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RAGE mediates airway inflammation via the HDAC1 pathway in a toluene

diisocyanate-induced murine asthma model

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Abstract

Background

Exposure to toluene diisocyanate (TDI) is a significant pathogenic factor for asthma. We previously reported that receptor for advanced glycation end products (RAGE) plays a key role in TDI-induced asthma; however, the mechanism is not clear. Epigenetic alterations of histone deacetylase (HDAC) are associated with allergic asthma. However, its effect in TDI-induced asthma is not known. The purpose of this study was to determine the role of RAGE and HDAC1 in the regulation of airway inflammation using a TDI-induced asthma model.

Methods

BALB/c mice were sensitized, and challenged by TDI to establish murine asthma models.

FPS-ZM1 (RAGE inhibitor), JNJ-26482585 and romidepsin (HDAC inhibitor) were given intraperitoneally before each challenge. The human bronchial epithelial cell line 16HBE was stimulated by TDI-human serum albumin (TDI-HSA) *in vitro*. RAGE knockdown cells were constructed and evaluated, and MK2006 (AKT inhibitor) was used in *in vitro* experiments.

Results

In the TDI-induced asthmatic mice, airway reactivity, the level of Th2 cytokines in lymph supernatant, IgE, airway inflammation, and goblet cell metaplasia were all significantly increased. The increases were suppressed by FPS-ZM1, JNJ-26482585, and romidepsin. The expression of HDAC1, RAGE, and p-AKT/t-AKT was also upregulated in TDI-induced asthmatic mice, and the expressions were attenuated by FPS-ZM1. Knockdown of RAGE attenuated the upregulation of HDAC1 and phospho-AKT (p-AKT) in 16HBE cells stimulated by TDI-HSA. Treatment with the AKT inhibitor MK2006 suppressed TDI-induced HDAC1 expression.

Conclusion: RAGE mediates airway inflammation in a TDI-induced murine asthma model, partly via the HDAC1 pathway.

Key words: Toluene diisocyanate, asthma, histone deacetylase 1, advanced glycosylation end product receptor

1. Introduction

Toluene diisocyanate (TDI) is a chemical widely used in many different industries, and is a common cause of occupational asthma (OA). Epidemiological studies have indicated that OA accounts for 10% to 25% of new-onset adult asthma cases (Tarlo and Lemiere 2014).

Receptor for advanced glycation end products (RAGE) is a cell surface receptor that belongs to the immunoglobulin superfamily; it recognizes endogenous ligands derived by pathogens and initiates an immune response to inflammation and tissue damage (Sukkar et al. 2012). Studies have indicated that RAGE is most highly expressed in lung tissues, and plays a vital role in pulmonary inflammatory responses (Oczypok et al. 2017; Brandt and Lewkowich 2019). RAGE has also been reported to be an important mediator of allergic inflammation and airway hyper-responsiveness (AHR) in house dust mite (HDM)- and fungal extract-induced murine asthma models (Milutinovic et al. 2012; Ullah et al. 2014; Oczypok et al. 2015). In our prior works, we found that RAGE and its ligands were increased in TDI-induced asthmatic mice, and that blocking RAGE signaling alleviated airway inflammation (Yao et al. 2016; Xiong et al. 2018; Zhao et al. 2018). However, the mechanism of these effects was not determined.

Epigenetic changes, including histone deacetylation, have been reported to be important in the pathogenesis of asthma (Alizadeh et al. 2017; van den Bosch et al. 2017; Papi et al. 2018). Histone deacetylases (HDACs) are classified into four main groups based on their homology with yeast orthologs^[13]. Clinical studies have found that HDAC activity is different between asthmatics and healthy people, and activity is associated with the response to corticosteroids (Ito et al. 2002; Kim et al. 2013; Wawrzyniak et al. 2017). In addition, histone deacetylase inhibitors have been shown to have therapeutic effects in ovalbumin (OVA)-induced asthma models (Banerjee et al. 2012; Ren et al. 2016). However, the role of HDAC in TDI-induced asthma requires further study.

RAGE is necessary for the pulmonary accumulation of group 2 innate lymphoid cells

(ILC2s), which promote allergic airway inflammation (Oczypok et al. 2015). A murine study reported that the HDAC inhibitor trichostatin A suppressed innate allergic inflammation by blocking ILC2 activation (Toki et al. 2016). Another study found that administration of the RAGE agonist glycated-albumin increased HDAC activity in retinal pigment epithelium (Desjardins et al. 2016). However, no similar studies have examined the relation between RAGE and asthma.

Based on prior research, we hypothesized that RAGE regulates airway inflammation through the HDAC pathway. Thus, the purpose of this study was study the regulatory effect of RAGE on airway inflammation using a murine TDI-induced asthma model.

2. Materials and Methods

Reagents

TDI (toluene-2, 4-diisocyanate, $\geq 98.0\%$), acetone, and methacholine were obtained from Sigma-Aldrich (Shanghai, China). The vehicle that TDI was dissolved in was a mixture of acetone and olive oil (AOO): the ratio of acetone to olive oil was 2:3 for sensitization, and 1:4 for challenge. FPS-ZM1 (RAGE inhibitor), JNJ-26482585 (HDAC inhibitor), romidepsin (HDAC inhibitor), and MK2206 (AKT inhibitor) were purchased from Selleck (SelleckChem, Shanghai, China). JNJ-26482585 is a novel second-generation HDACi with highest potency for HDAC1 and romidepsin is a selective inhibitor of HDAC1 and HDAC2. Anti-RAGE (ab37647), and anti-HDAC1 (ab109411) were from Abcam. Anti-total-AKT (#4691) and Phospho-Akt (#4060) were purchased from Cell Signaling Technology (Boston, MA, USA).

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

Male BALB/c mice, 6-8 weeks old, were purchased from Southern Medical University. The mice were housed in a specific pathogen-free (SPF) environment with a 12 hour dark-light cycle and free access to food and water. All animal experiments met the requirements of the Southern Medical University Committee on the Use and Care of Laboratory Animals.

A total of 50 mice were randomized to the following groups, with 10 mice in each group: (1) AOO; 2) TDI; 3) TDI+FPS-ZM1; 4) TDI+JNJ-26482585; 5) TDI+romidepsin.

TDI-induced murine asthma models were established as previously described (Yao et al. 2016). On day 1 and day 8, the mice were dermally sensitized with 0.3% TDI on the dorsum of both ears (20 μ l per ear). On day 15, 18, and 21 the mice were separately placed in a horizontal cylindrical chamber for an airway challenge with 3% TDI dissolved in acetone/olive oil (1:4). The TDI was dispersed by compressed air nebulization (NE-C28; Omron, Tokyo, Japan), and the mice remained in the chamber for 3 hours. Mice in the control group were sensitized by the same method, and challenged by the same method using the same volume of AOO rather than TDI. Before each challenge, mice were respectively injected with FPS-ZM1 (1.5 mg/kg, i.p.), JNJ-26482585 (5 mg/kg, i.p.), and romidepsin (2.4 mg/kg, i.p.) which were dissolved in DMSO and diluted with PBS. The control group received the same amount of vehicle.

Assessment of AHR

Airway responsiveness to methacholine was assessed by measuring lung resistance (RL) (Buxco Electronics, Troy, NY) on day 22, using a previously described method (Liang et al. 2015). Mice were placed in a barometric plethysmographic chamber and challenged with sterile saline, followed by increasing concentrations of aerosolized methacholine (3.125, 6.25, 12.5, and 25 mg/mL). RL was recorded every 5 minutes following each nebulization step according to manufacturer's protocol. RL was recorded as the percentage of baseline value (value for sterile saline) for each concentration of methacholine.

Measurement of interleukin (IL)-4, IL-5, and IL-13 in supernatants of cultured lymphocytes

Cervical lymph nodes were isolated from individual mice, and then pressed through a 40 μ m cell strainer (BD Falcon, USA) to obtain a cell suspension. The cells were counted using a hemocytometer, and inoculated into 48-well culture plates at a density of 10^6 cells/mL.

Lymphocytes were cultured in RPMI-1640 medium containing 10% fetal calf serum (Hyclone) with 5 μ g/mL concanavaline A (Sigma Aldrich) for 43 h, and then centrifuged ($1000\times g$, 10 min). Supernatants were collected and preserved at -80°C until use. The levels of IL-4, IL-5, and IL-13 in the supernatants were measured using an ELISA (Boster, Wuhan, China), according to manufacturer's instructions.

