



Shikonin alleviates allergic airway remodeling by inhibiting the ERK-NF- κ B signaling pathway



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ABSTRACT

Shikonin is a naphthoquinone extracted from the root of *Lithospermum erythrorhizon*, and has been reported to suppress allergic airway inflammation in mice. However, the underlying mechanisms are unclear and need to be further elucidated. In this study, shikonin (0.5, 2 or 4 mg/kg) was given intraperitoneally to ovalbumin (OVA)-challenged BALB/c mice. We found that the pathological airway remodeling of asthmatic mice was alleviated by shikonin, and the infiltrated inflammatory cells and collagen deposition in their lungs were reduced. Furthermore, the abnormal activation of extracellular signal-regulated kinase (ERK)/nuclear factor- κ B (NF- κ B) pathway and the elevation of matrix metalloproteinase 9 (MMP9) in the lung of asthmatic mice were suppressed by shikonin. The inactivation of NF- κ B by shikonin was at least in part via inhibiting I κ B α activation. In vitro, the treatment of shikonin inhibited the platelet-derived growth factor (PDGF)-induced proliferation of primary airway smooth muscle cells (ASMCs), and induced a G0/G1 arrest in these cells. In addition, ASMCs exposed to PDGF acquired an enhanced migratory ability, and the activities of MMP9 and matrix metalloproteinase 2 (MMP2) and expression of MMP9 of these cells were significantly up-regulated. These PDGF-induced alterations were also inhibited by shikonin. The inhibitory effects of shikonin on the proliferation and migration of ASMCs were comparable to pyrrolidinedithiocarbamate (PDTTC), an inhibitor of NF- κ B pathway. In conclusion, the present study sheds lights on how shikonin alleviates allergic airway remodeling.

1. Introduction

Bronchial asthma, characterized by airway hyper-responsiveness, is a chronic inflammatory disease that involves many inflammatory cells especially mast cells, eosinophils and T lymphocytes [1]. At present the main treatment for persistent asthma is inhalation of glucocorticoids, however hormones cannot fully exert the antioxidant effect. Hormone dependence or hormone resistance [2] may appear in some patients. Therefore it is very important to find reliable drugs for treatment of this disease.

Shikonin is one of the effective naphthoquinone compounds extracted from *boraginaceae* plants *Lithospermum erythrorhizon*'s dry root, which has many pharmacological effects such as anti-inflammatory [3], antineoplastic [4], antiviral [5], hypoglycemic [6] and so on. The research by Lee et al. showed that shikonin inhibited maturation of bone marrow-derived dendritic cells and suppressed allergic airway inflammation in a murine model of asthma, which hinted that shikonin may be an effective drug for the treatment of asthma [7]. Although a lot of biological and pharmacological activities of shikonin have been reported, possible mechanisms of shikonin on allergic inflammation

have rarely been determined, which need to be further studied. Several studies have shown that shikonin inhibited the activation of NF- κ B signaling pathway in different kinds of cells [8–11]. Therefore we speculate that shikonin may alleviate allergic airway inflammation and remodeling by inhibiting the NF- κ B signaling pathway.

Extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase (MAPK) family. The ERK is activated through Ras/Raf kinase/ERK pathway. The activation of ERK, characterized by phosphorylation, may cause the degradation of I κ B α and then activate the NF- κ B signaling pathway. Matrix metalloproteinases (MMPs) are the downstream targets of the NF- κ B signaling pathway, which play an important role in tissue repair, tumor invasion, and metastasis [12,13].

The aim of our study was to investigate the effects and mechanisms of shikonin against allergic airway inflammation and remodeling in a mouse asthmatic model in vivo and the PDGF-induced ASMCs in vitro.

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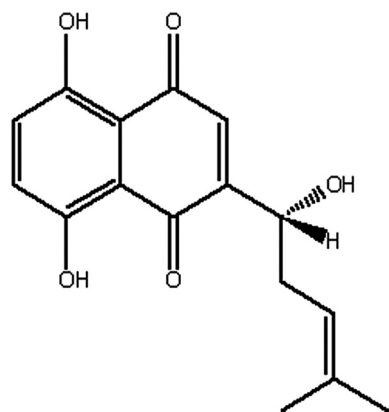


Fig. 1. Chemical structure of shikonin. The molecular formula of shikonin is $C_{16}H_{16}O_5$ and its molecular weight is 288.30.

2. Methods

2.1. Drugs and antibodies

The shikonin, PDGF, PD98059, PDTC were purchased from Meilun Biotech Co., Ltd. (Dalian, China), Uscn Life Science Inc. (Wuhan, China) and Beyotime (Haimen, China) respectively. The purity of shikonin was > 98% as detected by HPLC. These drugs were dissolved in DMSO as a stock concentration of 10 mg/ml, 50 µg/ml, 0.005 M, 1 M respectively and stored in the dark at -20°C . The hexadecadrol injection was obtained by prescription. The chemical structural of shikonin is showed in Fig. 1. The DMEM was used for diluting the drugs for different experiments. The antibodies used for western blotting and immunohistochemistry/-fluorescence were as follows: anti-p-IkB α , anti-IkB α , anti-NF- κ B, anti-p-ERK, anti-ERK, anti-MMP9, anti- α -SMA, anti- β -actin, anti-Histone H3. All the antibodies were purchased from WanLei Life Sciences Co., Ltd. (Shenyang, China).

