



Alleviating airway inflammation by inhibiting ERK-NF- κ B signaling pathway by blocking Kv1.3 channels

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ARTICLE INFO

Keywords:

Asthma
Airway inflammation
Kv1.3 channel
T lymphocyte
ERK
NF- κ B

ABSTRACT

Allergic asthma is a chronic inflammatory disease of the airways. T lymphocytes play an important role in the pathogenesis of asthma. The voltage-gated Kv1.3 potassium channel is a target for the preferential inhibition of T_{EM} cells. In this study, we investigate the effects of PAP-1, a selective inhibitor of Kv1.3 channel, on the treatment of the neutrophilic asthma model. PAP-1 (40 mg/kg) was injected intraperitoneally into ovalbumin (OVA)-lipopolysaccharide (LPS)-challenged BALB/c mice. We found that the expression of the Kv1.3 channel in the lung tissues, and the intensity of the Kv current in the asthmatic mice increased clearly compared with those in normal control. PAP-1 significantly reduced airway hyperresponsiveness (AHR), inflammatory cell count in the bronchoalveolar lavage fluids (BALF) and serum, and attenuated airway inflammation in a histological examination of the asthmatic mice. Moreover, PAP-1 inhibited the OVA-LPS-induced imbalance of Th1/Th2, Treg/Th17 lymphocytes, and reduced levels of IL-4 and IL-17, inducing an increase in the production of IFN- γ and IL-10. Furthermore, the activation of the extracellular signal-regulated kinase (ERK)/nuclear factor- κ B (NF- κ B) pathway in the lungs of the asthmatic mice was suppressed by PAP-1. We also found that PD-98059, an inhibitor of ERK, had a similar effect with PAP-1 in terms of regulating the imbalance of Th1/Th2, Treg/Th17 cytokines. However, PD-98059 could not further influence cytokine changes when the cells were treated with PAP-1. The results suggest that ERK acts as a downstream regulator of inhibitors of the Kv1.3 channel in neutrophilic asthma. In conclusion, the inhibitor of the Kv1.3 channel has therapeutic potential for treating asthma.

1. Introduction

Asthma is a chronic inflammatory disease of the airways. The most widely used mouse model of asthma involves allergic sensitization by intraperitoneal injections of ovalbumin (OVA) and aluminum hydroxide, challenged by an aerosol or intranasal instillation of OVA. Pulmonary inflammation here is mediated by Th2 cells [1]. However, studies have shown that some severe asthmas, and glucocorticoids-dependent or -resistant asthmas, are mediated by Th17 cells with the infiltration of neutrophils [2–4]. Therefore, it is important to seek reliable drugs for the treatment of Th17-mediated neutrophilic asthma.

Voltage-gated Kv1.3 potassium channel is a target for the preferential inhibition of T_{EM} cells [5], and has been reported to be involved in the pathogenesis of T-cell-mediated autoimmune diseases, such as autoimmune encephalomyelitis, rheumatoid arthritis (RA), inflammatory bowel disease, and psoriasis [6–9]. Koshy et al. investigated the effects of ShK-186, a selective Kv1.3 channel blocker, for the treatment of asthma, and found that ShK-186 can reduce Th2-mediated

response [10]. Although a large number of biological and pharmacological activities of the Kv1.3 channel have been reported, its mechanisms and possible effects on Th17-mediated neutrophilic asthma are still unknown. Several studies have shown that the Kv1.3 channel mediates autoimmune diseases through the activation of the extracellular signal-regulated kinase (ERK)-nuclear factor (NF)- κ B (NF- κ B) signaling pathway [11, 12].

ERK, a member of the mitogen-activated protein kinase (MAPK) family, is a key molecule that transfers signals from the cell surface to the nucleus. Phosphorylated ERK mediates the degradation of I κ B α and the activation of transcription factors, such as NF- κ B. Some researches have claimed that the activation of the ERK signaling pathway is related to the response of IL-17 [4, 13].

In this study, we investigate the expression and function of the Kv1.3 channel in lung tissues and the bronchoalveolar lavage fluid (BALF) in the Th17-mediated neutrophilic asthmatic mouse model. Furthermore, 5-(4-phenoxybutoxy) psoralen (PAP-1) is a potent and selective small molecule Kv1.3 blocker [14, 15], we investigate

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<https://doi.org/10.1016/j.intimp.2018.07.009>

Received 11 February 2018; Received in revised form 29 June 2018; Accepted 10 July 2018

1567-5769/© 2018 Published by Elsevier B.V.

