. RAGE knockdown 16HBE cells were exposed to TDI-HSA (60 μ g/ml) for 6 h, and lysates separated into cytosolic and membrane fractions followed by Western blotting. (H) PP2 restrained redistribution of β -catenin as examined with Western blotting. (E, G, I) Desitometric analysis of the blots. Data are mean $\pm$ standard deviation of three independent experiments. * $P < 0.05$ compared with control group; # $P < 0.05$ compared with TDI-HSA group or wild-type 16HBE cells.

Fig. 6. Cav-1 phosphorylation is required for TDI-HSA-induced β -catenin redistribution. (A) Non-phosphorylated Cav-1 mutant (Y14F-Cav-1) 16HBE cells restrained redistribution of β -catenin as analyzed by immunofluorescent examination. Wild-type, Cav-1-overexpression, or Y14F-Cav-1 16HBE cells were stimulated with TDI-HSA (60 μ g/ml) for 12 h, and then β -catenin was stained with the rabbit polyclonal antibody and Alexa 488-labeled goat anti-rabbit IgG antibody. The cell nuclei were stained with DAPI. (B) Detection of Cav-1 phosphorylation Y14F-Cav-1 16HBE cells by Western blotting. (C) Western blotting analysis of β -catenin distribution in wild-type, Cav-1-overexpression, or Y14F-Cav-1 16HBE cells. (D) Desitometric analysis of the blots. Data are mean $\pm$ standard deviation of three independent experiments. * $P < 0.05$ compared with wild-type 16HBE cells control group; # $P < 0.05$ compared with wild-type 16HBE cells TDI-HSA group.