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PACAP38 improves airway epithelial barrier destruction induced by house dust mites allergen

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ABSTRACT

Purpose: This study aimed to investigate the mechanism of PACAP38 on house dust mite (HDM)-induced asthmatic airway epithelial barrier destruction.

Methods: The HDM-induced asthma mice model and 16HBE cell model was established respectively. The enzyme linked immunosorbent assay (ELSIA), cell count and immunohistochemical assay were performed on mice in control group, HDM group and PACAP38 + HDM group.The cAMP/PKA activity, p-CREB and total CREB expression, TEER and the FITC-DX were investigated on cells in control-16HBE group, HDM-16HBE group and PACAP38 + HDM-16HBE group.

Results: The levels of IL-4 and IL-5 in the HDM group were significantly higher than those in the control group ($P < 0.05$), while the above indexes in the PACAP38 + HDM group were lower than those in the HDM group ($P < 0.05$). E-cadherin, β-catenin, ZO-1 and occludin in the control group were highly immunoreactive in airway epithelial cells, whereas connexin staining was attenuated after HDM induction. The TEER level, cAMP levels and PKA activity were decreased, while FITC-DX transmittance was increased in HDM-16HBE group ($P < 0.05$) compared with the control-16HBE group.

Conclusion: PACAP38 could reduce the airway inflammation, weaken the AJC protein heterotopia and activate cAMP/PKA signaling pathway in HDM-induced asthma, which indicate that PACAP38 may be an important contributor in HDM-induced asthma.

1. Introduction

Asthma is a lung airways disease characterized by airway hyperresponsiveness (AHR), airway inflammation and remodeling (Salehi et al., 2017). It affects approximately 330 million people worldwide, and causes 345,000 people die per year (O'Connell, 2015; Denning et al., 2013). House dust mite (HDM) is a common allergen that triggers human-specific and innate immune responses, which further lead to asthma (Ishii et al., 2018). Allergen-specific immunotherapy can inhibit allergy symptoms, reduce medication, prevent allergic rhinitis and asthma exacerbations (Lew et al., 2018; Hesse et al., 2018). Although some progress in therapy of asthma have been made, the prevention effect and recovery rate of asthma are still unsatisfactory (Beute et al., 2018).

Epithelial tight junction damage and airway cell apoptosis after allergen exposure take part in the development of asthma (Yuan et al., 2018). The permeability of the bronchial epithelial barrier and apical junctional complex (AJC) is very impoartant in regulating epithelial cell differentiation (Georas and Rezaee, 2014; Ivanov et al., 2010). Sweerus et al. have indicated that the increasing dysfunction of the epithelial barrier and permeability lead to antigen sensitization and disease progression in asthmatic patients (Sweerus et al., 2017). Importantly, the respiratory epithelium is not only a barrier against allergens, but also a target for the treatment of respiratory allergies and asthma (Waltl et al., 2018). Pituitary adenylate cyclase active peptide (PACAP) is a neuropeptide with various biological functions such as repair epithelial cells on the surface of the damaged cornea and promote epithelial cell proliferation and differentiation (Lusheng et al., 2015). PACAP38, an active form of PACAP, is a natural pleiotropic neuropeptide with immunomodulatory effects (Khan and Batuman, 2016). Previous studies indicate that PACAP38 play an import role in epithelial cells, inflammatory response, and vasodilation of multiple disorders (Amin and Schytz, 2018; Heppner et al., 2018; Kasica-Jarosz et al., 2018; Waschek et al., 2018). Actually, the biological function of PACAP38 is realized