Measurement of serum IgE level

Mice were sacrificed in a manner previously described (Liang et al. 2015). Blood samples were collected, allowed to remain at room temperature for 1 hour, and then centrifuged at

3000×g for 20 min. The supernatants were collected and stored at -80°C until use. IgE levels were measured by ELISA (Cusabio, Wuhan, China), according to manufacturer's instructions.

Analysis of bronchoalveolar lavage fluid (BALF)

BALF was collected, and a hemocytometer was used to count the total number of cells. The BALF was then centrifuged at 1,000×g for 10 minutes. A cytospin sample was prepared for differential cell counts. The cells were stained with hematoxylin and eosin (H&E) and examined under a light microscope to determine the differential cell counts. A total of 200 cells were counted, and the percentages of macrophages, lymphocytes, neutrophils, and eosinophils were determined.

Histopathological examination of lung tissue

The left lungs were fixed in 4% formaldehyde, and then embedded in paraffin. Lung sections (4 µm) were stained with H&E and periodic acid-Schiff (PAS) to show airway inflammation and mucus production. Airway inflammation was scored in a semi-quantified manner as previously described [8]. Briefly, peribronchial inflammation and perivascular inflammation were used to quantify pulmonary inflammation. A value of 0 was given when no inflammation was detectable; a value of 1 for occasional cuffing with inflammatory cells; a value of 2 for most bronchi or vessels surrounded by 1 layer (1 to 10 cells) of inflammatory cells; a value of 3 for most bronchi or vessels surrounded by 2 layers (10 to 20 cells) of inflammatory cells; a value of 4 for most bronchi or vessels surrounded by more than 2 layers

(more than 20 cells) of inflammatory cells. Twenty sections of 10 mice in each group were examined, and at least 40 image fields at 200× magnification were viewed and scored. A random code was assigned to each sample so the examiner was not aware of the source of each section.

Immunohistochemistry (IHC) evaluation of lung tissue

To examine lung tissue for HDAC1, lung slices were deparaffinized for 2 hours and then subjected to antigen retrieval. The tissue slices were treated with H₂O₂ for 15 min, and then incubated in recommended dilutions of anti-HDAC1 antibodies at 4°C overnight. After washing with PBS 3 times, the samples were incubated with secondary antibodies at room temperature for 20 min. The signals were displayed with DAB solution.

Preparation of TDI-human serum albumin (TDI-HSA) conjugates

TDI-HSA conjugates were prepared in a manner previously described (Zhao et al. 2009), which was a modification of the method described by Son (Son et al. 1998). Briefly, TDI was added to HSA in PBS with constant stirring. Then, the samples were centrifuged at 3000×g for 20 min at room temperature to remove unreacted TDI. The product was then dialyzed with PBS using cellulose membranes (Sigma Chemical Co., St. Louis, MO, USA) for 3 days.

Culture, transfection, and treatment of cells

Human bronchial epithelial cell line 16HBE14o-(16HBE) cells (Shanghai Fuxiang Biological Technology Co., ATCC, Portland, OR, USA) were cultured in RPMI-1640 medium

containing 10% fetal calf serum in an incubator at 37°C with 5% CO₂. When 90% confluence was reached, the cells were passaged and inoculated to new culture plates, and various concentrations of TDI-HSA conjugate (0-100 µg/mL) were added to the culture medium and cultured for different lengths of time. The TDI-HSA concentrations used were based on those used in prior studies (Song et al. 2013). The lentiviral system used to knockdown RAGE was developed by Applied Biological Materials Inc. (Nanjing, China). The expression of RAGE was determined by Western blotting.

Western blotting

In order to evaluate the expression of RAGE, HDAC1, AKT, and p-AKT *in vivo* and *in vitro*, whole lung tissue and cell protein extracts were mixed with 5× SDS loading buffer and separated by 10% SDS-polyacrylamide gel electrophoresis. The samples were then transferred to PVDF membranes (Millipore), and the membranes were probed with anti-RAGE, anti-HDAC1, anti-AKT, and anti-p-AKT antibodies using the recommended dilutions. After incubation with an IRDye® 680WC-conjugated secondary antibody (LI-COR Biosciences), immunoreactive bands were imaged using an Odyssey® CLx Imager. Odyssey software was used for data analysis, and Image J software was used for quantitative image analysis.

Statistical analysis

Comparisons among groups were analyzed by one-way analysis of variance (ANOVA), accompanied by Bonferroni post hoc tests for multiple comparisons. Data were presented as

mean \pm standard deviation, and values of $P < 0.05$ were considered statistically significant.

Statistical analysis was performed with SPSS version 20.0 software.

3. Results

3.1. RAGE inhibitor decreased expression of HDAC1 and phosphorylation of AKT, and ameliorated airway inflammation in TDI-induced asthmatic mice

First, the expression of HDAC1 and airway inflammation was determined in TDI-induced mice. In order to prove that murine asthmatic models were successfully established, we detected airway responsiveness and IL-4 level. The results indicated that mice treated with TDI exhibited increased airway inflammation (Fig. 1A) and AHR (Fig. 1B), and higher IL-4 levels (Fig. 1C). IHC staining showed increased expression of HDAC1, mainly in airway epithelium, with most immunostaining in the nucleus, while intervention with a RAGE inhibitor decreased HDAC1 expression (Fig. 1D). Western blotting showed up-regulated expression of HDAC1 in the TDI group, which was significantly inhibited by blocking RAGE signaling with FPS-ZM1 (Fig. 1E, F). In addition, phosphorylation of AKT was upregulated after TDI exposure compared with the AOO group, indicating the PI3K/AKT axis was activated. As expected, pretreatment with FPS-ZM1 attenuated these responses (Fig. 1G, H).

3.2 HDAC inhibition decreased AHR and Th2 cytokines in TDI-induced asthmatic mice

In order to evaluate the role of the HDAC pathway in TDI-induced asthma, airway responsiveness was assessed 24 h after the last challenge. The results showed that AHR

increased significantly in TDI-sensitized mice challenged by methylcholine as compared with the AOO group (6.25, 12.5, and 25 mg/mL; Fig. 2A). AHR was partially alleviated by intraperitoneal injection of an HDAC inhibitor, suggesting a prophylactic effect of HDAC inhibitors on TDI-induced AHR. To evaluate the role of HDAC inhibitors on allergic airway inflammation induced by TDI, we examined the secretion of Th2 cytokines in the supernatants of cultured lymphocytes. The results indicated that JNJ-26482585 and romidepsin reduced the levels of IL-4, IL-5, and IL-13 in TDI group (Fig. 2B). Similarly, treatment with HDAC inhibitors markedly suppressed the increase of the serum total IgE level induced by TDI (Fig. 2C).

3.3 HDAC inhibitors attenuated airway inflammation and goblet cell metaplasia in TDI-induced asthma

The total numbers of cells, and the percentages of different inflammatory cells in BALF were determined (Fig. 2D-F). Consistent with total cell counts (Fig. 2D), higher percentages of neutrophils and eosinophils were found in the TDI group, and these percentages were markedly decreased by administration of JNJ-26482585 and romidepsin. Examination of H&E stained tissue showed typical pathological features of asthma induced by TDI. The bronchi were infiltrated with numerous inflammatory cells compared with AOO group. Pretreatment of mice with JNJ-26482585 and romidepsin resulted in a significant decrease in the exosmosis of inflammatory cells in the peribronchial and perivascular regions, and a decrease in the proliferation of airway epithelium (Fig. 3A, B). Examination of PAS stained tissue showed goblet cell metaplasia, and pretreatment with JNJ-26482585 and romidepsin

eliminated the development of metaplasia (Fig. 3A).

3.4 RAGE blockade suppressed the expression of HDAC1 stimulated by TDI-HSA in 16HBE cells

The expression of HDAC1 in 16HBE cells was examined after stimulation with TDI-HSA. Western blotting showed the expression of HDAC1 was markedly increased after stimulation by TDI-HSA (Fig. 4A), and this effect peaked within 3 to 12 hours after TDI-HSA stimulation (Fig. 4B). In order to identify whether RAGE signaling affects the upregulation of HDAC1 stimulated by TDI-HSA *in vitro*, 16HBE cells were transfected with lentiviral-expressed RAGE shRNA and then selected using a proper concentration of puromycin (Fig. 4E). RAGE-shRNA 16HBE cells were stimulated by 60 µg/mL TDI-HSA for 3 h and 6 h. Silencing of RAGE markedly inhibited the increased expression of HDAC1. These results suggest that RAGE may be involved in the regulation of HDAC1 in TDI-exposed 16HBE cells (Fig. 4F, G).