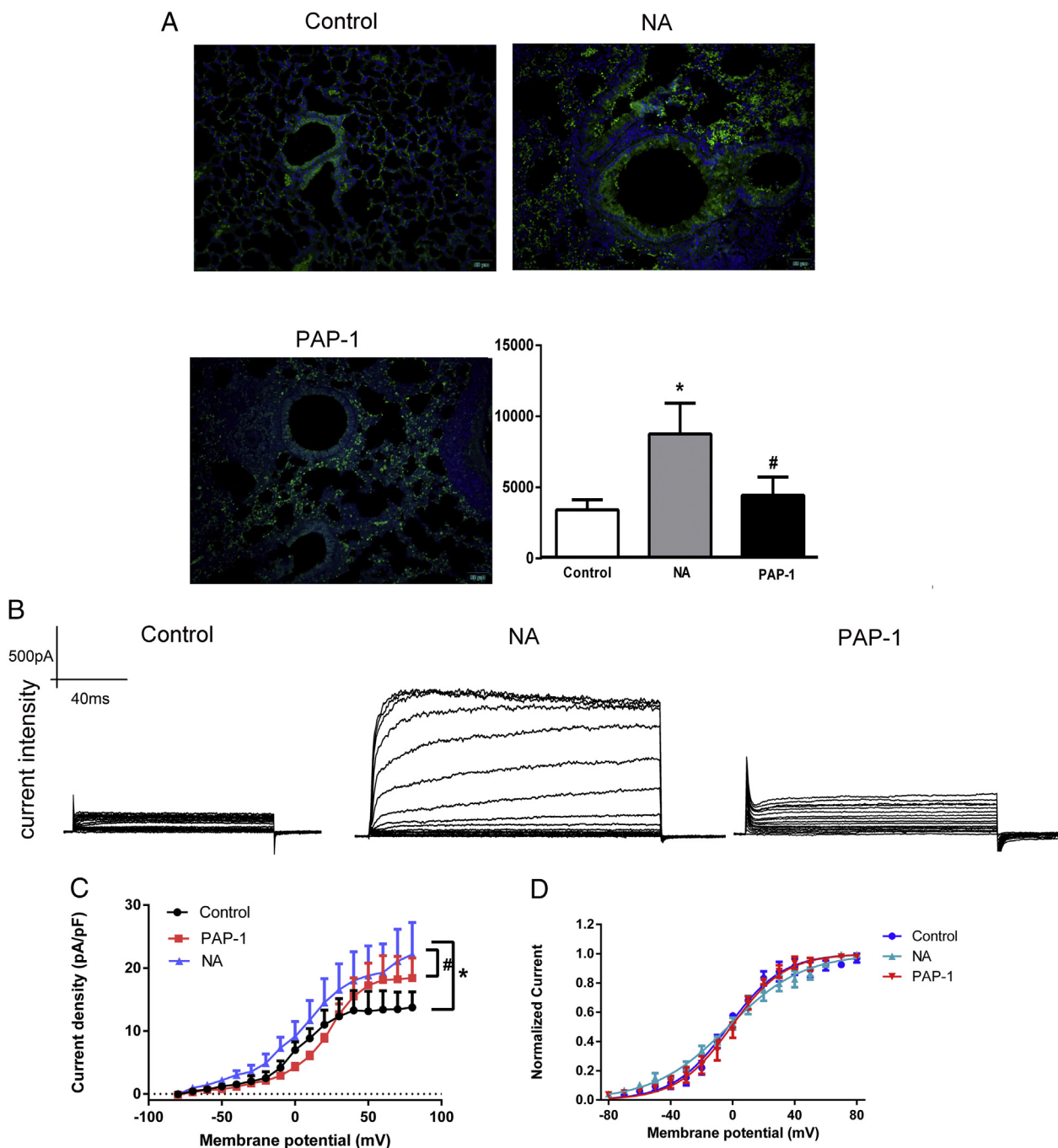


Fig. 1. The expression and activities of Kv1.3 increase in the lung tissues and BALF of in OVA-LPS-induced neutrophilic asthmatic mice. (A) Kv1.3 protein in lung tissues increased in the OVA-LPS-induced neutrophilic asthmatic mice model detected by immunofluorescence. (B) Representative currents obtained by step pulses from -80 to 80 mV in 10 mV steps. (C) The peak current to voltage relation (I - V curve) of I_{Kv} . The intensity of the I_{Kv} increased clearly in the OVA-LPS-induced neutrophilic asthmatic mice model, which was significantly reduced in PAP-1-treated mice. (D) The voltage-dependence of KV activation was estimated by fitting normalized conductance against clamp voltages using the Boltzmann distribution equation. There was no difference in the voltage-dependence of I_{Kv} in different groups.

BALF: Bronchoalveolar lavage fluid. MFI: Mean fluorescence intensity. NA: neutrophilic asthmatic group. Each value represents the mean $\pm$ SD ($n = 8$).

* $P < 0.01$ versus the control group.

$P < 0.01$, versus the NA group.

mechanisms of PAP-1, and its effects on the Th17-mediated neutrophilic asthmatic mouse model.

2. Materials and methods

2.1. Animals and groups

Female BALB/c mice (eight–12 weeks old, 20 ± 2 g) were obtained from the China Medical University (Shenyang, Liaoning, China). The animals were used in accordance with the regulations of the Ethics

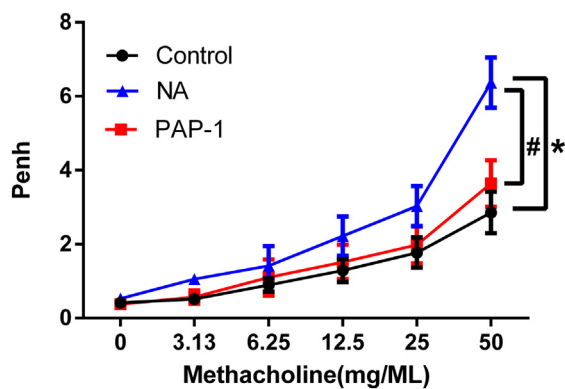


Fig. 2. PAP-1 reduces airway hyperresponsiveness of mice in response to inhaled methacholine. The airway responsiveness monitoring index-enhanced pause (Penh) was recorded. NA: neutrophilic asthmatic group. Each value represents the mean $\pm$ SD ($n = 6$).

* $P < 0.01$ versus the control group.

$P < 0.01$, versus the NA group.

Committee of Shengjing Hospital of the China Medical University (2015PS261K). The mice were free fed in cages in a temperature range of 20–25 °C and a relative humidity of 40%–70%, and the cages were lit 12 h per day.

2.2. NA mouse model

After acclimatization for one week, the animals were randomly

divided into three groups using a random number table, including the (1) control group, (2) the NA (neutrophilic asthmatic) group, (3) and the PAP-1 (5-(4-phenoxybutoxy) psoralen, a selective Kv1.3 channel blocker) group. We used $n = 10$ for each group.

Mice in the NA and PAP-1 groups were given 20 μ L of sensitizing solution containing 100 μ g of OVA and 0.1 μ g of LPS by dipping into the airways after anesthetization on days 0, 6, and 13. Mice in all groups except the control group were 2% OVA-challenged daily from days 21 through 35, where each session lasted for 30 min [16, 17].

At 30 min before stimulation, mice in the PAP-1 group received PAP-1 intraperitoneal injections (40 mg/kg) for 14 days.

All mice were anesthetized with 5% chloral hydrate 48 h after the last OVA challenge to collect blood, BALF, and lung tissues.

2.3. Airway responsiveness detection

Airway responsiveness was determined for a separate subset of six mice from each experimental group. Twenty-four hours after the last OVA atomization stimulation, the mice were made to inhale 0, 1.56, 3.125, 6.25, 12.5, 25, and 50 g/L methacholine (Mch) successively, followed by airway responsiveness detection for three minutes. The noninvasive pulmonary function test was performed using a small-animal lung function detection system. The airway responsiveness monitoring index-enhanced pause (Penh) was recorded. Those parameters were used to calculate Penh according to the equation: $\text{Penh} = (\text{Pi}/\text{Pe}) * [(\text{Te}-\text{Tr})/\text{Tr}]$, in which Pi is the chamber pressure change during inspiration, Pe is the chamber pressure change during expiration, Te is the expiratory time, and Tr is the relaxation time.