2.2. Animals and experimental protocol

BALB/c mice were purchased from Beijing Vital River Laboratory Animal Co., Ltd. (Beijing, China). BALB/c mice (6–8 weeks old) were randomly divided into control, OVA (Sigma-Aldrich, Beijing, China) model, OVA + shikonin (0.5 mg/kg), OVA + shikonin (2 mg/kg), OVA + shikonin (4 mg/kg), OVA + hexadecadrol (5 mg/kg) groups. The hexadecadrol was used as a positive control. The asthmatic mice model was built by modified OVA inhalation method. Firstly, on the days 1, 14, 28, the mice except control group were sensitized with intraperitoneal injection of 50 µg OVA mixed with 10% aluminium hydroxide solution and then from the day 21, the mice were challenged by an aerosol of 2% OVA in normal saline for 20 min, twice a week for 8 weeks. In control group, OVA was replaced by saline during sensitization and challenge. Half hour before each challenge the mice except control and OVA group were intraperitoneally injected with 0.5, 2, 4 mg/kg shikonin and 5 mg/kg hexadecadrol respectively, and the mice in control and OVA groups were intraperitoneally injected with 1% DMSO in the same volume. All animal experiments were carried out strictly in accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals. The experiments were approved by the Institutional Animal Care and Use Committee of China Medical University.

2.3. Histological examinations

After bronchoalveolar lavage, lung tissue samples were fixed in 10% buffered formalin for 48 h and were then dehydrated by washing with ascending grades of ethanol. Then samples were sectioned and

embedded in paraffin wax. 5 µm lung sections were prepared for routine hematoxylin-eosin (H & E), Masson trichrome and periodic acid-schiff (PAS) stains. H & E stain was used to observe the morphologic changes of lung tissue, which reflected the inflammation of bronchus. Evaluations were performed and scored semi-quantitatively in a blinded manner using an arbitrary scale [14]. Inflammation was graded as follows: 0, none; 1, < 25% of inflammatory cells; 2, 25–50%; 3, 50–75%; and 4, > 75% of inflammatory cells.

Goblet cell hyperplasia was assessed by stained with PAS. To determine the extent of mucus production, the percentage of PAS staining-positive cells in the airway epithelium was quantified.

The Masson trichrome stain was performed to observe the collagen formation in bronchial airway. Quantitative assessment was carried out by calculating the percentage of Masson trichrome staining positive area in bronchial airway. Under 200 $\times$ magnification, each section was randomly selected and photographed for 5 fields.

2.4. Isolation of airway smooth muscle cells (ASMCs)

ASMCs were isolated from SD rats. Briefly, the sterile bronchial tissue of rat was washed by PBS for several times to remove the impurities in the bronchus and then cut into small pieces of 1–3 mm². They were then placed upside down in culture flask (Corning, USA) supplemented with high-glucose DMEM (containing 10% FBS) (Gibco, USA) at 37 $^{\circ}\text{C}$, under a 5.0% CO₂ atmosphere until single smooth muscle cells relaxed. Then cells were trypsinized and plated into 12-well plates (Corning, USA). The cells at passage 3–6 and grown to 80% confluence were chosen for the following experiments. ASMCs were identified by immunofluorescence using anti- α -SMA (1:200 diluted in PBS) and Cy3-conjugated goat-anti-rabbit (1:200 diluted in PBS) antibodies and observed under a fluorescence microscope.

2.5. Bronchoalveolar lavage fluid (BALF) and cell counting

BALF was concentrated by centrifugation (1000 rpm, 10 min at 4 $^{\circ}\text{C}$), and the cell pellet was resuspended in 0.5 ml PBS. Total cell count in BALF was determined with a haemocytometer. To perform the differential leukocyte cell count, 10 µl of the cell suspension was transferred to a glass slide and stained with Giemsa stain.

2.6. ELISA

The concentrations of HYP in tissue and IgE in BALF were detected using ELISA kits (Jiancheng, China), following the instructions of the manufacturer. HYP, and IgE concentrations were calculated by generating a standard curve using standard proteins and analyzed using curve expert 1.3 software.