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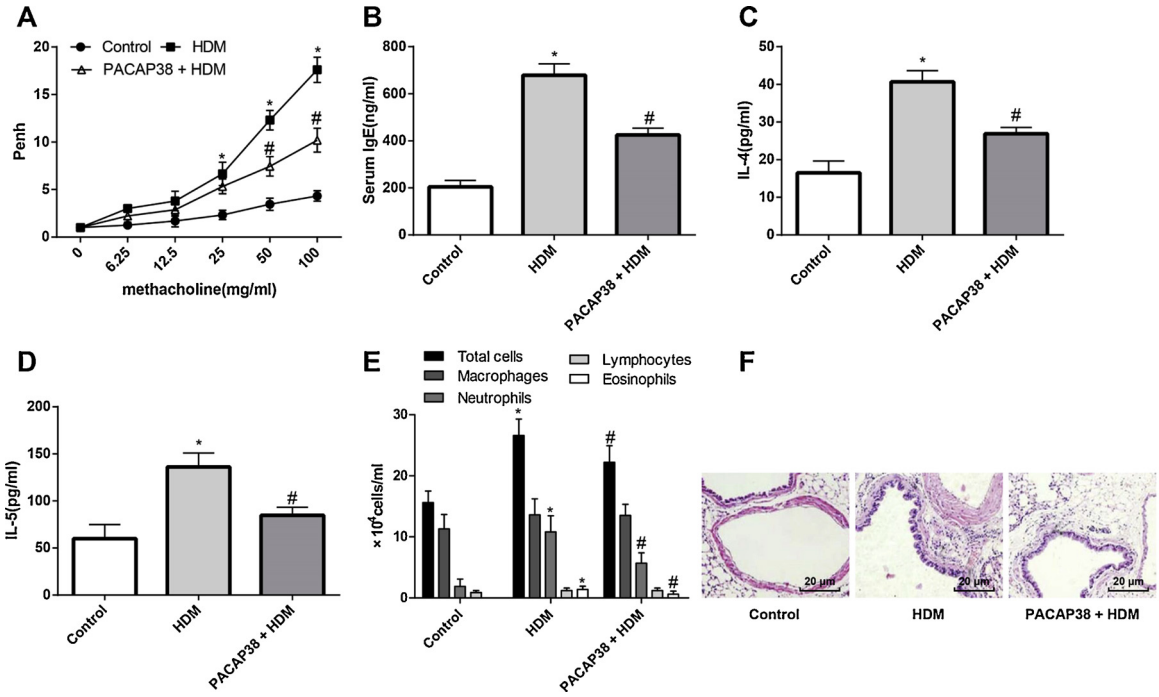

Fig. 1. PACAP38 attenuated AHR and airway inflammation in house dust mites (HDM)-induced asthmatic mice.
(A) Penh value detected by airway reactivity measurement; (B) content of serum lgE detected by ELISA; (C) content of IL-4 in alveolar lavage fluid detected by ELISA; (D) content of IL-5 in alveolar lavage fluid detected by ELSIA; (E) the number of white blood cells and classified cells in alveolar lavage fluid; (F) HE staining of lung tissue in different groups (200×); black arrow represented the inflammatory infiltration around the airway; the triangle symbol represented epithelial shedding; *, $P < 0.05$ versus control group; #, $P < 0.05$ versus HDM group.

through certain pathways such as cAMP/PKA signaling pathway (Ji et al., 2013; Monaghan et al., 2010). However, despite of these sporadic researches of PACAP38 on asthma, the detail mechanism of PACAP38 on HDM-induced asthma is still unclear.

In the current study, HDM-induced asthma mice model and 16HBE cell model was established respectively. Then, the enzyme linked immunosorbent assay (ELSIA), and immunohistochemical assay were performed. We found that PACAP38 could attenuate AHR and airway inflammation in HDM-induced asthmatic mice and inhibit HDM-induced destruction of airway epithelial barrier. This study hoped to reveal the potential mechanism of PACAP38 on HDM-induced airway epithelial barrier destruction in asthma, which might provide a new sight for asthma thearpy.

2. Materials and methods

2.1. HDM model construction

A total of 30 male BALB/c mice (20-24 g, 6–8 weeks, SPF grade) were purchased from Animal Research Institute of Shandong University, China. All mice were randomly divided into control group (n = 10, intranasal given 10 μL PBS), asthma model group (n = 10, intranasal given 10 μg HDM (Greer, Lenoir, NC, USA), dissolved in deionized water) and PACAP38 + HDM group (n = 10, intravenous injection with 0.2 μL of 1 μmol/L PACAP38 (Sigma, USA), followed by intranasal given 10 μg HDM, dissolved in deionized water). After 8 intervention cycles of 5 consecutive days' drug treatment and 2 days' rest, the blood was collected after eye enucleation of each anesthetized (1% pentobarbital) mouse. Then, the left lung of mice were injected with paraformaldehyde fixative solution, followed by paraffin embedding, sectioning, hematoxylin-eosin (HE) staining and preservation after dehydration. This study was approved by the local ethics committee, all experiments were in accordance with the ARRIVE guidelines, which carried out in accordance with National Institutes of Health guide for the care and use of Laboratory animals.