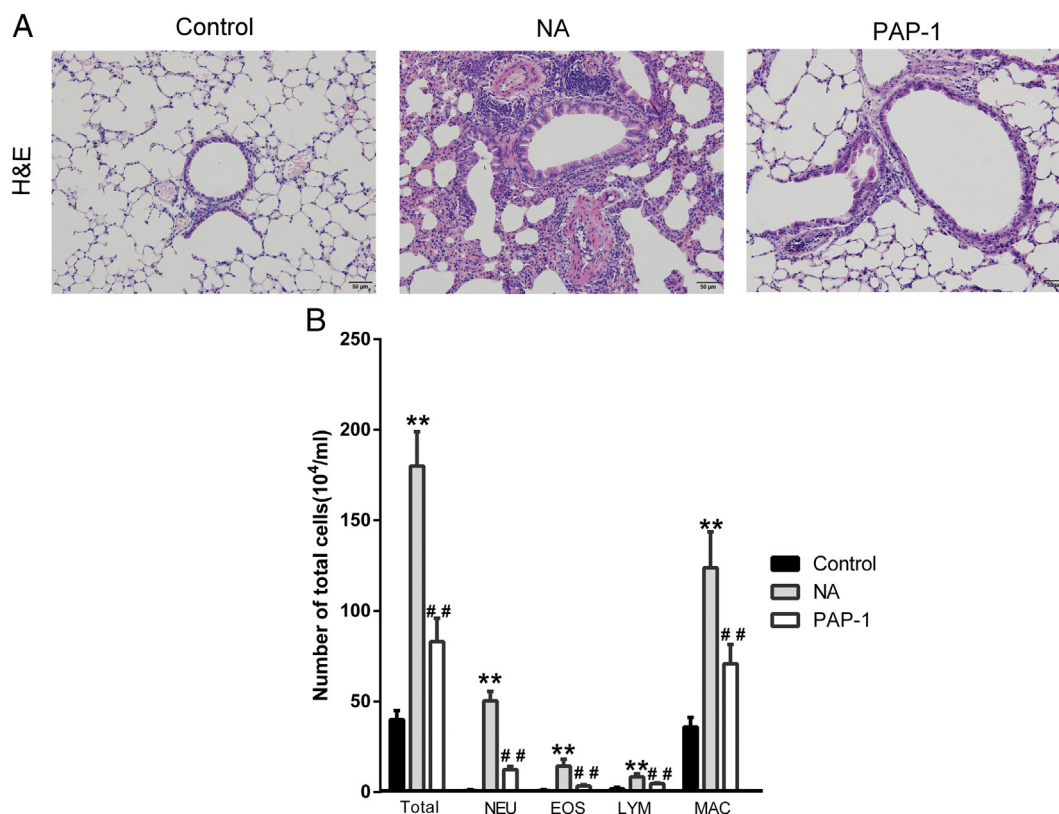


Fig. 3. PAP-1 alleviates airway inflammation in vivo. (A) Representative images of H&E staining of lung tissues. (B) Total and differential cell counts of BALF were determined by Giemsa staining. NEU: Neutrophils. EOS: Eosinophils. LYM: Lymphocytes. MAC: Macrophages. The results shown are representative of at least five independent experiments. NA: neutrophilic asthmatic group. Each value represents the mean $\pm$ SD ($n = 5$).

* $P < 0.05$,

** $P < 0.01$ versus the control group.

$P < 0.05$,

$P < 0.01$, versus the NA group.

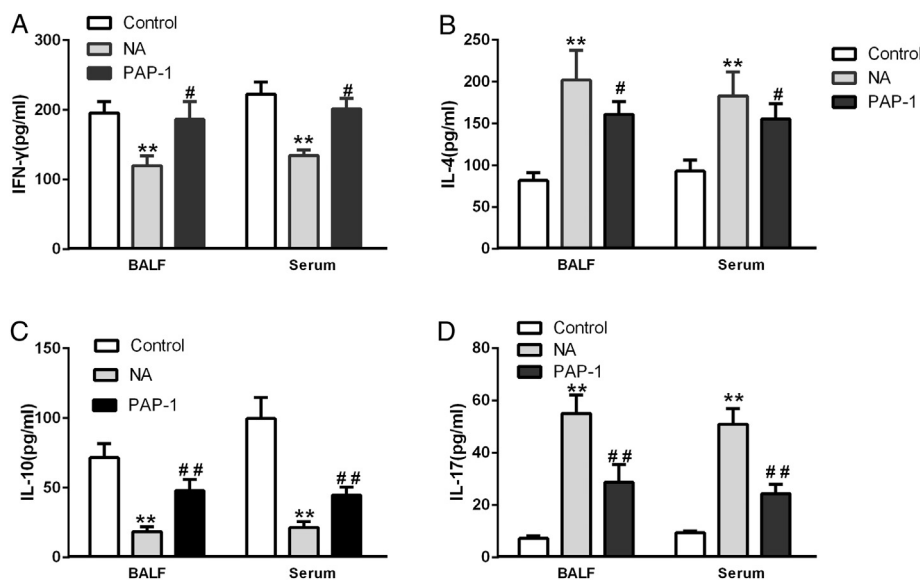


Fig. 4. PAP-1 regulates the imbalance of Th1/Th2 and Treg/Th17 cytokines. ELISA assay for the cytokine levels of IFN- γ (A), IL-4 (B), IL-10 (C), and IL-17 (D) in mice BALF and serum. NA: neutrophilic asthmatic group. Each value represents the mean $\pm$ SD (n = 8).

*P < 0.05,

**P < 0.01 versus the control group.

#P < 0.05, ##P < 0.01, versus the NA group.