3.5 Inhibition of the PI3K/AKT axis suppressed the increased expression of HDAC1 induced by TDI-HSA in 16HBE cells

To determine the mechanism by which RAGE mediates the upregulation of HDAC1 induced by TDI, we detected the relevant signaling pathways. Consistent with the *in vivo* experiments, the PI3K/AKT axis was activated following stimulation of TDI-HSA in 16HBE cells (Fig. 5A). Western blotting showed that AKT phosphorylation was upregulated, and this upregulation was inhibited by RAGE silencing (Fig. 5B). In order to further elucidate

PI3K/AKT pathway involvement in the regulation of HDAC1, 16HBE cells were treated with an AKT inhibitor (MK2206, 1 μ M) before stimulation by TDI-HSA. Pretreatment with MK2206 inhibited the TDI-induced upregulation of HDAC1 (Fig. 5C). These findings suggest that RAGE may regulate the expression of HDAC1 through the PI3K/AKT pathway.

4. Discussion

In this study, we found for the first time that RAGE is a potential positive regulator of HDAC1 in a TDI-induced murine asthma model, and the effect may be related to the PI3K/AKT pathway. Additionally, we also demonstrated that inhibition of HDAC prophylactically prevented TDI-induced airway inflammation.

RAGE has been shown to play an important role in asthma in studies using several types of asthma models. Recently, Perkins TN et al. reports that RAGE is a critical component of type 2 cytokine signaling, which is a driving force behind type 2-high asthma (Perkins et al. 2019). Consistent with a previous study, TDI-induced mice in this study exhibited typical features of asthma (AHR, a Th2 response, and airway inflammation), while treatment with FPS-ZM1 attenuated the response (Yao et al. 2016; Zhao et al. 2018). However, the mechanism by which RAGE mediates TDI-induced airway inflammation remains to be explored.

Subtype HDAC1 has been shown to play an important role in asthma. Wawrzyniak et al. observed that the expression of HDAC1 was significantly elevated in human bronchial epithelial cells (HBECs) from asthmatic patients (Wawrzyniak et al. 2017). Similarly, another study reported that a single nucleotide polymorphism (SNP) in HDAC1 (rs1741981) was

significantly related to asthma severity and response to corticosteroids (Kim et al. 2013). In OVA-induced asthmatic mice, HDAC1 activity in lung tissue was greater than in lung tissue from control mice (Su et al. 2018). Previous studies have shown that administration of a RAGE agonist such as glycated-albumin or vascular endothelial growth factor (VEGF) increased HDAC activity in retinal pigment epithelium (Desjardins et al. 2016). Sundar et al. reported that electronic cigarettes caused a reduction of HDAC2 in gingival epithelial cells via a RAGE-dependent pathway (Sundar et al. 2016). In this study, we found that expression of HDAC1 was increased in TDI-induced asthmatic mice, and the increase was attenuated by blocking RAGE signaling with FPS-ZM1, indicating that RAGE may regulate airway inflammation via HDAC1.

Consistent with previous studies, we found that the levels of p-A in whole lung tissues and airway epithelium were significantly upregulated in TDI-induced asthmatic mice as compared with the control (AOO) group (Yao et al. 2015; Wang et al. 2017), and this upregulation was blocked when mice were pretreated with FPS-ZM1. In addition, we found that pretreatment with the AKT inhibitor MK2006 suppressed TDI-induced upregulation of HDAC1 in 16HBE cells. Taken together, these results suggest that RAGE may regulate expression of HDAC1 in TDI-induced asthma via the PI3K/AKT pathway. Other studies using experimental models have also indicated that the PI3K/AKT pathway is involved in the regulation of HDAC (Kim et al. 2017; Woo et al. 2018). Whether there are other signaling pathways are involved, however, requires further research.

In order to further examine the relation of HDAC1 and asthma, TDI-induced asthmatic mice were treated with JNJ-26482585 and romidepsin, relatively specific inhibitors of

HDAC1. Treatment with HDACs inhibitors inhibited the increase in AHR, the airway inflammatory response, and the increase in Th2 cytokines. Ren et al. reported that inhibition of HDAC suppressed suppress airway remodeling, AHR, airway inflammation in an OVA-exposed asthma model (Ren et al. 2016). Another study demonstrated that treatment with HDAC inhibitors suppressed airway inflammation in a murine model of chronic allergic airway disease (Royce et al. 2012). Blocking HDAC activity may be a novel target for epithelial barrier defects in patients with asthma and allergic rhinitis (Wawrzyniak et al. 2017; Steelant et al. 2019). As such, the results of the current study also suggest that blocking HDAC activity may be a novel therapeutic intervention for asthmatic patients. However, it is important to develop more specific inhibitors of HDACs.

In summary, our results revealed that RAGE modulates the expression of HDAC1 via the PI3K/AKT pathway, and inhibition of HDAC can prevent TDI-induced airway inflammation. These results help to understand how RAGE mediates airway inflammation, and may provide insights for novel treatments of asthma.

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Availability of data and materials

The data set during and/or analyzed during the current study available from the corresponding author on reasonable request.

Author contributions

Haijin Zhao and ShaoxiCai conceived the study, and supervised the scientific work. Xianru Peng, Wenqu Zhao and Minyu Huang¹ performed the experiments, and wrote the first draft of the manuscript. Zihan Lan, Xiaohua Wang, Yafei Yuan, Bohou Li,Changhui Yu, Laiyu Liu and Hangming Dong contributed to the research plan and design. All of the authors contributed to, and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

All the authors declare that they are consent for the publication.

Ethics approval and consent to participate

Not applicable.