2.2. Detection of AHR

At 24 h after the last nasal drip, AHR was evaluated by detecting the enhanced pause (Penh) value of mice using a barometric plethysmograph (Buxco Electronics, Troy, NY). Brirfly, the Penh values of the mice were determined in response to baseline (non-atomized) and atomized saline with stimulated using gradient methacholine (0, 6.25, 12.5, 25, 50, 100 mg/ml). Each measurement was performed with 1 min nebulization and 3 min monitoring.

2.3. ELSIA assay

The levels of serum lgE, and IL-4 and IL-5 in alveolar lavage fluid were measured in strict accordance with the operating instructions of the corresponding kit (Wuhan Dr. Biological Reagent Co., Ltd., China). The absorbance value was measured by spectraMAX 340 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 450 nm and 550 nm, followed by the levels calculation of lgE, IL-4 and IL-5.

2.4. Blood cells count and lung histological examination

The alveolar lavage fluid was collected, then a total blood cell count and the number of sorted cells were counted by a fully automated blood analyzer SYSMEX XE-2100 (Sysmex Corp. Kobe, Japan). Furthermore, the HE staining was used for the lung histological examination. Briefly, the lung tissues sections of mice in each group were dewaxed with xylene, dehydrated with ethanol of different gradient concentration, and then stained with hematoxylin. The pathological changes were observed under light microscope.

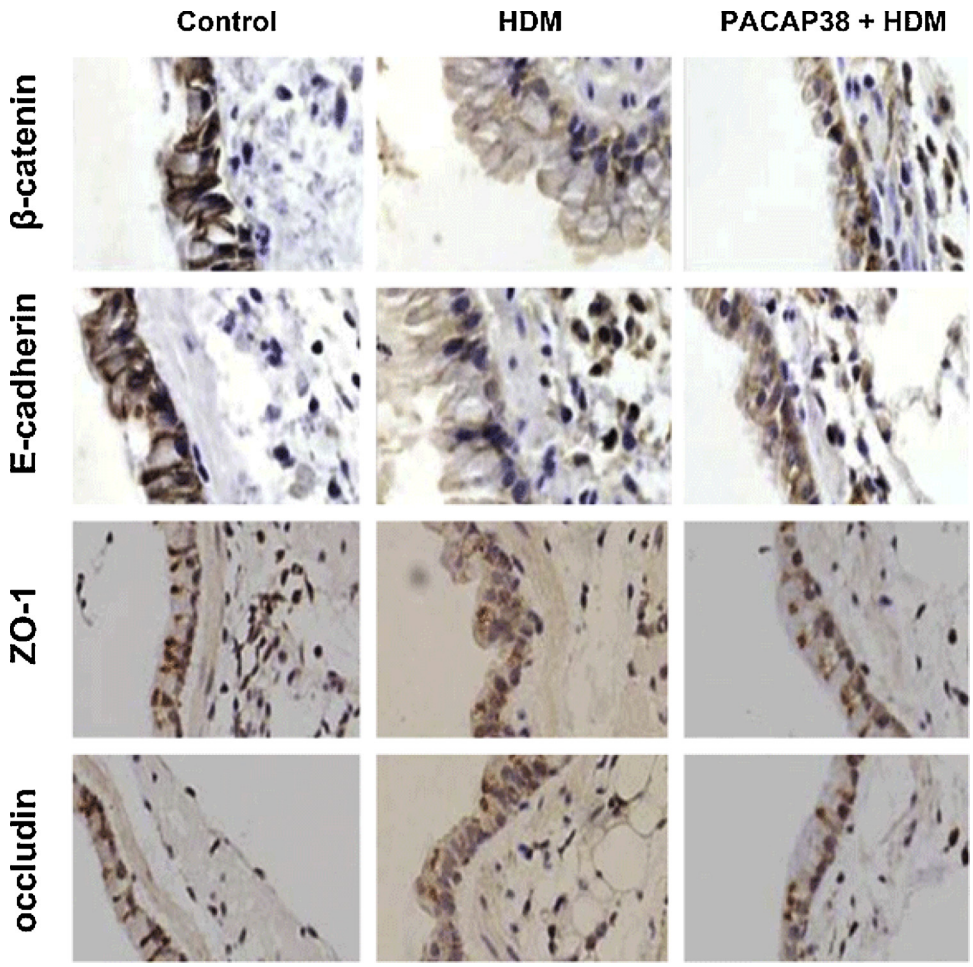

Fig. 2. Effect of PACAP38 on apical junctional complex (AJC) distribution in airway epithelium.
The expression of E-cadherin, beta-catenin, ZO-1 and occludin were all presenting brown (400×).