2.4. Bronchoalveolar lavage (BAL) and counting cells

Following the measurement of lung responsiveness, the mice were killed with an overdose of chloral hydrate. A catheter was then inserted into the trachea and BAL was performed using three aliquots of 0.5 mL PBS (pH 7.2) and withdrawn three times each. The qualified fluid recovery rate was over 80%. Cell suspension was concentrated by centrifugation (1000 rpm, 10 min. at 4 °C), and the cell pellet was resuspended in a 1-mL saline solution. To perform the differential leukocyte cell count, 0.1 mL of the cell suspension was dropped on a glass slide and stained with a Wright–Giemsa stain. A microscope was then used to examine 400 nucleated cells.

2.5. Hematoxylin and eosin staining

Routine histological staining methods were applied. The middle lobe of the right lung sections of 4 mm were stained with hematoxylin and eosin (HE) for general histological evaluation.

2.6. Patch-clamp electrophysiology

The BALF of mice in each group was concentrated by centrifugation (1000 rpm, 10 min. at 4 °C) and resuspended in 10% fetal bovine serum albumin. The cells were stained with CD4-FITC (BD Biosciences, San Jose, CA) for four hours at room temperature in the dark. Whole-cell currents of CD4⁺ cells were recorded using the patch-clamp technique in the whole-cell configuration in the dark.

Micropipettes were prepared from borosilicate glass capillaries (Sutter Instruments, GER) using a P-97 puller (Sutter Instruments, GER) and fire polished. The pipettes had a resistance of 2–5 M Ω . For the cells, pipettes were filled with a solution containing (in mM) 5NaCl, 0.1 CaCl₂, 1 MgCl₂, 10 HEPES, 1 EGTA, 2 Mg-ATP, and 140 K-Glucose (pH 7.2 with KOH). The extracellular solution contained (in mM) 140 NaCl, 3.5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES 1.25NaH₂PO₄, and 10 K-Glucose (pH 7.4 with NaOH).

The cells were clamped at a holding potential of –60 mV. To invoke voltage-gated currents, all cells were stimulated with a 250-ms square pulse from –80 to +80 mV. The peak amplitude (pA) was normalized using the capacitance values (pF). Data acquisition and analysis were performed using the HEKA EPC10 USB amplifier (HEKA Elektronik, GER) and PatchMaster software (HEKA, GER).

2.7. Immunofluorescence of lung tissues

The expressions of the Kv1.3 protein were identified by immunofluorescent stainings. Briefly, paraffin-embedded lung sections (4 mm thick) were cut and kept in an oven at 60 °C for 24 h to increase section adherence to the slide. The slides were incubated with anti-Kv1.3 (1:200, Abcam Inc., England) overnight at 4 °C, and with secondary antibodies for 60 min at room temperature. Rinsing was performed by using PBS. The nucleus was stained with DAPI. At a magnification of 200 $\times$, five microscopic fields were randomly chosen.

2.8. Flow cytometry

Cells isolated from single-cell suspensions prepared from the spleens of the mice were washed with FACS-PBS (calcium-free PBS + 10% fetal bovine serum albumin).

The activation strategy was as follows: 2 $\times$ 10⁶/mL cells were stimulated for three to five hours (100% humidity, 37 °C, 5% CO₂) with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) and ionomycin (1 μ g/mL) in the presence or absence of brefeldin A (BFA) (1 μ g/mL) (all three from Sigma-Aldrich, USA).

After being washed with FACS-PBS three times, the cells were stained with rat antimouse CD4-FITC (BD Biosciences, USA) for 30 min at 4 °C in the dark. They were then washed and centrifugated (1000 rpm, 5 min, at 4 °C). BD A total of 250 μ L of Cytofix™ Fixation Buffer was added for 10 min at 37 °C. After washing, 500 μ L of BD Phosflow Perm Buffer II was added for 20 min at 4 °C in the dark.

After three washes with FACS-PBS, the cells were stained with rat antimouse IFN- γ -PE, rat antimouse IL-4-PE, rat antimouse IL-17A-PerCP-Cy™5.5 (BD Biosciences, USA), respectively, for 30 min at 4 °C in the dark.

Regulatory T-cells (Tregs) were defined as CD4 + CD25 + FoxP3⁺. For nuclear staining of Treg cells, cells were isolated from single-cell suspensions prepared from the spleens of the mice were washed with FACS-PBS, then surface-labeled with rat antimouse CD4-FITC (BD Biosciences, USA) and rat antimouse CD25-APC for 30 min at 4 °C in the dark after washing. After washing with FACS-PBS three times, the cells were fixed for 30 min at 37 °C, followed by permeabilization, and intracellular staining with rat antimouse Foxp3-PE (BD Biosciences, USA) for 30 min at 4 °C in the dark.

After washing again, the cells were resuspended with FACS-PBS and analyzed by flow cytometry with a FACSC alibur (BD FACSCanto™ II, USA). The results were analyzed on the Cell Quest software (BD