2.7. MTT assay

Cell proliferation was performed using the MTT assay. Briefly, the ASMCs were trypsinized and plated into 96-well plates (Corning, USA). The various drugs were added to cells as follows. Overall, total 13 groups, including control, shikonin (0.1, 0.3, 1, 3, 5 µM), PDGF (25 ng/ml), PDGF + 0.3 µM shikonin, PDGF + 1 µM shikonin, PDGF + PD98059 (10 µM), PDGF + PD98059 + 1 µM shikonin, PDGF + PDTC (100 µM), PDGF + PDTC + 1 µM shikonin. The control group was added DMSO in the same volume. After treatment, directly added 20 µl of MTT (Sigma-Aldrich, USA) dissolved in PBS at 5 mg/ml to all the wells, and the plates were incubated for 4 h at 37 $^{\circ}\text{C}$. After removal of the supernatant 100 µl of DMSO was used to dissolve the formed formazan crystals. The result was recorded at 490 nm on a microplate reader (Bio-Tek, USA).

2.8. Analysis of the cell cycle distribution

When the ASMCs from the different groups had grown to 80% confluency, they were collected and fixed with cold 70% ethanol and stored at -20°C . The cells were then washed and resuspended in cold PBS and incubated at 37°C for 30 min with 10 mg/ml RNase and 1 mg/ml propidium iodide (Sigma-Aldrich, USA). Cell cycle analysis was performed by flow cytometry (BD, USA). The percentage of cells in the different cell cycle phases was determined using the Cell Quest acquisition software.

2.9. Transwell assay

The ASMCs in medium without serum were placed on the membrane in the top chamber (Corning, USA). Medium containing 20% FBS was placed in the lower chamber to act as a chemoattractant. After incubation for 24 h, a cotton swab was used to remove the cells that did not migrate through the pores. Then cells were fixed with 10% methanol and stained with 0.1% crystal violet. Under a microscope ($\times 200$) five random fields were photographed for each well and migrating cells were quantified by counting stained cells.

2.10. Western blot analysis

The tissue and ASMCs from the different groups were lysed in a sample buffer and then denatured. The total, cytoplasmic and nuclear protein concentration was determined by the BCA protein estimation kit (Beyotime, China). Equal quantities proteins samples were separated by SDS-PAGE. The proteins were then transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk, and incubated with anti-p-IkB α (1:500), anti-IkB α (1:1000), anti-NF- κ B (1:1000), anti-p-ERK (1:1000), anti-ERK (1:1000) anti-MMP9 (1:1000), anti- β -actin (1:1000), anti-Histone H3 (1:1000) antibodies respectively, at 4°C overnight. The goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:5000) was incubated. Immunodetection was carried out with ECL detection reagent (Beyotime, China). Photographs were taken and the optical densities of the bands were scanned and quantified with the Gel Doc 2000 (BioRad, USA).

2.11. Reverse transcription and quantitative real-time polymerase chain reaction (qPCR)

qPCR was used to quantify the expression of MMP9 mRNA in the experimental groups. Total RNA was isolated using the RNA easy kit (Invitrogen, USA). RNA was converted into complementary DNA using a Reverse Transcriptase (BioTeke, China). MMP9 and β -actin were amplified using SYBR Green PCR Master Mix (BioTeke, China). The primer sequences were as follows: MMP9 (forward-5'-GAATCACGGAG-GAAGCCAAT-3', reverse-5'-TAGCCCCAACCTTATCCAGACT-3'), and β -actin (forward-5'-GGAGATTACTGCCCTGGCTCCTAGC-3', reverse-5'-GGCCGGACTCATCTACTCTGCTT-3'). β -Actin was used as an internal reference gene to normalize the expression of MMP9. Relative quantification of gene was analyzed by the comparative threshold cycle (Ct) method.

2.12. Gelatin zymography assay

This is an assay using gelatin-substrate gel electrophoresis to measure the MMP9 and MMP2 activities in tissue and ASMCs. 30 μ g protein samples were electrophoresed without reduction on 8% SDS polyacrylamide gels copolymerized with 0.1% gelatin. When the tracking dye at the front reached the bottom of the gel, the SDS was removed by wash buffer (2.5% Triton X-100, 50 mM Tris-HCl, 5 mM CaCl₂, 1 μ M ZnCl₂, pH 7.6) for 40 min twice and then the gel was incubated for 18 h at 37°C in incubation buffer (50 mM Tris-HCl, 5 mM

CaCl₂, 1 μ M ZnCl₂, 0.02% Brij, 0.2 M NaCl). The gel was stained for 3 h with 0.05% (w/v) Coomassie brilliant blue R-250 and destained with destaining solution containing methanol-acetic acid-water (50:10:40). Protease activity was detectable as a clear zone against the background.

2.13. Electrophoretic mobility shift assay (EMSA)

The tissues were homogenized and nuclear protein was extracted with a Nuclear and Cytoplasmic kit (Beyotime, China). Protein concentration was determined by the BCA protein estimation kit (Beyotime, China). The levels of NF- κ B in nucleus were then measured by EMSA. Briefly, nuclear protein samples were mixed with labeled DNA probe. The samples were loaded and run at 180 V for 80 min. Following electrophoresis, protein was transferred from the gel to a positively charged nylon membrane by electroblotting. UV-cross linked and biotin-labeled DNA was detected by chemiluminescence.