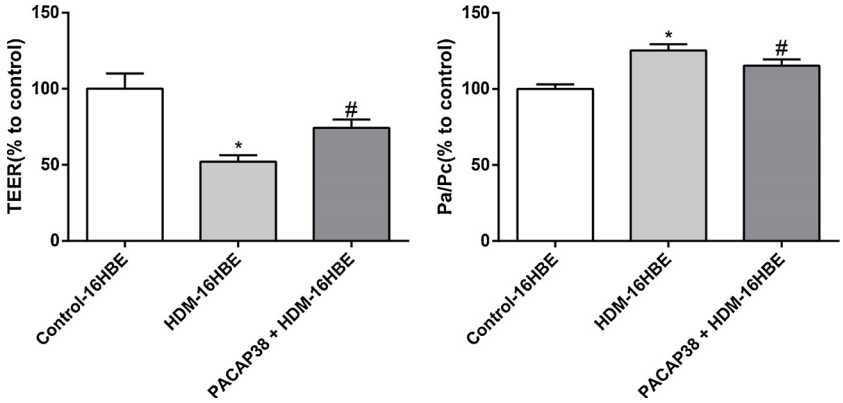

Fig. 3. Effect of PACAP38 on the transepithelial electrical resistance (TEER) and the permeability of fluorescein isothiocyanate labeled dextran (FITC-DX).
(A) the value of TEER in different groups; (B) the value of FITC-DX in different groups; *, $P < 0.05$ versus Control-16HBE group; #, $P < 0.05$ versus HDM-16HBE group.

2.5. Immunohistochemical assay

The expression of E-cadherin, β-catenin, ZO-1 and occludin were detected by immunohistochemical assay. After paraffin section dewaxing of xylene and gradient alcohol rehydration, sections were placed in EDTA antigen repair buffer (pH 9.0 and 3% H2O2) for 20 min. Then the samples were performed with primary antibodies (E-cadherin, β-catenin, ZO-1, occludin, 1:100, Santa Cruz Biotechnology Inc., USA) overnight at 4 °C. Subsequently, the samples were treated with HRP-labeled secondary antibody (goat anti-mouse, 1:500, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) for 15 min at room temperature. The slides were subjected to DAB chromogenic reaction and counterstained with hematoxylin for 30 s, dehydrated, and neutral gums were used for sealing. Five unfolded fields of view were taken from each slice under the microscope for observation and photographing.