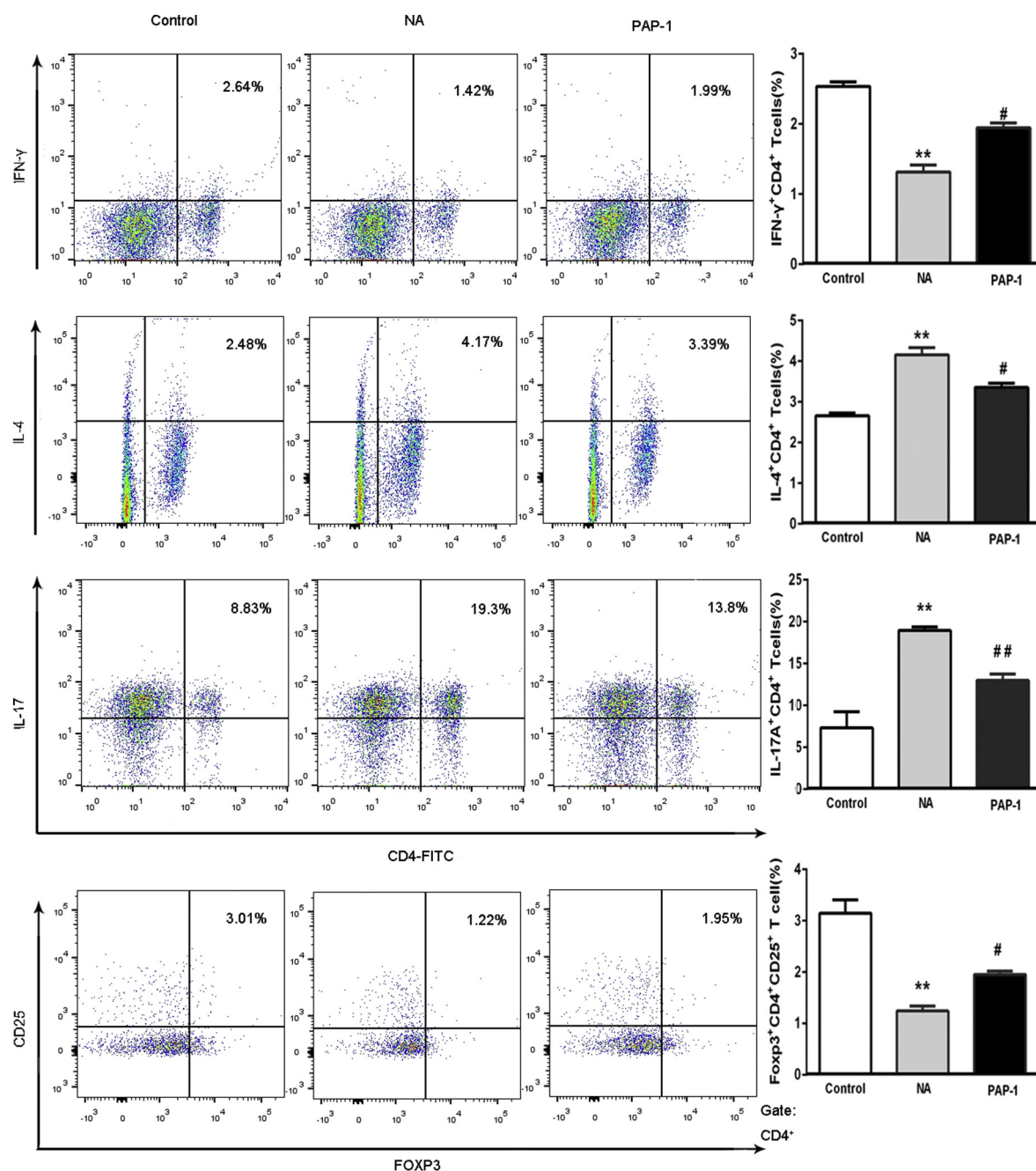


Fig. 5. PAP-1 modulates the imbalance of Th1/Th2 and Treg/Th17 cells in OVA-LPS-induced neutrophilic asthmatic mice. Intracellular expression of IFN- γ by Th1 cells, IL-4 by Th2 cells, and CD25 and Foxp3 by Treg cells among CD4⁺ T cells was determined by FACS. NA: neutrophilic asthmatic group. Each value represents the mean $\pm$ SD ($n = 5$).

* $P < 0.05$,

** $P < 0.01$ versus the control group.

$P < 0.05$, ## $P < 0.01$, versus the NA group.

Biosciences, USA).

2.9. Detection of cytokine levels by ELISA

Kits to measure cytokine levels in the BALF from mice were purchased from R&D Systems (Minneapolis, MN) for IFN- γ , IL-4, IL-10, and IL-17, and were used following the manufacturer's instructions.

2.10. Western blot analyses

The lungs of mice in each group were collected 24–48 h after the

final OVA challenge and stored at -80°C . The total cytoplasmic and nuclear protein concentrations were determined by the BCA protein estimation kit (Beyotime, China). The samples were incubated at $95-100^{\circ}\text{C}$ for five minutes. Samples containing $50\text{ }\mu\text{g}$ of proteins each were then resolved on 10% acrylamide Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gels and transferred to the polyvinylidene difluoride (PVDF) membrane, and were blocked for two hours in a 5% skimmed milk powder blocking buffer and washed with PBST four times. The blots were incubated with the anti-Kv1.3 (1: 1000), anti-ERK (1: 1000), anti-p-ERK (1: 1000), anti-I κ B α (1: 1000), anti-p-I κ B α (1: 1000), anti-NF- κ B (1:1000), anti- β -actin (1: 1000)

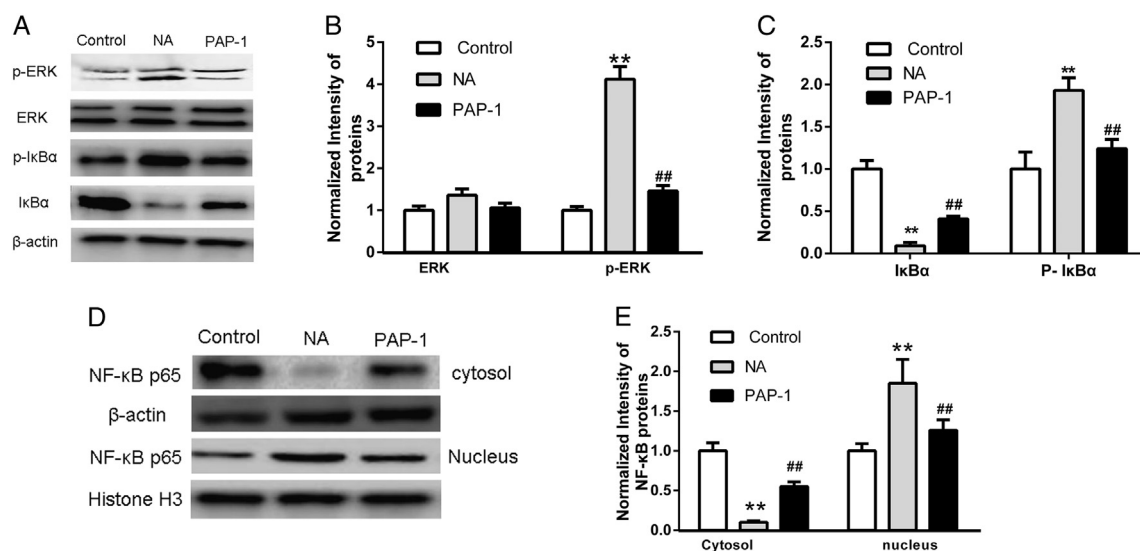


Fig. 6. PAP-1 suppresses the ERK-NF- κ B pathway. The protein expressions of ERK and p-ERK (A) & (B), I κ B α and p-I κ B α (A) & (C), in lung tissue were detected by western blot. (D) & (E) The expression of NF- κ B in cytosol and the nucleus of the lung tissues were detected by western blot. NA: neutrophilic asthmatic group. Each value represents the mean $\pm$ SD ($n = 5$).