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Figure Legends

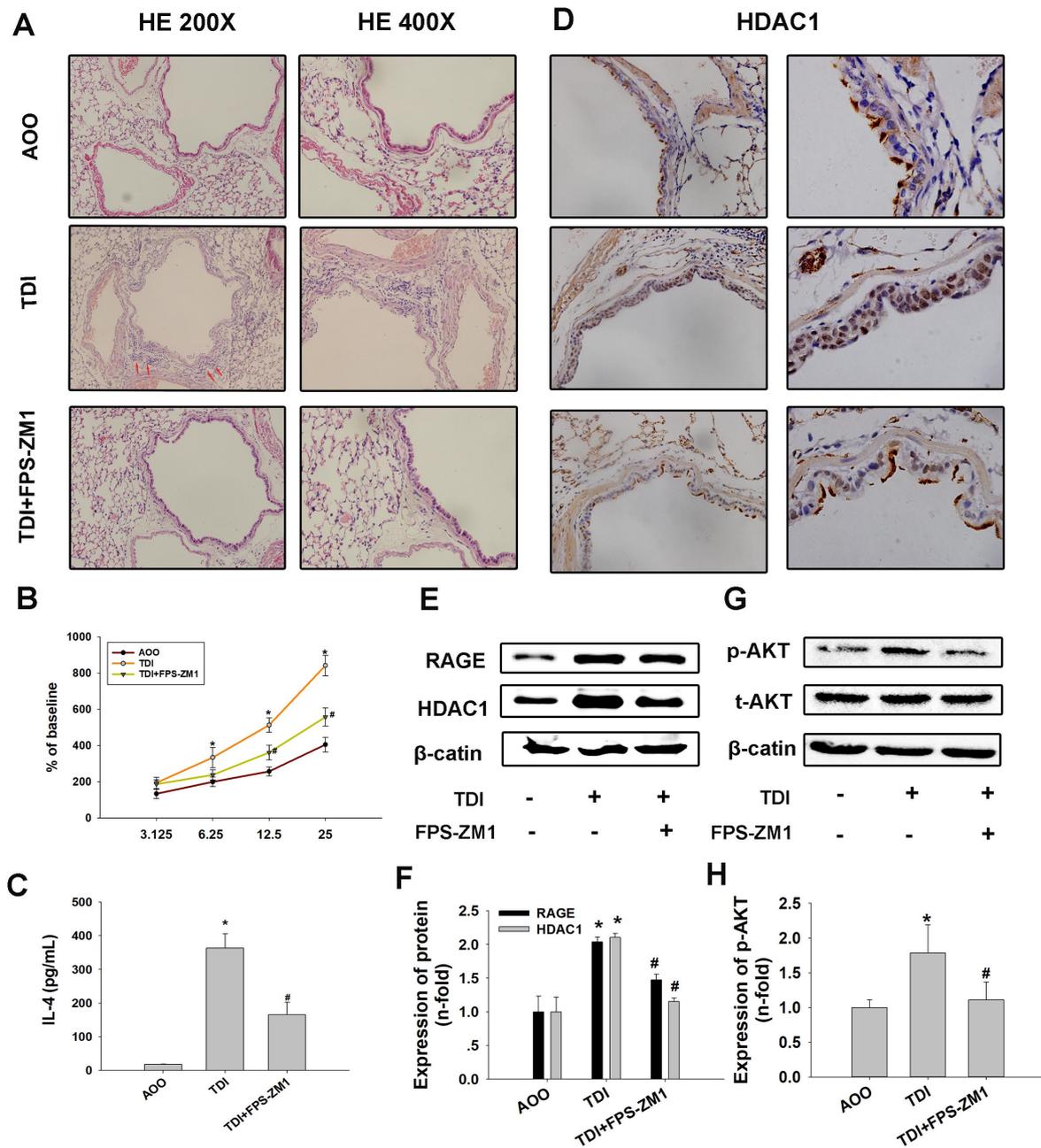


Fig. 1. Effects of RAGE inhibitors on the expression of HDAC1 and p-AKT in

TDI-induced asthmatic mice

(A) Immunohistochemistry analysis of the expression and distribution of HDAC1 in airway epithelium. (B) Western blotting for examination of RAGE and HDAC1 expression in whole lung tissue. (C) Western blotting for examination of AKT and p-AKT expression in whole lung tissue. (D, E) Densitometric analysis of Western blots. * $P < 0.05$ compared with AOO group; # $P < 0.05$ compared with TDI group. (A) Representative lung sections stained with

H&E of each group at 200× and 400× original magnification.(B) Measurement of airway hyperresponsiveness by lung resistance (RL). Data were expressed as a percentage of the baseline value (n=5). (C) Detection of IL-4 in the supernatant of cultured lymphocytes by ELISA.(D) Immunohistochemistry revealed the expression and distribution of HDAC1 in the airway epithelium; (E,G): Western blot showed the expression of RAGE, HDAC1, AKT and p-AKT in whole lung tissue of mice; (F,H): Subsequent densitometric analysis of the blots. *P < 0.05 compared with AOO group; #P < 0.05 compared with TDI group.

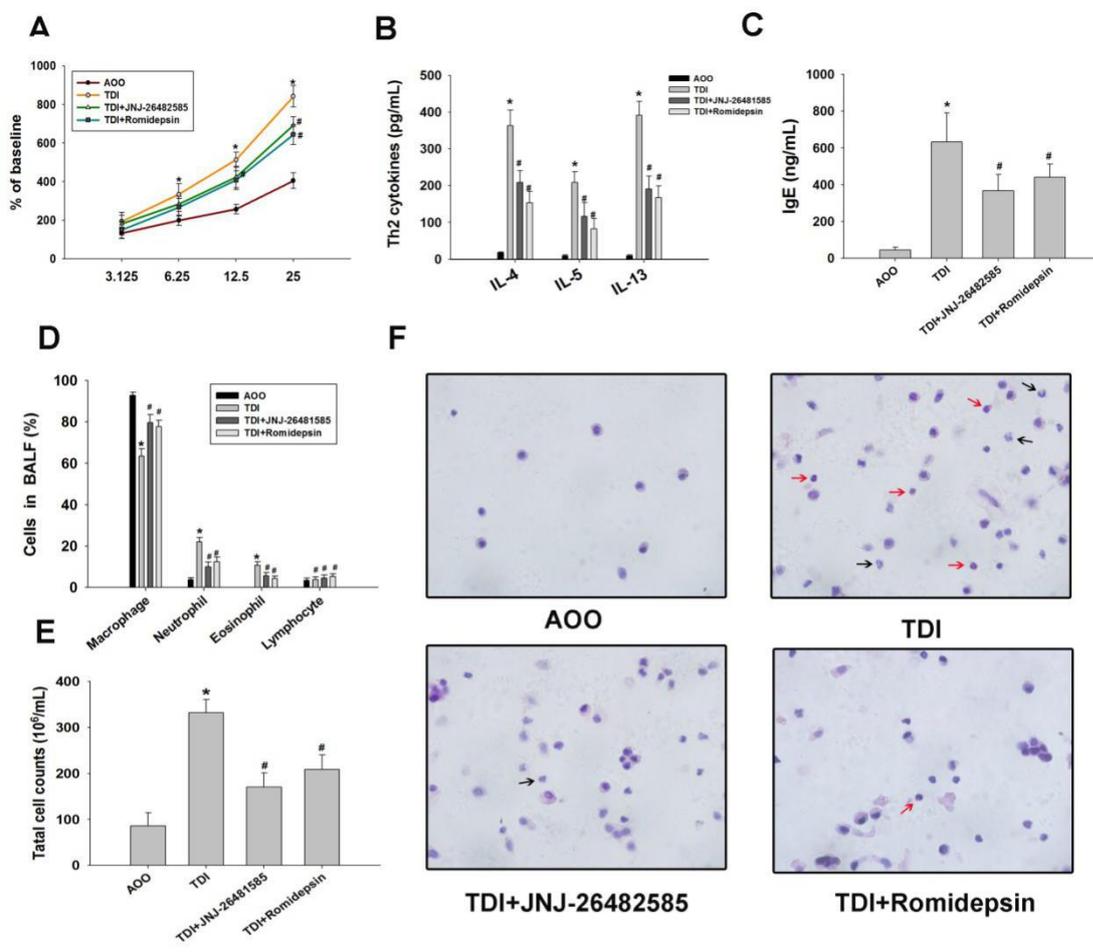


Fig. 2. Effects of HDAC inhibitors on the asthmatic response in TDI-exposed mice

(A) Measurement of airway hyper-responsiveness by lung resistance (RL). Data were expressed as percentage of baseline value (n = 5). (B) Detection of IL-4, IL-5, and IL-13 in the supernatants of cultured lymphocytes by ELISA. (C) Measurement of IgE in serum by

ELISA. (D-F) Total inflammatory cell count and differential cell counts in the BALF of mice.

A total of 200 cells stained with hematoxylin and eosin (H&E) in cytospun samples were

counted to calculate the percentages of different inflammatory cells. n=6~8. *P < 0.05

compared with AOO group; #P < 0.05 compared with TDI group.