2.14. Immunohistochemistry/fluorescence

The expression of airway alpha smooth muscle actin (α -SMA) in tissue from different groups was detected by immunohistochemistry. The lung tissue was fixed in 4% paraformaldehyde, then imbedded in paraffin. Lung sections of 5 μ m were sectioned and placed on poly-L-lysine-coated slides, then kept in an oven at 60°C for 24 h to increase section adherence to the slide. The slides were deparaffinized using xylene and dehydrated by graded concentrations of alcohol, then placed in a retrieval solution and incubated in a microwave at high power for 15 min. The slides were then cooled and washed with wash buffer and incubated sequentially with primary antibody, biotin-labeled secondary antibody. Finally, the sections were stained with DAB, counterstained with hematoxylin, dehydrated, cleared in xylene, and fixed. Under $400\times$ magnification, 5 different microscopic fields were randomly chosen. Quantitative analysis of α -SMA expression was performed by calculating average optimal density of α -SMA positive staining.

The expression of α -SMA and the distribution change of NF- κ B between cytosol and nucleus in ASMCs were identified by immunofluorescent stainings. The ASMCs were rinsed three times in PBS before being fixed in 4% paraformaldehyde for 15 min, and then the cells were rinsed another three times in PBS. The slides were incubated with primary antibodies over night at 4°C and with secondary antibodies for 60 min at room temperature. Rinsing steps were performed by PBS. The nucleus was stained with DAPI. Under $400\times$ magnification, 5 different microscopic fields were randomly chosen.

2.15. Statistical analysis

All data are given as means $\pm$ SD. The significance of differences among experiment groups was analyzed by one-way ANOVA followed by Bonferroni's Multiple Comparison Test using GraphPad Prism software. A p -value of < 0.05 was considered significant.

3. Results

3.1. Effect of shikonin on alleviating airway inflammation and remodeling in vivo

Histological examination of lung sections from the asthmatic group showed a large number of infiltrating inflammatory cells around the airway, compared to control group, as shown in the H & E-stained lung sections (Fig. 2(A)). The shikonin group showed clearly less inflammatory cell infiltration than asthmatic group. PAS staining was performed to determine whether shikonin inhibited the increased mucus formation, goblet cell hyperplasia in the airway epithelium induced by OVA (Fig. 2(B)). The percentage of PAS positive cells in the asthmatic group was significantly higher than that in the control group, but this increase was significantly weakened by shikonin treatment.