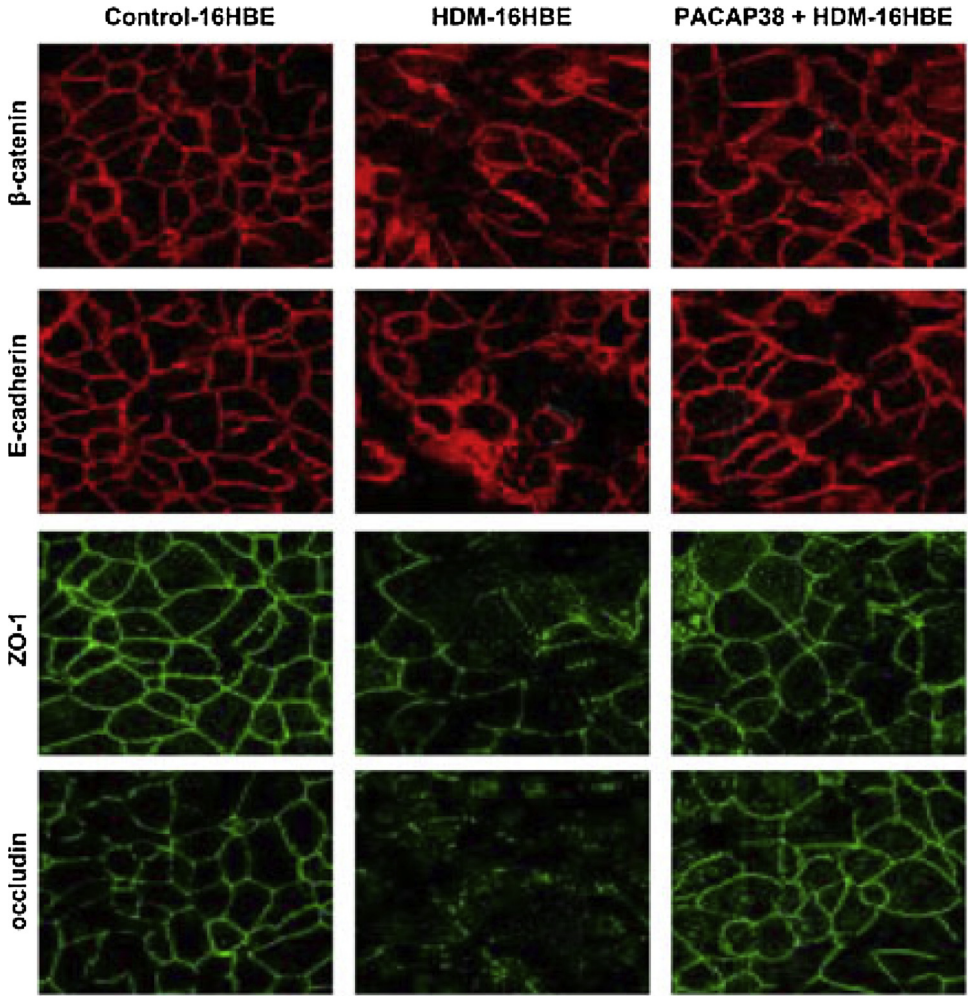

Fig. 4. PACAP38 inhibits house dust mites (HDM)-induced destruction of airway epithelial barrier in asthma.
The white arrow represented the expression of E-cadherin, beta-catenin, ZO-1 and occludin.

2.6. Cell culture and grouping

The 16HBE cells (normal human bronchial epithelial cell line, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China) were added to RPMI-1640 complete medium (with 10% fetal bovine serum, HyClone, USA) and penicillin (Gibco, USA), and cultured in an incubator with 37 °C and 5% CO_2. After were digested with 0.25% trypsin, all cells were divided into Control-16HBE group, HDM-16HBE group (stimulated with 400 U/mL HDM (ALK, Denmark)) and PACAP38 + HDM-16HBE group (treated with 100 nmol/l PACAP38 (Sigma, USA) for 1 h, followed by stimulation with 400 U/mL HDM).

2.7. Detection of bronchial airway epithelial membrane resistance (TEER) and dextran permeability (FITC-DX)

For TEER detection, a total of 500 μL cell was suspended and placed in a 12-well plate chamber of Transwell (2 × 10 (Lew et al., 2018) cells/mL for a well), followed by TEER (Ax) value measurement with Millicell transmembrane resistance meter ERS-2 (Millipore, USA). Furthermore, for FITC-DX detection, cells were added to a 24-well plate chamber of Transwell (1 × 10 (Lew et al., 2018) cells/mL, 200 μL/well). Then, a total of 200 μL phenol red-free RPM ll640 (Sigma, USA) containing 0.5 mg/mL luciferin isothiocyanate (FITC-DX) was added to the upper chamber, and 500 μL of phenol red-free RPM ll640 without FITC-DX was added to the lower chamber. A 24-well plate containing cells and drugs was placed in the incubator for 90 min. A total of 100 μl of the liquid from the upper and lower chambers of the Transwell were transferred to a black 96-well plate in dark, respectively. Fluorescence values were measured using a TECAN Infinite M200-Multichannel Microplate Reader (TECAN, Switzerland) and FITC-DX permeability was calculated.

2.8. Immunofluorescence

The expression of E-cadherin, β-catenin, ZO-1 and occludin were detected by immunofluorescence assay. Simply, 16HBE cells were fixed with 4% paraformaldehyde, washed by PBS, treated with 0.2% Triton X-100 and blocked with 5% bovine serum albumin for 3 h. Then, primary antibodies (E-cadherin, β-catenin, ZO-1, occludin, p-CREB, Santa Cruz Biotechnologies, USA) were added overnight at 4 °C. Following the addition of Alexa 488-labeled goat anti-rabbit or Alexa 568-labeled goat anti-rabbit secondary antibody (Life Inc., USA), incubation was carried out for 1 h at 37 °C. Subsequently, DAPI (Sigma, USA) was added and stained for 10 min. The treated cells were photographed under a laser confocal microscope (Olympus, Tokyo, Japan) and images were processed using FV10-ASW 2.0 Viewer software (Olympus, Tokyo, Japan).