* $P < 0.05$,

** $P < 0.01$ versus the control group.

$P < 0.05$, ## $P < 0.01$, versus the NA group.

antibodies, respectively (Abcam Inc., England) overnight at 4 °C. After washing with PBST four times, the blots were incubated with secondary antibodies for two hours at room temperature. Immunodetection was carried out with an ECL detection reagent (Beyotime, China). Photographs were taken, and the optical densities of the bands were scanned and quantified using Gel Doc 2000 (BioRad, USA).

2.11. Statistical analysis

The data were evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test using the GraphPad Prism software. $P < 0.05$ was considered significant. The data were presented as $x \pm sd$ (mean $\pm$ standard deviation). Statistical analysis was performed using the SPSS22.0 software.

3. Results

3.1. Expression of Kv1.3 channel, and Kv current intensity are higher in lung tissues of in OVA-LPS-induced neutrophilic asthmatic mice

We analyzed the Kv1.3 protein in lung tissues by using immunofluorescent stainings (IF). As shown in Fig. 1A, IF revealed the presence of Kv1.3 protein mainly in bronchial epithelial cells and around the alveoli. The expression of Kv1.3 in the NA group was significantly higher than that in the control group ($P < 0.05$). The expression was significantly reduced in PAP-1-treated mice.

We then used the whole-cell technique of patch-clamp electrophysiology to detect the function of Kv1.3 channels in the CD4⁺ T lymphocytes in BALF. Repetitive application of a pulse (from -80 to $+80$ mV in 10 mV steps from a holding potential of -80 mV) induced an increase of Kv channel currents (I_{kv}) over 20–40 s of the initial amplitude. The intensity of the I_{kv} increased clearly in the OVA-LPS-induced neutrophilic asthmatic mice model ($P < 0.01$). The intensity was significantly reduced in PAP-1-treated mice (Fig. 1B). The peak current to voltage relation (I–V curve) of I_{kv} was obtained from responses to pulses in CD4⁺-T cells; as displayed in Fig. 1C. I_{kv} increased greatly in the NA group, when treated with PAP-1, I_{kv} seemed to decrease.

Analyses of the voltage-dependence of outward currents derived

from I–V curve in Fig. 1D. Half-activation voltage ($V_{1/2}$) values were -19.58 ± 2.09 mV for the control group, while 24.14 ± 3.21 mV for the NA group, and 19.35 ± 2.14 for the PAP-1 group ($P > 0.05$). There was no difference in the voltage-dependence of I_{kv} in different groups.

3.2. PAP-1 reduces AHR in OVA-LPS-induced neutrophilic asthmatic mice

Airway hyperresponsiveness (AHR) increased with the growing of concentration of methylcholine inhaled in each group. Compared with the control group, AHR significantly increased in the OVA-LPS-induced neutrophilic asthmatic mice, and was attenuated by treatment with PAP-1. In particular, AHR was significantly reduced in PAP-1-treated mice than that in untreated asthmatic mice exposed to 25 and 50 mg/mL of methylcholine (Fig. 2).

3.3. PAP-1 alleviates airway inflammation in OVA-LPS-induced neutrophilic asthmatic mice

A histological examination of lung sections of mice in NA group showed a large number of infiltrating inflammatory cells around the airway (Fig. 3A). In contrast, there was no airway inflammation in the control group. However, this increase in the number of inflammatory cells was significantly weakened by PAP-1 treatment.

The number of inflammatory cells in BALF was measured in different groups. Compared with the control group, the numbers of total inflammatory cells, neutrophils, eosinophils, lymphocytes and macrophages in BALF from OVA-LPS-induced neutrophilic asthmatic mice were higher, particularly neutrophils. By contrast, the number of inflammatory cells in BALF from PAP-1-treated mice was significantly lower than that in untreated OVA-LPS-induced neutrophilic asthmatic mice (Fig. 3B).

3.4. PAP-1 modulates the imbalance of Th1/Th2 and Treg/Th17 in OVA-LPS-induced neutrophilic asthmatic mice

As PAP-1 reduced airway inflammation, and as the imbalance of Th1/Th2 and Treg/Th17, especially the latter, contributed to the initiation and mediation of the OVA-LPS-induced neutrophilic asthma, we

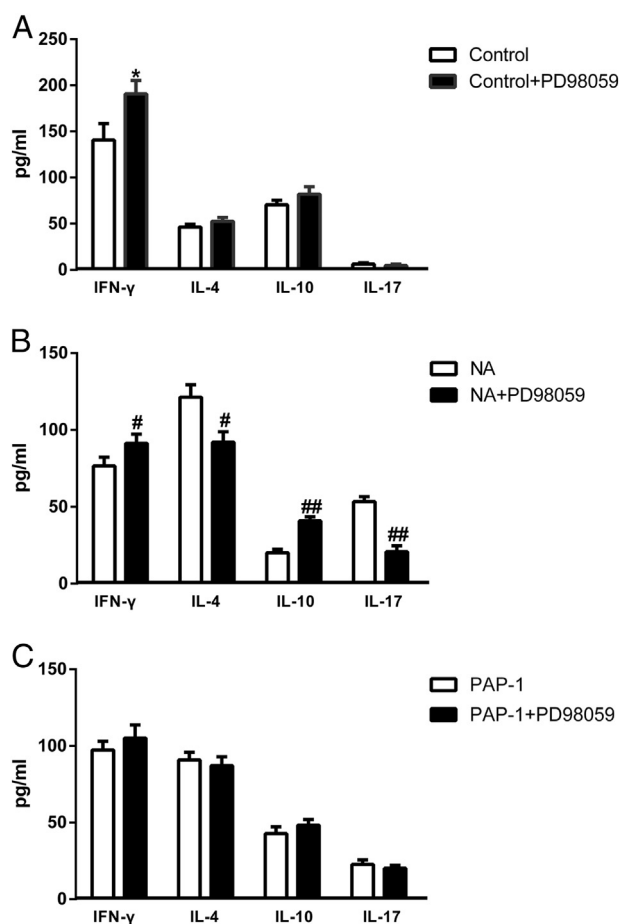


Fig. 7. Inhibition of ERK activity has no effect on PAP-1-induced cytokine changes. (A) PD98059 influenced cytokine levels in the control group. (B) PD98059 could mitigate cytokine changes induced by OVA-LPS in neutrophilic asthmatic group. (C) PD98059 had no effect on cytokine levels in the PAP-1 group. NA: neutrophilic asthmatic group. Each value represents the mean $\pm$ SD ($n = 5$).