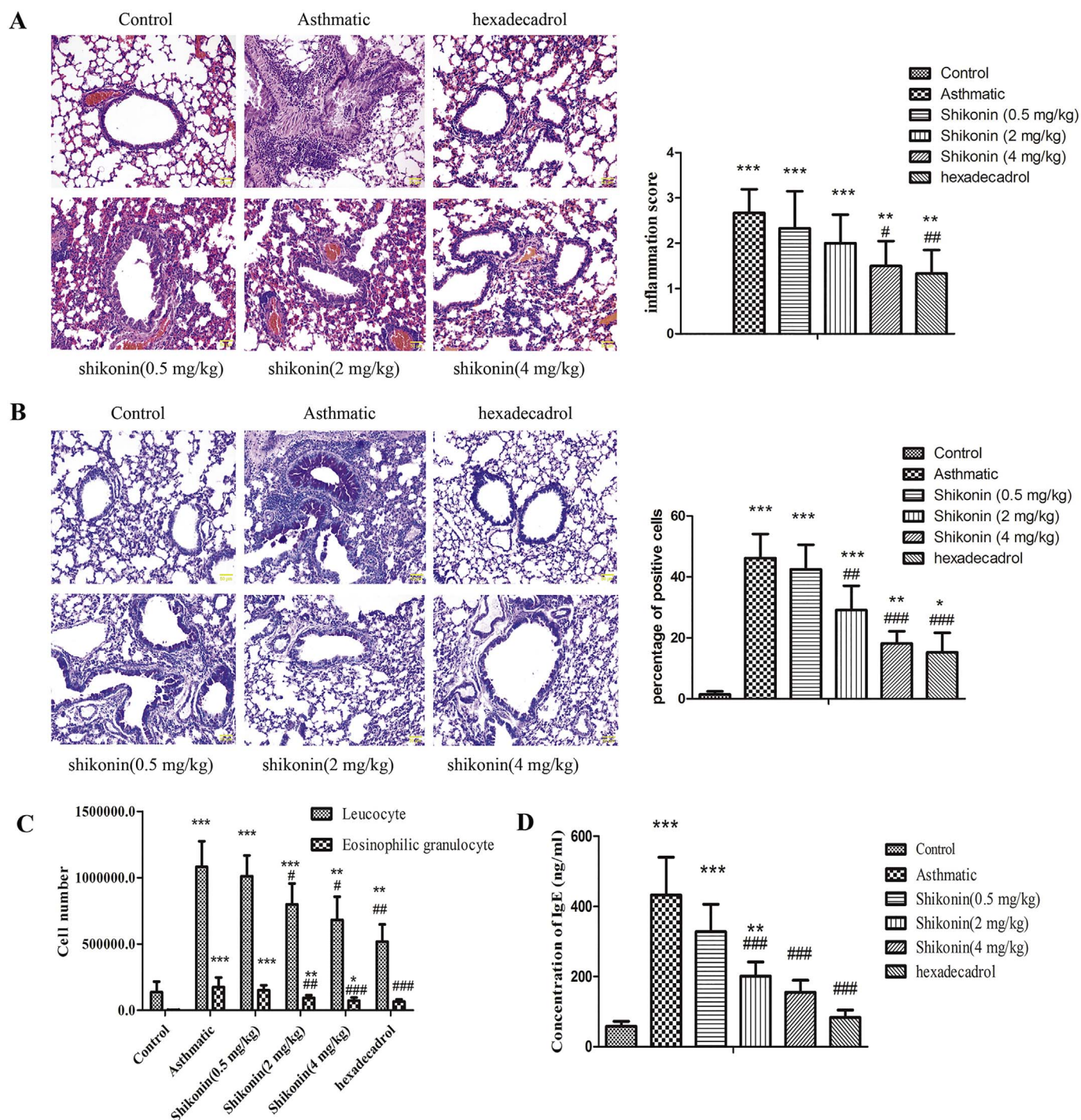


Fig. 2. Shikonin alleviates airway inflammation in vivo. (A) & (B) Lung sections were stained with H & E for measurement of inflammatory cells and PAS for measurement of mucus production around the airways. (C) Total and differential cell counts of BALF were determined by Giemsa staining. (D) The concentration of IgE in BALF was measured by ELISA. The results shown are representative of at least three independent experiments. Each value represents the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, versus the asthmatic group.

The number of inflammatory cells and concentration of IgE in BALF was measured among different groups. As shown in Fig. 2(C) & (D), the numbers of total leucocyte, eosinophils and concentration of IgE in asthmatic group were significantly higher than that from control group. Conversely, shikonin dose-dependently inhibited the increases in leucocyte, eosinophils counts and IgE level.

The Masson trichrome stain was performed to observe the collagen formation in bronchial airway. The shikonin group showed clearly less collagen formation than asthmatic group (Fig. 3(A)). Immunohisto-

chemistry and western blot were employed to investigate the expression of α -SMA protein in lung tissue. As shown in Fig. 3(B) & (C), the expression of α -SMA protein in the asthmatic group was significantly higher than that in the control group, but this increase was dose dependently abrogated by shikonin treatment. As shown in Fig. 3(D), the concentration of HYP in asthmatic group was significantly higher than that from control group. Conversely, shikonin induced a dose-dependent decrease in the concentration of HYP.