2.9. The activity analysis of cAMP/PKA kinase

The level of cAMP and the activity of PKA in 16HBE cells were

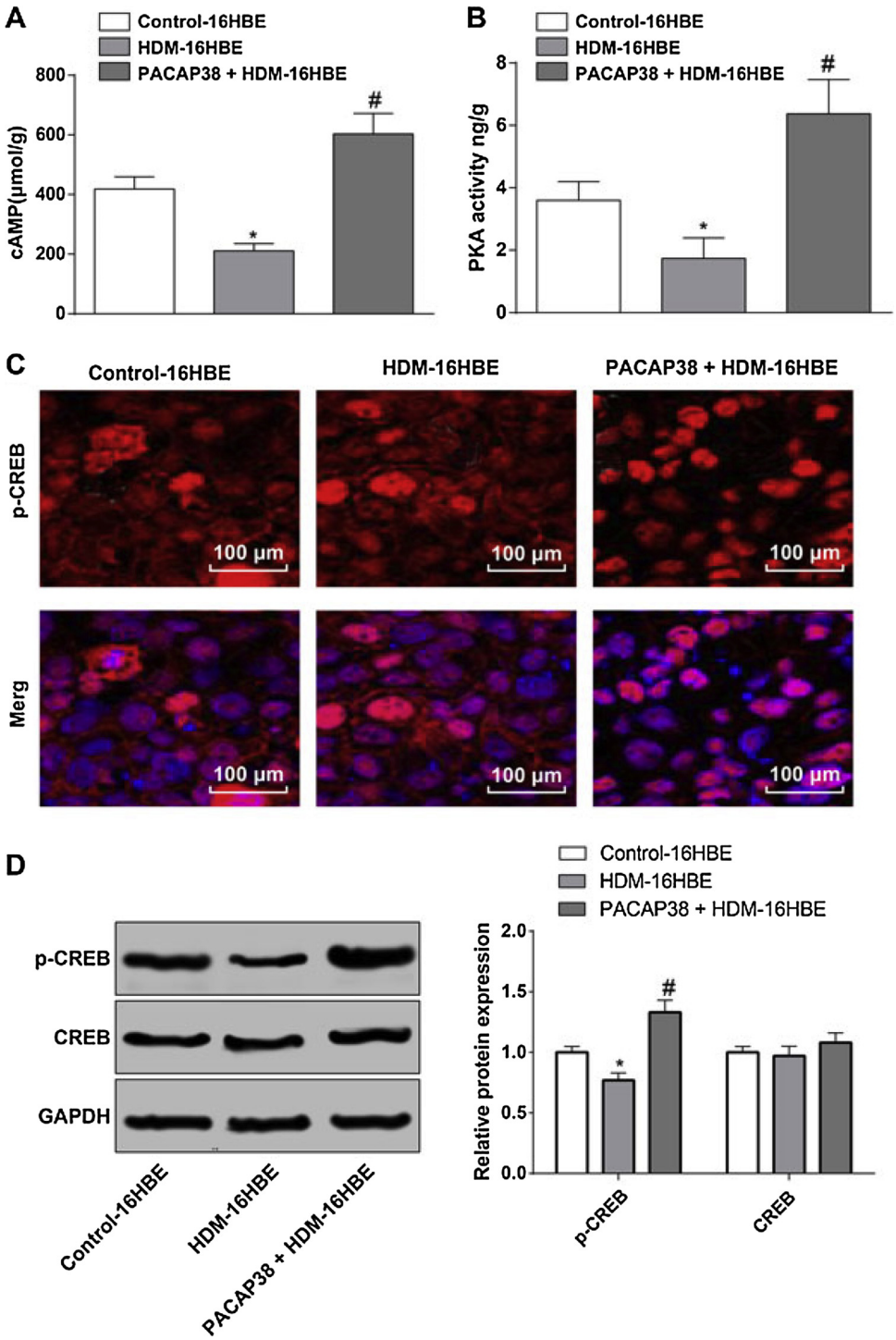

Fig. 5. PACAP38 activated the cAMP/PKA signaling pathway.
(A) the level of cAMP under PACAP38 treatment; (B) the activity of PKA under PACAP38 treatment; (C) the localization of p-CREB (red) and nuclei (blue) by immunofluorescence and confocal microscopy; arrow represented the nuclear localization of p-CREB in PACAP38-treated cells (40 μm); (D) the expression of p-CREB and total CREB protein detected by Western blot; *, $P < 0.05$ versus control-16HBE group; #, $P < 0.05$ versus HDM-16HBE group.

determined by cAMP enzyme immunoassay and PKA kinase activity kit measurement (Enzo Life Sciences, Farmingdale, NY).