* $P < 0.05$, ** $P < 0.01$ versus the control group.

$P < 0.05$, ## $P < 0.01$, versus the NA group.

determined the effects of PAP-1 on Th1, Th2, Treg, and Th17 cytokines: IFN- γ , IL-4, IL-10 and IL-17 respectively in the BALF and serum. As shown in Fig. 4A–D, the upregulation of IL-4, IL-17 levels inducing by OVA-LPS was significantly reduced by PAP-1, whereas the levels of IFN- γ and IL-10 were upregulated by PAP-1 in the BALF and serum of mice. The downregulation of IL-4, IL-17 levels was more significant.

Flow cytometric analysis was carried out to assess the Th1, Th2, Treg, and Th17 cells in the spleen. The level of the Th1 cells was lower in the NA group than in the control group (1.42% vs 2.46%, $P < 0.01$). PAP-1 could increase the level of the Th1 cells (1.99% vs 1.42%, $P < 0.05$). The level of the Th2 cells was significantly higher in the NA group than in the control group (4.17% vs 2.18%, $P < 0.01$). while PAP-1 reduced the level of the Th2 cells (3.39% vs 4.17%, $P < 0.05$). Furthermore, a decrease in the Treg cells induced by OVA-LPS was relieved by PAP-1. The levels of the Treg were significantly lower in the NA group than in the control group (1.22% vs 3.01%, $P < 0.01$). Treatment with PAP-1 increased the level of the Treg cells (1.95% vs 1.22%, $P < 0.05$). The levels of the Th17 were significantly higher in the NA group than in the control group (19.30% vs 8.83%, $P < 0.01$). Treatment with PAP-1 reduced the levels of the Th17 cells (13.8% vs 19.3%, $P < 0.01$). (Fig. 5).

These results suggest that PAP-1 regulates the balance of Treg/Th17 and Th1/Th2 in neutrophilic asthma mouse models.

3.5. PAP-1 suppresses the ERK-NF- κ B pathway in OVA-LPS-induced neutrophilic asthmatic mice

To assess the mechanism of PAP-1 in OVA-LPS-induced neutrophilic asthmatic mice, we analyzed the levels of a number of related proteins in ERK and the NF- κ B signaling pathway by using the western blot. Compared with the control group, the protein levels in the phosphorylated ERK1/2, phosphorylated I κ B α and NF- κ B levels in the nucleus of the neutrophilic asthmatic group significantly increased, whereas the levels of the I κ B α protein level and NF- κ B protein in cytosol significantly decreased. However, these changes induced by OVA-LPS were relieved by PAP-1 treatment (Fig. 6A–E). These results demonstrated that the activation of ERK/NF- κ B pathway was elevated in OVA-LPS-induced neutrophilic asthmatic mice, and PAP-1 suppressed ERK/NF- κ B activation by inhibiting, at least in part, I κ B α activation.

3.6. Inhibiting ERK activity has no effect on PAP-1-induced cytokine changes

To confirm whether PAP-1 performed an anti-inflammatory function in OVA-LPS-induced neutrophilic asthmatic mice by suppressing the ERK signaling pathway, we determined the effects of the ERK inhibitor PD98059 on cytokine levels. Single spleen cells were isolated after being cultured for four hours in 10 μ mol/L of PD98059, and the cytokine levels of IFN- γ , IL-4, IL-10, and IL-17 were detected using ELISA. Compared with Control group, Control + PD98059 could only induced the elevating of IFN- γ . (IFN- γ : 140.69 ± 17.9 pg/mL vs 190.71 ± 14.64 pg/mL, $P < 0.05$. IL-4: 46.37 ± 2.80 pg/mL vs 52.62 ± 4.13 pg/mL, $P > 0.05$. IL-10: 70.55 ± 4.93 pg/mL vs 82.03 ± 8.17 pg/mL, $P > 0.05$. IL-17: 6.28 ± 1.50 pg/mL vs 4.76 ± 1.32 pg/mL, $P > 0.05$.) PD98059 mitigated the cytokine changes induced by OVA-LPS just as PAP-1 did. Compared with NA group, IFN- γ and IL-10, levels in NA + PD98059 group were clearly higher, while IL-4 and IL-17 levels were obviously decreased. (IFN- γ : 76.58 ± 5.69 pg/mL vs 91.34 ± 5.92 pg/mL, $P < 0.05$. IL-4: 121.35 ± 8.10 pg/mL vs 92.09 ± 6.77 pg/mL, $P < 0.05$. IL-10: 20.14 ± 2.27 pg/mL vs 40.96 ± 2.58 pg/mL, $P < 0.01$. IL-17: 53.45 ± 3.23 pg/mL vs 20.75 ± 3.88 pg/mL, $P < 0.01$.) However, it was unable to further effect cytokine changes that had occurred when the cells were treated with PAP-1 (IFN- γ : 97.39 ± 5.66 pg/mL vs 105.24 ± 8.35 pg/mL, $P > 0.05$. IL-4: 90.89 ± 5.07 pg/mL vs 87.16 ± 5.83 pg/mL, $P > 0.05$. IL-10: 42.77 ± 4.58 pg/mL vs 48.36 ± 3.64 pg/mL, $P > 0.05$. IL-17: 22.70 ± 2.98 pg/mL vs 20.15 ± 2.08 pg/mL, $P > 0.05$.)

Taken together, these results suggest that ERK is the downstream signal of Kv1.3 channels in OVA-LPS-induced neutrophilic asthmatic inflammation (Fig. 7A–C). And that PAP-1 attenuates the imbalance of Treg/Th17 and Th1/Th2 via suppressing the activation of ERK/NF- κ B pathway.