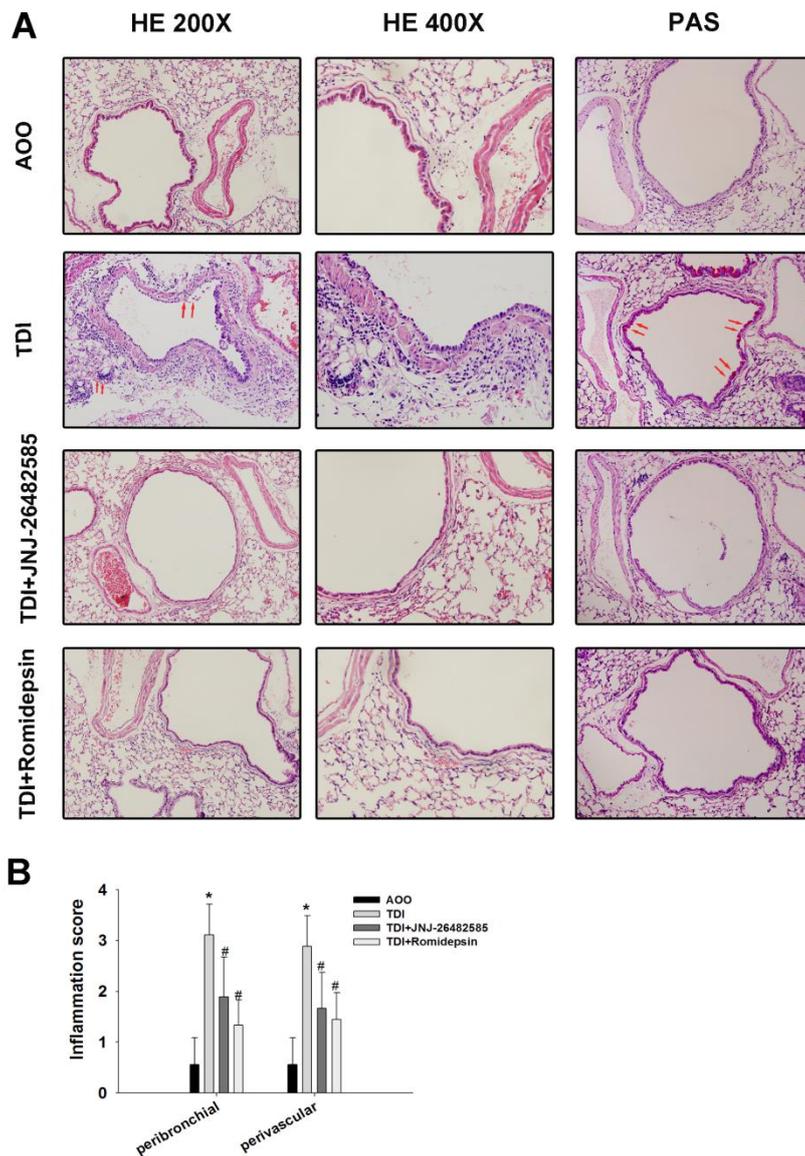


Fig. 3. Effects of HDAC inhibitors on lung tissue of TDI-exposed asthmatic mice

(A) Representative lung sections of each group stained with hematoxylin and eosin (H&E) at

200× and 400× magnification, and stained with PAS at 400× magnification. (B)

Semi-quantitative analysis of airway inflammation. n=8~10. *P < 0.05 compared with the AOO group; #P < 0.05 compared with the TDI group.

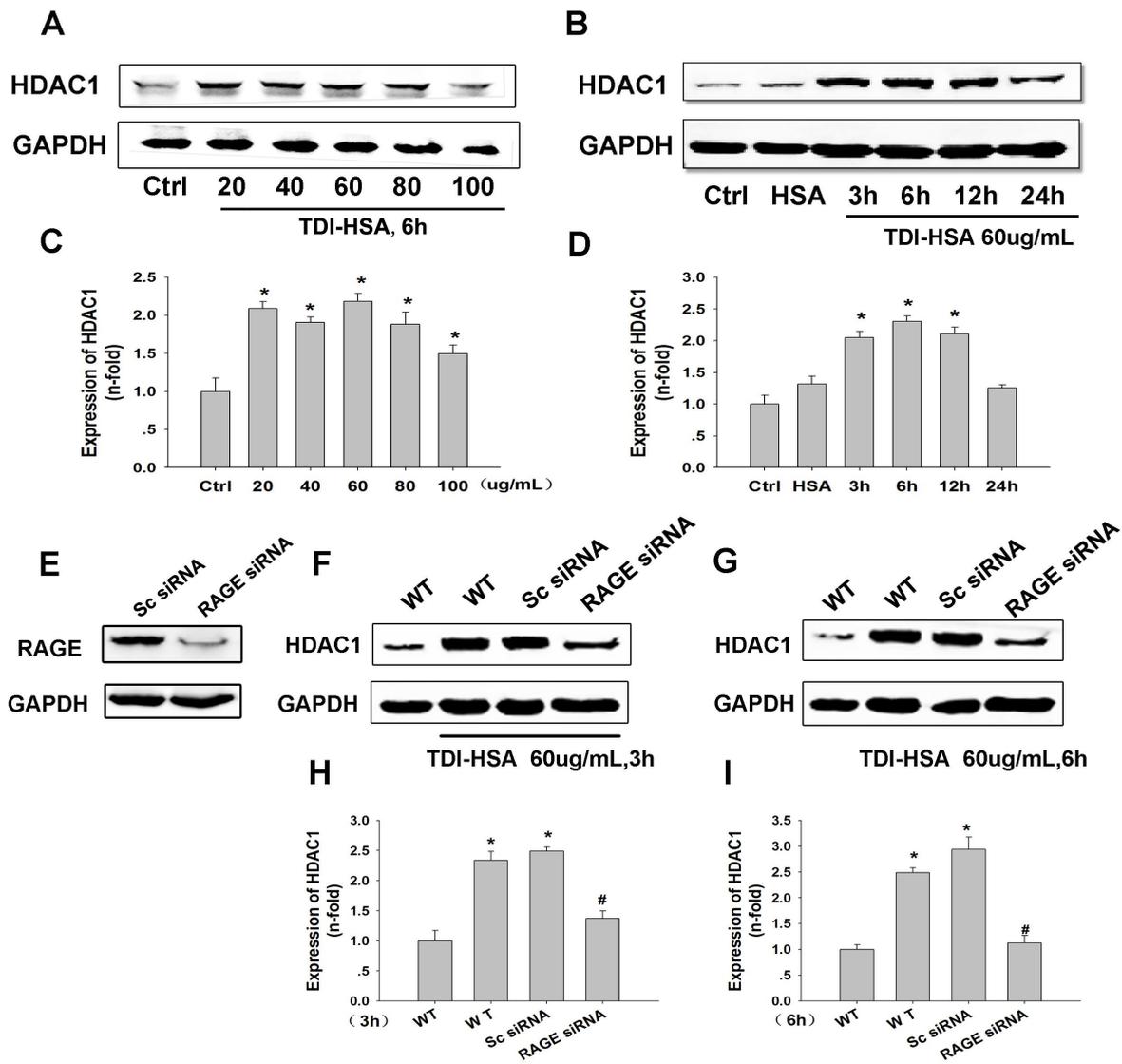


Fig. 4. Effects of RAGE knockdown on the expression of HDAC1 stimulated by TDI-HSA in 16HBE cells.

(A) 16HBE cells were stimulated with various concentrations of TDI-HSA (0, 20, 40, 80, 100 µg/mL) for 6 h, and the expression of HDAC1 was detected. (B) 16HBE cells were stimulated with 60 µg/mL TDI-HSA several times to examine the expression of HDAC1. (E)

Verification of RAGE knockdown in 16HBE cell via Western blotting analysis. (F) Effect of 60 $\mu\text{g}/\text{mL}$ TDI-HSA stimulation for 3 h on the expression of HDAC1 in 16HBE cells. (G) Effect of 60 $\mu\text{g}/\text{mL}$ TDI-HSA stimulation for 6 h on the expression of HDAC1 in 16HBE cells. (C, D, H, I) Desitometric analysis of Western blots. * $P < 0.05$ vs. control. # $P < 0.05$ compared with the Sc siRNA group.