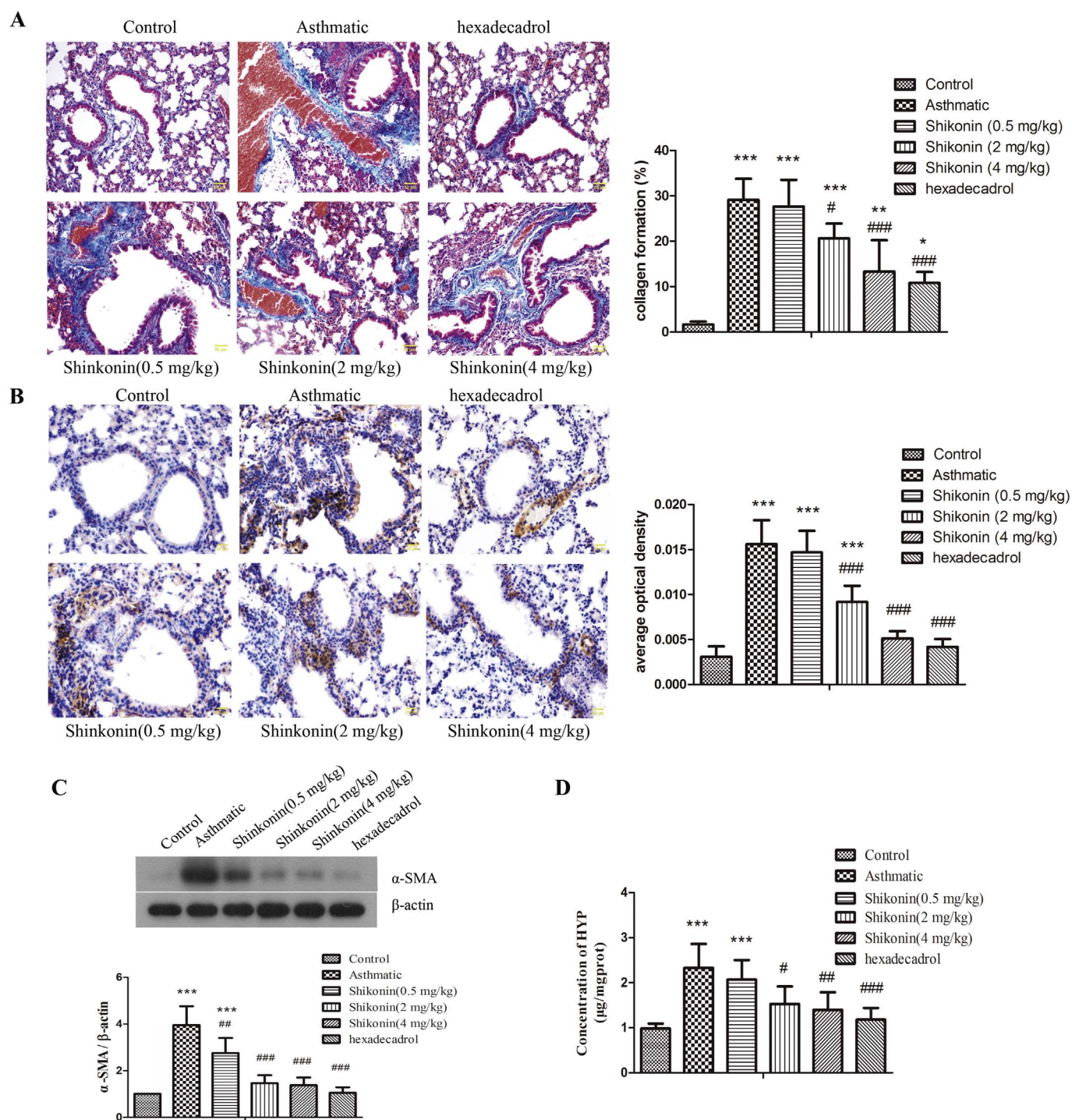


Fig. 3. Shikonin alleviates airway remodeling in vivo. (A) Lung sections were stained with Masson trichrome stains for measurement of collagen protein production around the airways. (B) & (C) α-SMA protein expression was measured by Immunohistochemistry and western blot in lung tissue. (D) The concentration of HYP in lung tissue was measured by ELISA. The results shown are representative of at least three independent experiments. Each value represents the mean $\pm$ SD ($n = 3$). *** $P < 0.001$, versus the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, versus the asthmatic group.

3.2. Effect of shikonin on ASMCs proliferation induced by PDGF *in vitro*

ASMCs were identified by immunofluorescence stain using anti α-SMA (1:200 diluted in PBS) and Cy3 conjugated goat-anti-rabbit (1:200) antibodies and observed under a fluorescence microscope. As shown in Fig. 4(A), the immunofluorescence of α-SMA suggested that the red-stained cells were ASMCs.

The effect of shikonin on the growth of ASMCs induced by PDGF in

vitro was tested by MTT assay. First, shikonin with different concentrations (0, 0.1, 0.3, 1, 3, 5 μM) was added to ASMCs for 48 h to determine the cytotoxicity, as shown in Fig. 4(B), the shikonin < 1 μM had no effect on proliferation. PDGF could significantly promote the proliferation of ASMCs, while incubation of shikonin (0.3, 1 μM) for 24 h and 48 h could inhibit the proliferation induced by PDGF remarkably (Fig. 4(C)).