2.10. Western blot assay

After 48 h of cell stimulation, Total proteins of different groups were harvested and lysed in cold RAIA buffer. After centrifugation, 20 μg protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis, and transferred to a poly (vinylidene fluoride) (PVDF) membrane. Then the membrane was blocked with 5% skim milk for 2 h. Then, all samples were treated with the primary antibodies (rabbit anti-human, E-cadherin, β-catenin, ZO-1, occludin, PKA, p-CREB, CREB, β-actin, GAPDH, 1:1000, Cell Signaling, Boston, USA). Finally, the membrane was incubated with HRP-labeled goat anti-rabbit IgG secondary antibody (1:5000, Cell Signaling, Boston, USA) for 1 h. Protein brands were visualized using Gel imaging system (Thermo Fisher

Scientific).

2.11. Statistical analysis

All data were processed using SPSS 21.0 statistical software and expressed as mean ± SD. For data between two groups, the *t*-test and one-way ANOVA followed by Fisher's least significant difference (LSD) were used for the data analysis. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. PACAP38 attenuated AHR and inflammation in HDM-induced asthmatic mice

The AHR assay showed that compared with the control group, the Penh value of airway responsiveness index in HDM group was significantly higher ($P < 0.05$) (Fig. 1A). Moreover, the ELISA assay showed that compared with the control group, the serum lgE in HDM group increased significantly ($P < 0.05$) (Fig. 1B), and the expression of inflammatory factors IL-4 and IL-5 in alveolar lavage fluid increased ($P < 0.05$) (Fig. 1C-D). Furthermore, the calculation of leukocyte and classified cell showed that compared with the control group, the total white blood cells, neutrophils and eosinophils in HDM group increased significantly ($P < 0.05$) (Fig. 1E). Compared with HDM group, the peribronchial inflammation and cells accumulated in the airway cavity were significantly alleviated in PACAP38 + HDM group ($P < 0.05$). HE staining assay showed that compared with the control group, the inflammatory cell infiltration of airway epithelium in HDM group was observed in HDM group ($P < 0.05$) (Fig. 1F), but alleviated in PACAP38 + HDM group.

3.2. PACAP38 inhibited AJC protein translocation in airway epithelium

Immunohistochemical staining analysis showed that the E-cadherin, β-catenin, ZO-1 and occludin have strong immunoreactivity at the lateral and apical borders of airway epithelial cells. However, the induction of HDM resulted in a decrease in connexin staining in epithelial cells at the pericapsular contact site, while treatment with PACAP38 partially could reverse these effects (Fig. 2).

The expression of E-cadherin, beta-catenin, ZO-1 and occludin were all presenting brown (400×).

3.3. PACAP38 improved TEER values and reduced FITC-DX transmittance

The detection of TEER and FITC-DX showed that compared with the control group, the compared with the control group, HDM could decrease TEER ($P < 0.05$) (Fig. 3A) and increase FITC-DC transmittance (Pa/Pc) ($P < 0.05$) (Fig. 3B). After pretreatment with PACAP38, TEER and FITC-DC transmittance (Pa/Pc) was significantly increased ($P < 0.05$) and decreased ($P < 0.05$) respectively in PACAP38 + HDM-16HBE group when compared with HDM-16HBE group.

3.4. PACAP38 reduced the abnormal distribution of AJC

The immunolabeling of 16HBE cells was used to detect E-cadherin, β-catenin, ZO-1 and occludin proteins. Immunofluorescence was used to observe the abnormal distribution and destruction of these proteins on the cell membrane. The results showed that PACAP38 treatment could partially improve the abnormality of the above indicators caused by HDM (Fig. 4).

3.5. PACAP38 activated cAMP/PKA signaling pathway

Compared with Control-16HBE group, the levels of cAMP and PKA in HDM-16HBE group were significantly decreased ($P < 0.05$), while the levels of cAMP and PKA in PACAP + HDM-16HBE group were significantly higher than those in HDM-16HBE ($P < 0.05$) (Fig. 5A-B). Immunofluorescence results showed that phosphorylated CREB expression was observed in PACAP + HDM-16HBE group (Fig. 5C). The Western blot assay showed that compared with control-16HBE group, phosphorylated CREB protein in HDM-16HBE group decreased significantly ($P < 0.05$). Meanwhile, compared with HDM-16HBE group, the amount of phosphorylated CREB in PACAP + HDM-16HBE group increased significantly ($P < 0.05$) (Fig. 5D).