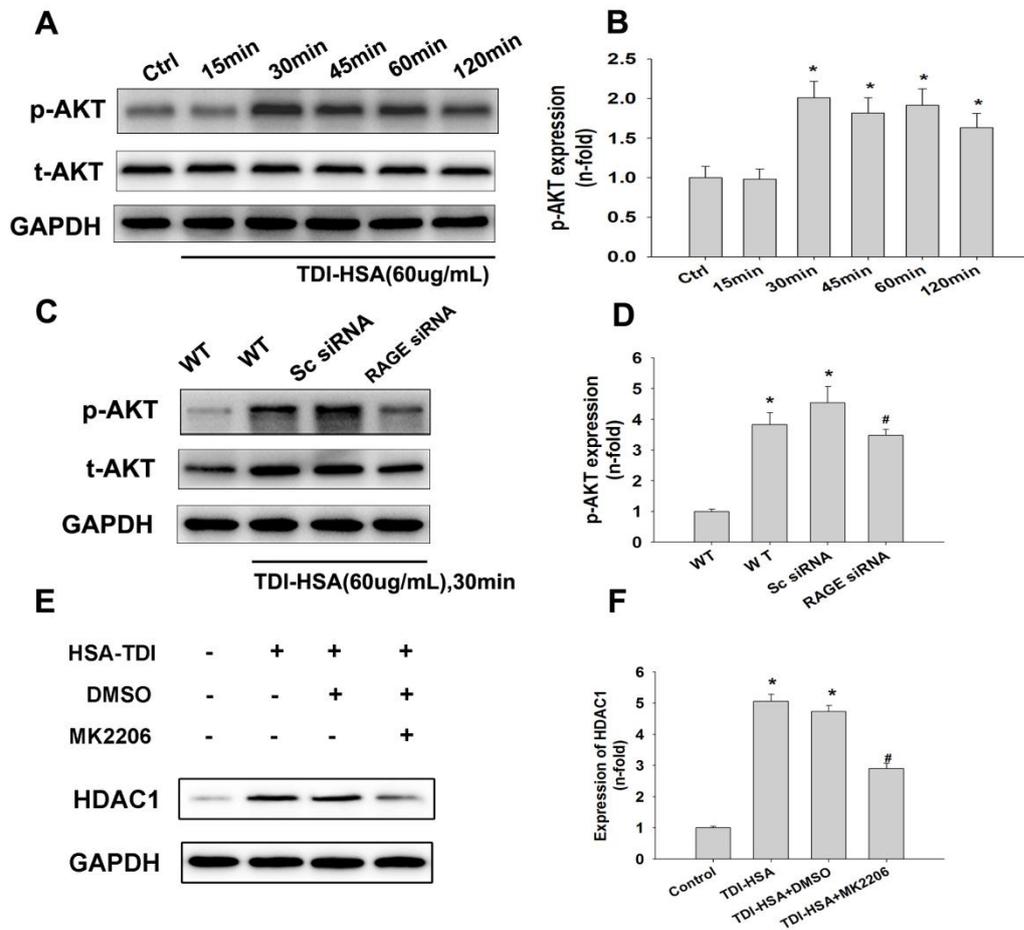


Fig. 5. Effects of AKT pathway intervention on HDAC1 expression induced by TDI-HSA

(A) Western blotting revealed that the phosphorylation of AKT was increased by 60 µg/mL TDI-HSA stimulation. (C) Western blotting showed that RAGE knockdown decreased the expression of p-AKT induced by TDI-HSA in 16HBE cells. (E) Western blotting revealed that AKT inhibition decreased TDI-HSA induced expression of HDAC1 in 16HBE cells. (B, D, F) Desitometric analysis of Western lots. * $P < 0.05$ vs. control. # $P < 0.05$ compared with the Sc siRNA group (D) or TDI-HSA+DMSO group (F).

Figures

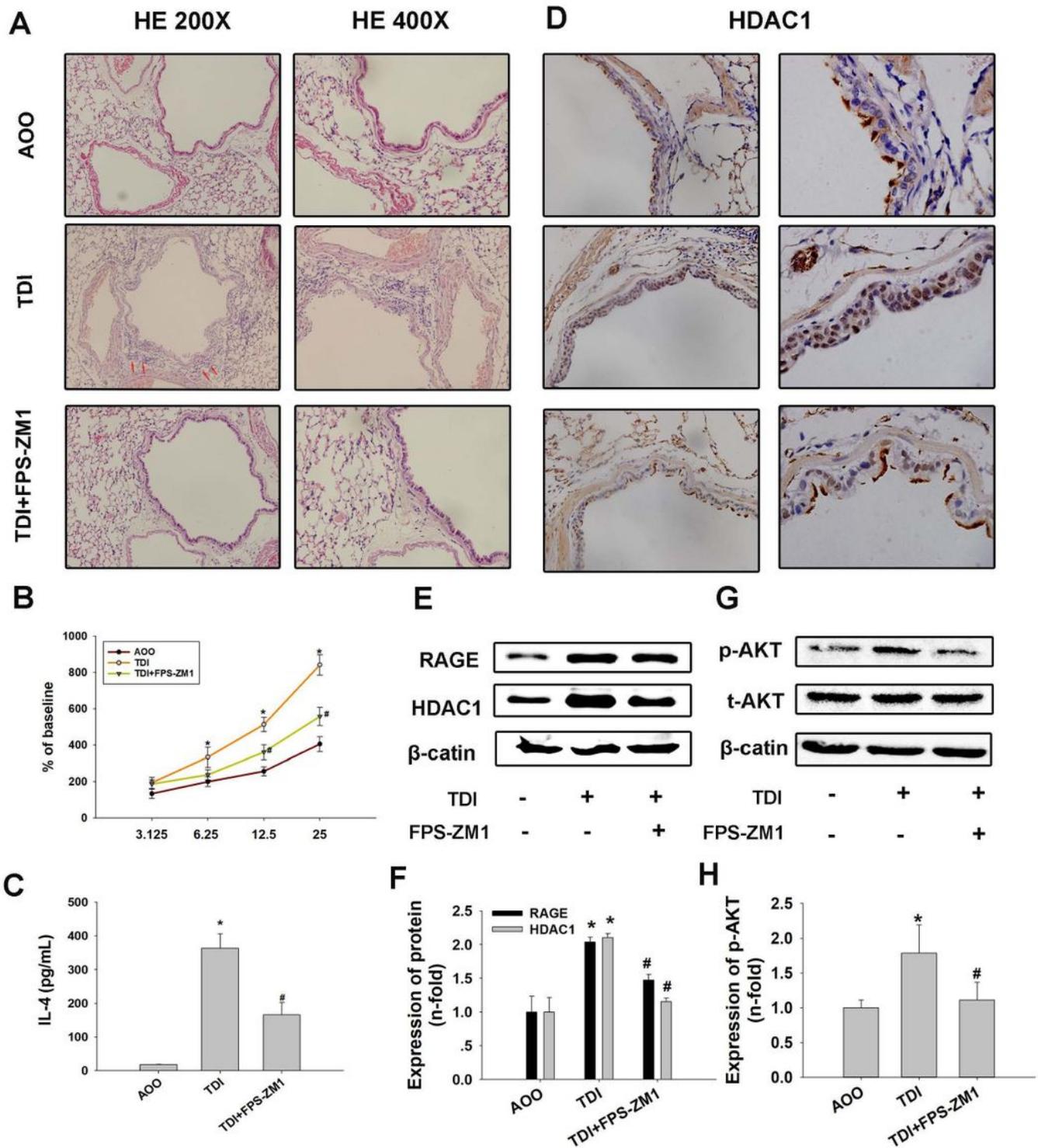


Figure 1

Effects of RAGE inhibitors on the expression of HDAC1 and p-AKT in TDI-induced asthmatic mice (A) Immunohistochemistry analysis of the expression and distribution of HDAC1 in airway epithelium. (B) Western blotting for examination of RAGE and HDAC1 expression in whole lung tissue. (C) Western

blotting for examination of AKT and p-AKT expression in whole lung tissue. (D, E) Densitometric analysis of Western blots. * $P < 0.05$ compared with AOO group; # $P < 0.05$ compared with TDI group. (A) Representative lung sections stained with H&E of each group at 200 \times and 400 \times original magnification. (B) Measurement of airway hyperresponsiveness by lung resistance (RL). Data were expressed as a percentage of the baseline value ($n=5$). (C) Detection of IL-4 in the supernatant of cultured lymphocytes by ELISA. (D) Immunohistochemistry revealed the expression and distribution of HDAC1 in the airway epithelium; (E,G): Western blot showed the expression of RAGE, HDAC1, AKT and p-AKT in whole lung tissue of mice; (F,H): Subsequent densitometric analysis of the blots. * $P < 0.05$ compared with AOO group; # $P < 0.05$ compared with TDI group.