Flow cytometric analysis was carried out to assess cell cycle

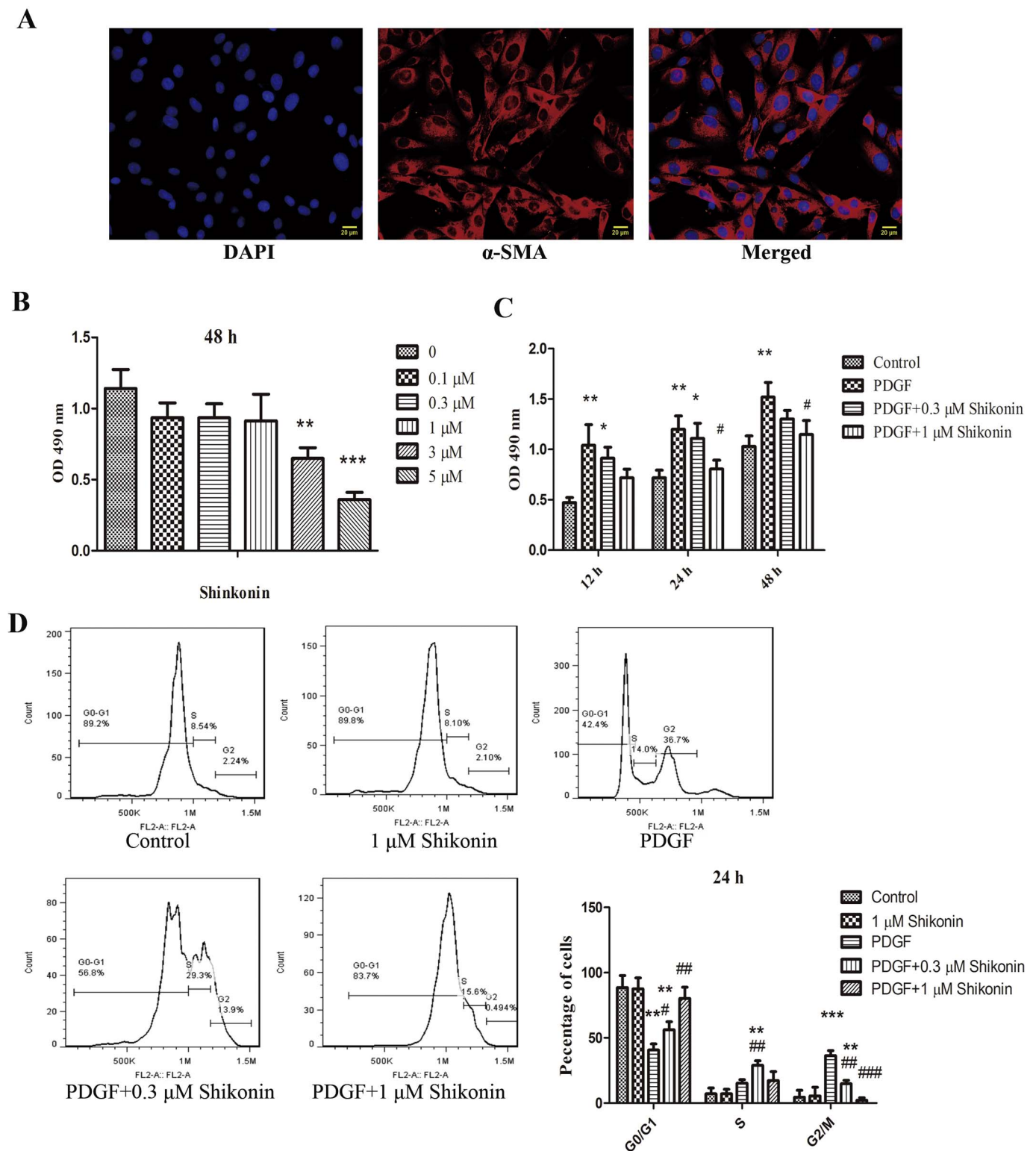


Fig. 4. Shikonin inhibits the proliferation of ASMCs induced by PDGF in vitro. (A) To identify the ASMCs the expression of α -SMA was determined by immunofluorescent staining. (B) ASMCs were treated with varying concentrations of shikonin and the toxicity of shikonin in ASMCs was detected by MTT assay. (C) Shikonin inhibited the proliferation of ASMCs induced by PDGF by MTT assay at 12 h, 24 h, and 48 h. (D) Shikonin affected the change of cell cycle progression induced by PDGF. ASMCs were treated with PDGF and shikonin for 24 h and the DNA content was analyzed by flow cytometry. The percentage of cells in the G1, S, and G2/M phases of the cell cycle was shown. The results shown are representative of at least three independent experiments. Each value represents the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, versus the PDGF group.

progression. After treatment with PDGF for 24 h, a significant decrease in the number of cells in the G0/G1 phase and a significant increase in the number of cells in the G2/M phase was found. However, shikonin (0.3, 1 μ M) could reverse the change induced by PDGF (Fig. 4(D)).

3.3. Effect of shikonin on ERK-NF- κ B signaling pathway

To assess the possible mechanism of shikonin against allergic inflammation and remodeling, we analyzed a number of related