4. Discussion

Asthma is an inflammatory disease serious affect the quality of life (Nurmagambetov et al., 2018). Although some influence factors such as PACAP38 take part in the process of the inflammatory response (Khan and Batuman, 2016), the detail mechanism of PACAP38 in asthma is still unclear. This study revealed the potential biological function of PACAP38 based on HDM-induced asthma mice model and 16HBE cell model. The result showed that by activating the cAMP/PKA signaling pathway, PACAP38 might alleviate airway hyperresponsiveness and inflammation induced by HDM in mice model, inhibit the translocation of AJC protein in airway epithelium, and alleviate the changes of TEER/FITC-DX transmittance in 16HBE cells.

The cAMP/PKA signaling can regulate excessive cellular function and cascade controlled many biological properties in almost all eukaryotic cells (Castro et al., 2016). Specifically, PACAP reversed AHR induced by environmental pollution not only through its bronchodilating activity, but also by counteracting pro-inflammatory and pro-oxidative effects (Tlili et al., 2015). Mei et al. showed that PACAP inhibited apoptosis of cerebellar granule cells and promoted cell survival by activating the cAMP/PKA transduction pathway (Mei et al., 2015). Actually, the intracellular cAMP has an opposite effect on airway smooth muscle, and the receptor stimulating cAMP pathway was the therapeutic target of asthma (Dale et al., 2018). Despite of that, a previous study indicates that concentrations of serum IL-4 in asthmatic mice was elevated (Fan et al., 2015). George et al. (George et al., 2017) reported an increase in the number of neutrophils and eosinophils in asthmatic mice compared to normal mice, and the release of cytokines IL-4 and IL-5 was also increased by HDM exposure. In the current study, the immunofluorescence and Western blot indicated that PACAP38 down-regulated the expression of AHR in HDM-induced asthmatic mice via activating cAMP/PKA pathway. Meanwhile, the signaling pathway analysis showed that PACAP38 reduced the up-regulation of cAMP, PKA activity and phosphorylated CREB protein in 16HBE cells induced by HDM. Moreover, the ELISA assay showed that PACAP38 treatment significantly reduced IL-4 and IL-5 levels. Thus, based on these results in this study, we speculated that PACAP38 might reduce the airway inflammation via cAMP/PKA pathway in HDM-induced asthma.

Structural and functional deficiencies in airway epithelial cells increase susceptibility to asthma and exacerbate the severity of asthma (Liu et al., 2018). A previous study shows that AJC maintained the integrity of the epithelial monolayer through cell contact (Yuki et al., 2008; Tamar et al., 2008). The maintenance of AJC mainly involved the actin cytoskeleton, including E-cadherin, β-catenin, ZO-1 and occludin (Yuki et al., 2008; Laukoetter et al., 2006). A previous study shows that the TJS integrity of bronchial epithelial cells in asthmatic patients is significantly reduced in healthy lung injury culture compared to healthy subjects (Wawrzyniak et al., 2016). Le et al. showed that HDM caused abnormal distribution of adhesion-linked protein E-cadherin and β-catenin above the cell membrane, which was diffused from the cell membrane to the cytoplasm (Yanqing, 2017). Dong et al. indicated that epithelial barrier disruption and adhesion of connexins E-cadherin and β-catenin were lost in HDM-treated 16HBE cell membrane (Dong et al., 2017). In this study, the TEER and FITC-DX value was decreased and increased respectively under high permeability of epithelial cell monolayer induced by HDM stimulation. Thus, we speculated that

PACAP38 might play an important role in maintaining the normal distribution of AJC protein, which further reduce the damage of airway epithelial cell barrier. However, there were some limitations in current study such as small simple size and lack of verification analysis. Thus, a further verification analysis based on a large sample size is needed.

5. Conclusions

In the present study, our results demonstrate that PACAP38 could reduce the airway inflammation via cAMP/PKA signaling pathway in HDM-induced asthma. PACAP38 might play an important role in maintaining the normal distribution of AJC protein, which further reduce the damage of airway epithelial cell barrier. Findings of this study may provide additional insights into the importance of PACAP38 in HDM-induced asthma and PACAP38 maybe serev as a potential therapeutic target. However, the exact effects of PACAP38 alone on the anti-inflammatory and anti-asthmatic effects, and on the activation of cAMP/PKA signaling pathway in mice or/and in epithelial cells remain to be further investigated in future studies.

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Declaration of Competing Interest

None.

Acknowledgments

None.

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