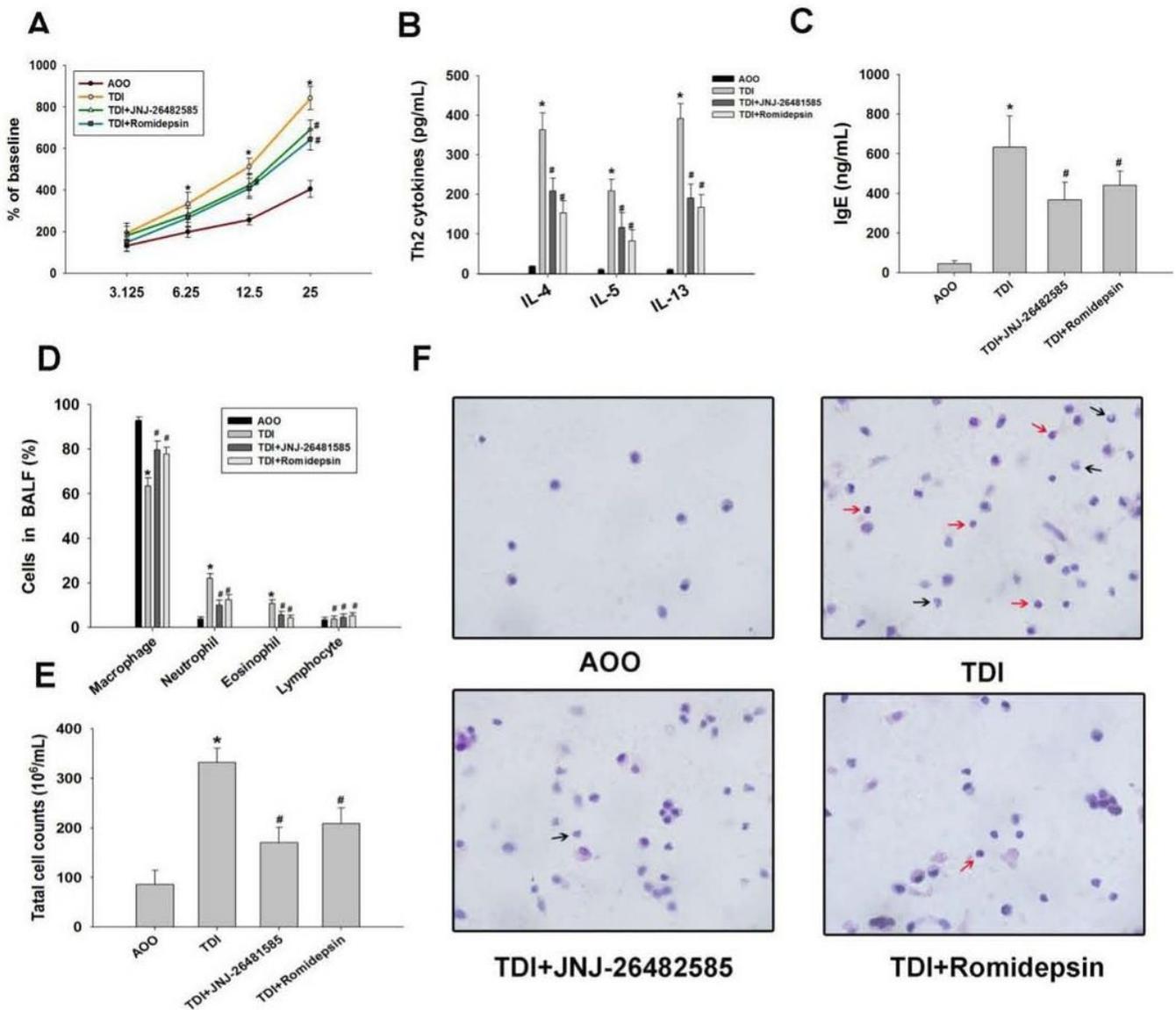


Figure 2

Effects of HDAC inhibitors on the asthmatic response in TDI-exposed mice (A) Measurement of airway hyper-responsiveness by lung resistance (RL). Data were expressed as percentage of baseline value (n = 5). (B) Detection of IL-4, IL-5, and IL-13 in the supernatants of cultured lymphocytes by ELISA. (C) Measurement of IgE in serum by ELISA. (D-F) Total inflammatory cell count and differential cell counts in the BALF of mice. A total of 200 cells stained with hematoxylin and eosin (H&E) in cytopspin samples were counted to calculate the percentages of different inflammatory cells. n=6~8. *P < 0.05 compared with AOO group; #P < 0.05 compared with TDI group.

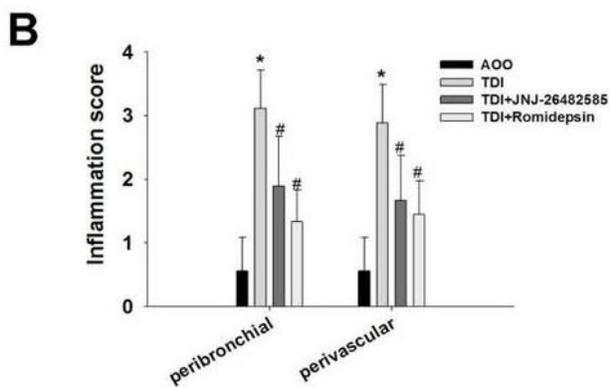
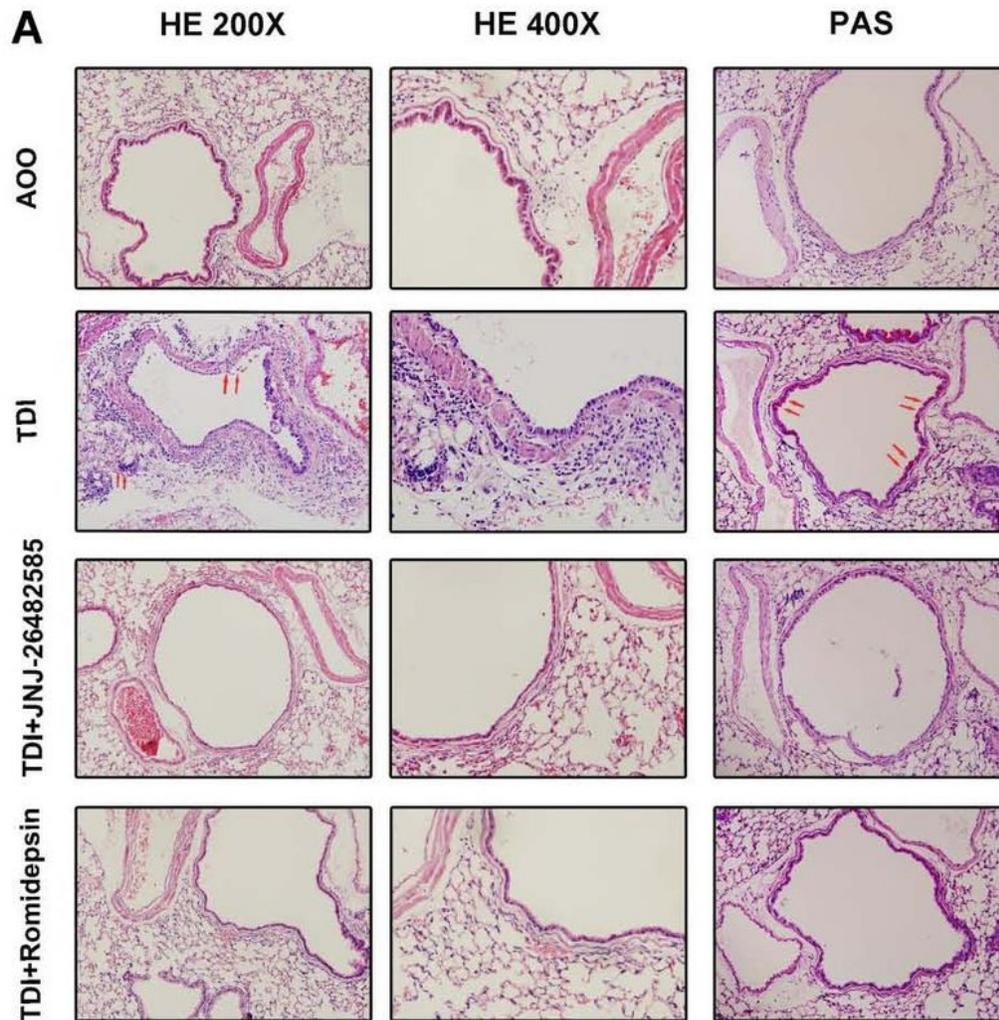


Figure 3

Effects of HDAC inhibitors on lung tissue of TDI-exposed asthmatic mice (A) Representative lung sections of each group stained with hematoxylin and eosin (H&E) at 200× and 400× magnification, and stained with PAS at 400× magnification. (B) Semi-quantitative analysis of airway inflammation. n=8~10. *P < 0.05 compared with the AOO group; #P < 0.05 compared with the TDI group.