4. Discussion

In this study, we made an interesting discovery pertaining to PAP-1 treatment to inhibit the ERK-NF- κ B signaling pathway, which is a mechanism to reduce airway inflammation in case of OVA-LPS-induced neutrophilic asthma.

We found that the expression of the Kv1.3 channel in the lung tissues of OVA-LPS-induced neutrophilic asthmatic mice was higher than that in the control group in our experiment. Moreover, the current intensity of Kv increased clearly compared with those in the control group. However, there was no difference in the voltage-dependence of I_{kv} in different groups.

The Kv1.3 channel modulates many calcium-dependent cellular processes in T lymphocytes, especially the activation and proliferation of T lymphocytes [18, 19]. In many chronic inflammatory diseases mediated by T lymphocytes, expression and activation are higher in the

T lymphocytes than normal [5, 8]. The inflammation of asthma is characterized by an increase in CD4⁺ T lymphocytes and related pro-inflammatory cytokines. Koshy et al. recently showed the increased expression and activity of Kv1.3 channels in T lymphocytes from airways in OVA-induced rat models and peripheral blood of patients with asthma [10], results that coincide with those of our study.

Moreover, we found that PAP-1 reduced airway reactivity and airway inflammation in the mice model of OVA-LPS-induced neutrophilic asthma. The characteristics of asthma include AHR and chronic airway inflammation, which involve of a variety of forms of cell infiltration, inflammatory mediators, cytokines, and adhesion molecules, all of which result in airway obstruction and limited flow of air. The PAP-1 downregulated the AHR, diminished histological changes, and reduced airway inflammation by attenuating the infiltrating inflammatory cells.

Our results show that PAP-1 regulates the imbalance in Th1/Th2, Treg/Th17 lymphocytes. The traditional view is that the imbalance in Th1/Th2 response is the essential pathology of allergic asthma [20, 21]. IL-4 is a vital Th2 cytokine that triggers the differentiation of Th2 cells, and thus induces the enhancement of Th2 cytokines, including IL-4, IL-6, and IL-13 [22]. IFN- γ is a crucial cytokine secreted by Th1 cells that can regulate Th2 responses. However, the theory of an imbalance in Th1/Th2 cannot explain all the phenomena occurring in asthma patients. Thus, Treg and Th17 cells that form subsets of CD4⁺ T cells have emerged as popular subjects in asthma research. Treg cells secreting IL-10 can inhibit inflammatory activity [23], whereas Th17 cells and the related IL-17 play an important role in the development of glucocorticoids-insensitive airway inflammation in asthma patients, and are related to the airway infiltration of neutrophils [4, 24–26]. Recent studies have shown that the expression and function of the Kv1.3 channel vary in immune cells under different conditions. In healthy people, the inhibitor of the Kv1.3 channel decreases calcium influx in the Th1, Th2, and Th17 cells, whereas in Treg cells, it has had no reported significant effects [27].

In RA patients, the inhibition of Kv1.3 channels has resulted in a larger decrease in calcium entry to Th2 and CD4 than in Th1 and CD8 cells [8]. Tregs from patients of multiple sclerosis have been recorded to have fewer K(V)1.3 channels than naive cells [28]. The inhibition of the Kv1.3 channel mediates the activation of differentiated Th17 cells through the CCR7(–) phenotype [29]. Koshy et al. found that the Kv1.3 channel blocker reduces levels of IL-4 and IL-5, and induces an increase in the production of IL-10 in the OVA-induced rat model of asthma [10]. In this study, the number of Th2 and Th17 cells increased while the number of Th1 and Treg cells significantly decreased in the OVA-LPS-induced neutrophilic asthmatic mice model, causing an imbalance in both Th1/Th2 and Treg/Th17. This imbalance was confirmed by the related cytokines secreted by these types of cells in terms of increased expression of IL-4 and IL-17, which are mainly secreted by Th2 and Th17 cells, and decreased secretion of IFN- γ and IL-10, which are mainly secreted by Th1 and Treg cells. This imbalance in T lymphocytes can be attenuated by treatment by PAP-1. However, the I_{KV} in special T-cell types (Th1, TH2, Treg and Th17 cells) and the cytokine in vitro need to be tested in some following researches.

To seek further molecular mechanisms underlying the effect of PAP-1 on regulating the imbalance in the functions of Th1/Th2, Treg/Th17, we focused on the ERK-NF- κ B signaling pathway. ERK-NF- κ B is regarded as a critical signaling pathway of inflammation in asthma [30]. The activation of ERK is a critical signal for cell processes; such as activation, proliferation, and differentiation; which is characterized by phosphorylation [31]. NF- κ B is a nuclear transcription factor that plays a key role in regulating several pro-inflammatory genes related to immune response and inflammatory reactions. In un-stimulated cells, NF- κ B is sequestered by I κ B α proteins in the cytoplasm, and upon activation by a variety of stimuli, they are rapidly phosphorylated and degraded to activate NF- κ B [32]. The activation of NF- κ B regulates the expression of such cytokines as IL-8 and G-CSF, which help neutrophil

chemotaxis and survival. In this study, we found that ERK, NF- κ B and I κ B α were activated in the lung tissues of OVA-LPS-induced neutrophilic asthmatic mice, where the activation of ERK, NF- κ B, and I κ B α can be inhibited by PAP-1. These results suggest that PAP-1 can inhibit the ERK-NF- κ B signaling pathway. Moreover, PD-98059, a specific inhibitor of ERK that prominently represses NF- κ B activity, had a similar effect with PAP-1 on regulating the imbalance of Th1/Th2, Treg/Th17 cytokines. However, it could not further effect changes in the cytokine when cells were treated it. These results suggested that ERK acts as a downstream regulator of the inhibitor of the Kv1.3 channel in neutrophilic asthma.

5. Conclusion

In this study, we revealed that the Kv1.3 channel participates in airway inflammation by activating the ERK-NF- κ B signaling pathway. These results suggest that the Kv1.3 channel inhibitor can be applied as an anti-inflammatory agent for asthma treatment. The I_{KV} activity in Th1, TH2, Treg and Th17 cells and the related cytokine in vitro should be tested in some following researches.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

The authors are grateful to Professor Heike Wulff from the Department of Pharmacology at the University of California for his kind donation of 5-(4-phenoxybutoxy) psoralen (PAP-1) to support this study.

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