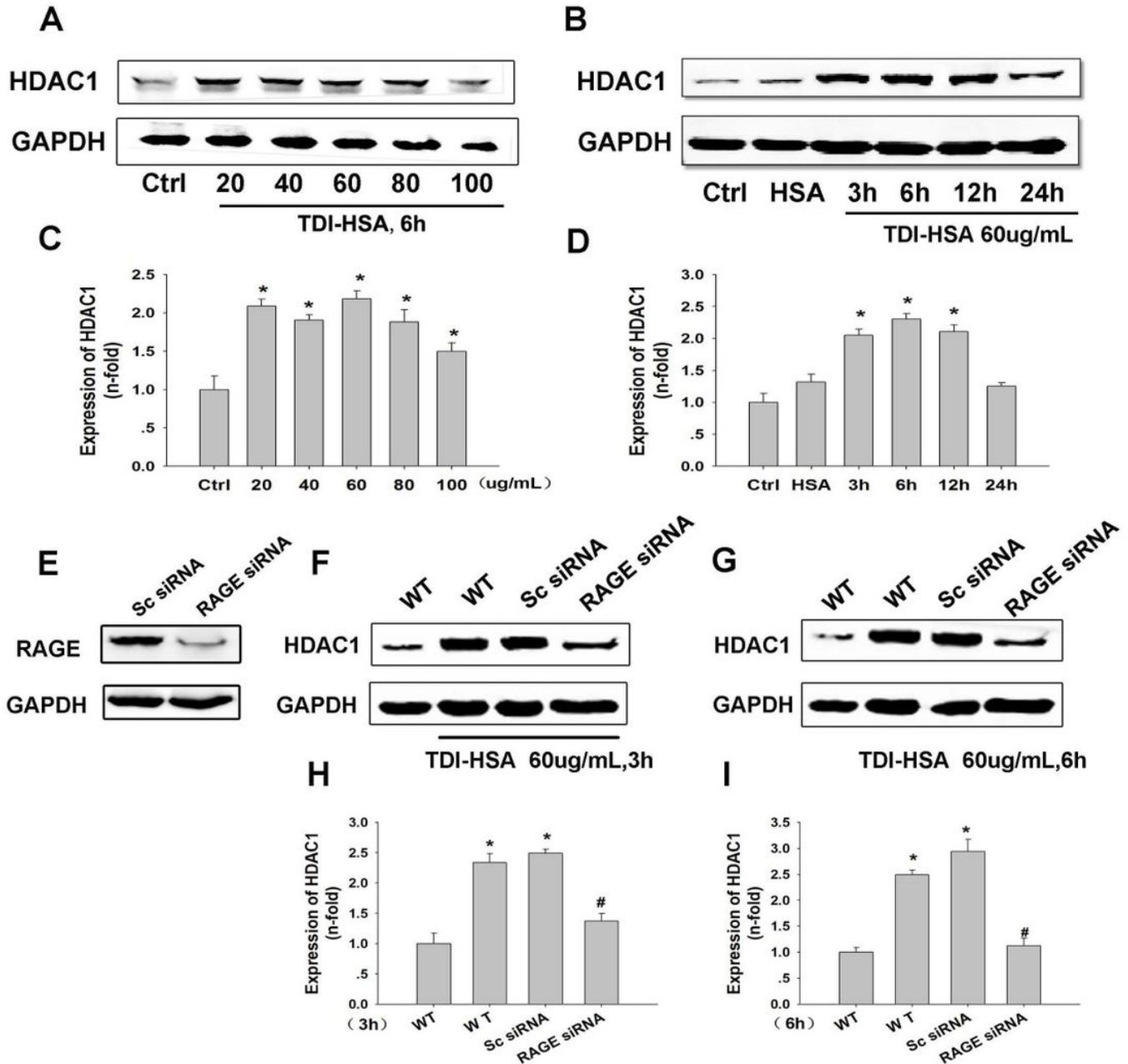


Figure 4

Effects of RAGE knockdown on the expression of HDAC1 stimulated by TDI-HSA in 16HBE cells. (A) 16HBE cells were stimulated with various concentrations of TDI-HSA (0, 20, 40, 80, 100 μ g/mL) for 6 h,

and the expression of HDAC1 was detected. (B) 16HBE cells were stimulated with 60 $\mu\text{g}/\text{mL}$ TDI-HSA several times to examine the expression of HDAC1. (E) Verification of RAGE knockdown in 16HBE cell via Western blotting analysis. (F) Effect of 60 $\mu\text{g}/\text{mL}$ TDI-HSA stimulation for 3 h on the expression of HDAC1 in 16HBE cells. (G) Effect of 60 $\mu\text{g}/\text{mL}$ TDI-HSA stimulation for 6 h on the expression of HDAC1 in 16HBE cells. (C, D, H, I) Desitometric analysis of Western blots. * $P < 0.05$ vs. control. # $P < 0.05$ compared with the Sc siRNA group.

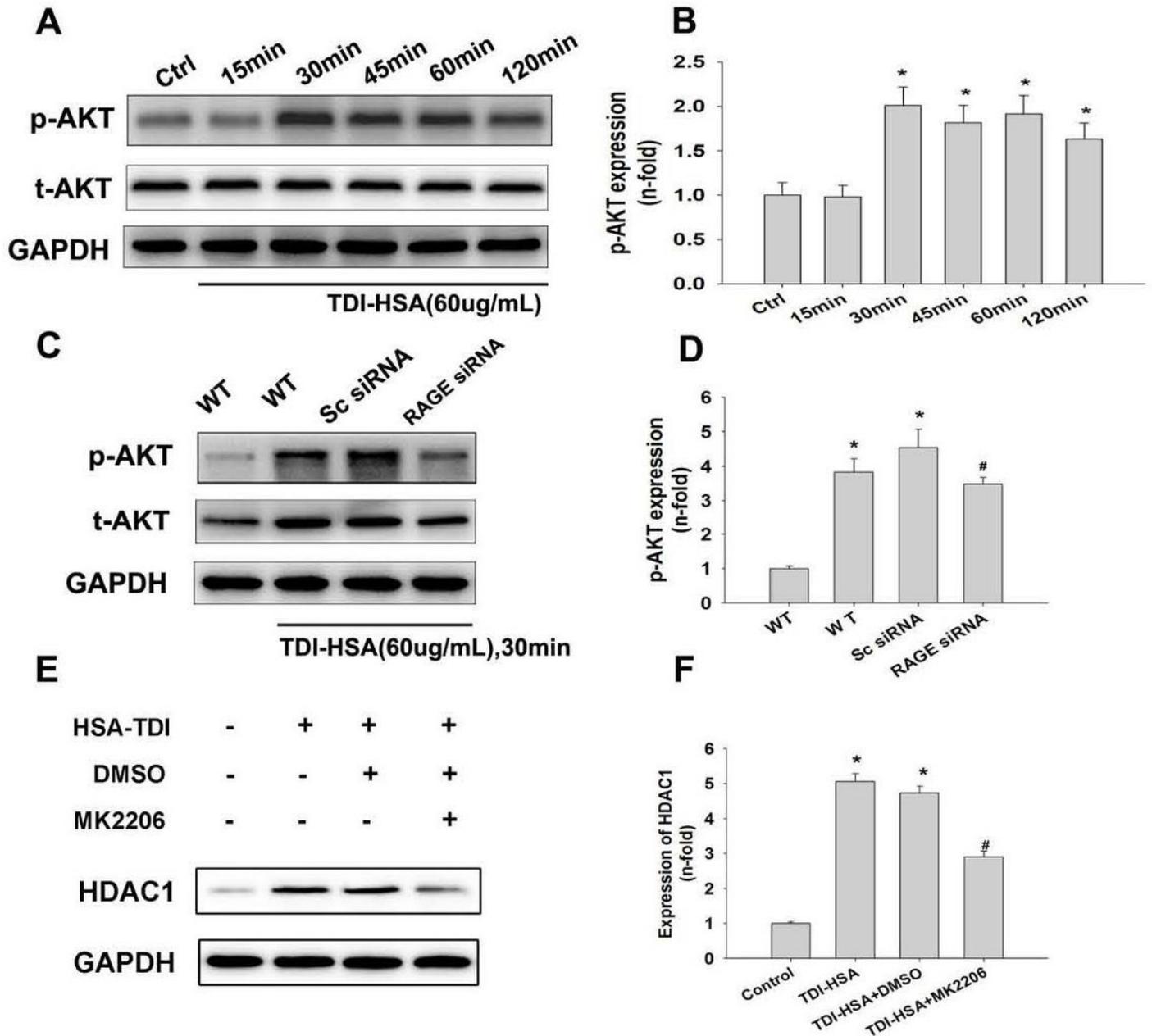


Figure 5

Effects of AKT pathway intervention on HDAC1 expression induced by TDI-HSA (A) Western blotting revealed that the phosphorylation of AKT was increased by 60 $\mu\text{g}/\text{mL}$ TDI-HSA stimulation. (C) Western blotting showed that RAGE knockdown decreased the expression of p-AKT induced by TDI-HSA in 16HBE

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