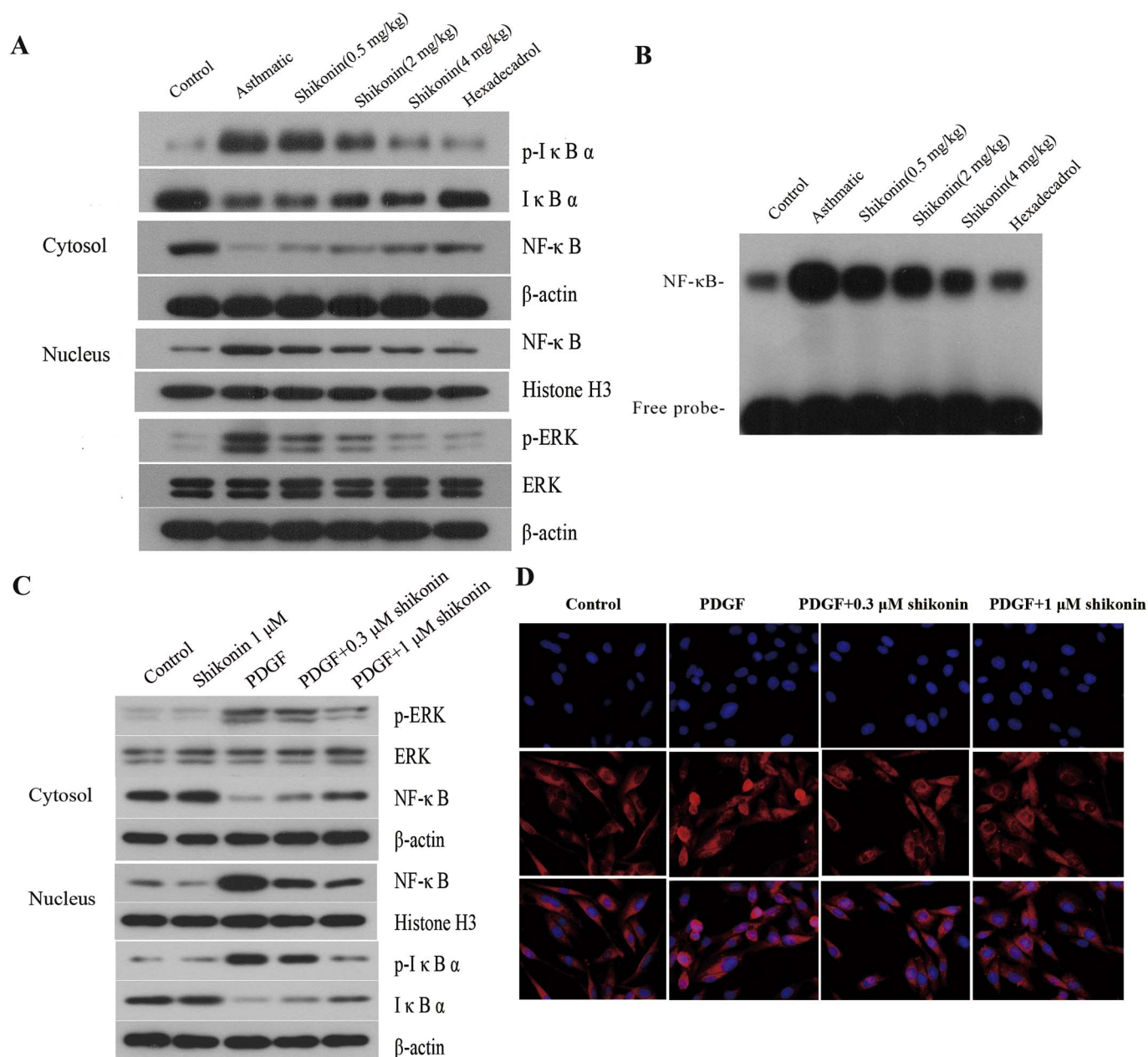


Fig. 5. Shikonin inhibits the ERK/NF-κB signaling pathway. (A) & (C) Shikonin affected the protein expressions of ERK/NF-κB signaling pathway. Lung tissues and ASMCs from different treatment groups were prepared and western blot analysis was performed against p-IκBα, IκBα, NF-κB, p-ERK, ERK, β-actin, Histone H3. β-Actin and Histone H3 were used as loading controls. (B) The transcription activity of NF-κB inhibited by shikonin in lung tissue was detected by EMSA assay. (D) The translocation of NF-κB in ASMCs was determined by immunofluorescent staining.

proteins in ERK and NF-κB signaling pathway by western blot. As illustrated in Fig. 5(A), the expressions of NF-κB in nucleus, phosphorylated ERK1/2, and phosphorylated IκBα protein in the asthmatic group were significantly higher than that in the control group, while the expressions of NF-κB in cytosol and IκBα protein were significantly decreased. However, these changes induced by OVA were dose dependently inverted by shikonin treatment. These results demonstrated that shikonin suppressed NF-κB activation by inhibiting, at least in part, IκBα activation. To further demonstrate the effect of shikonin on the activity of NF-κB binding to DNA, EMSA assay was initiated. The EMSA results clearly showed the activation of NF-κB in the asthmatic group, while shikonin could dose dependently inhibit the activation of NF-κB (Fig. 5(B)).

Similarly in ASMCs the expressions of NF-κB in nucleus, phosphorylated ERK1/2, and phosphorylated IκBα protein were enhanced by PDGF,

while the expressions of NF-κB in cytosol and IκBα protein were significantly decreased. However, shikonin inverted these changes induced by PDGF (Fig. 5(C)). The inactivation of NF-κB induced by shikonin was partly by inhibiting IκBα activation. Moreover the distribution of NF-κB in ASMCs was detected by immunofluorescent staining. As shown in Fig. 5(D), the distribution of NF-κB changed from cytosol to nucleus induced by PDGF, while shikonin could inhibit this change.

3.4. Effect of shikonin on migration in vitro and in vivo

MMPs are the downstream targets of NF-κB, which play an important role in degrading extracellular matrix components and promoting tumor cellular invasion in vitro and in vivo. Thus we used various assays to evaluate the effects of shikonin on the migration potential. As shown in Fig. 6(A), cellular migration detected by

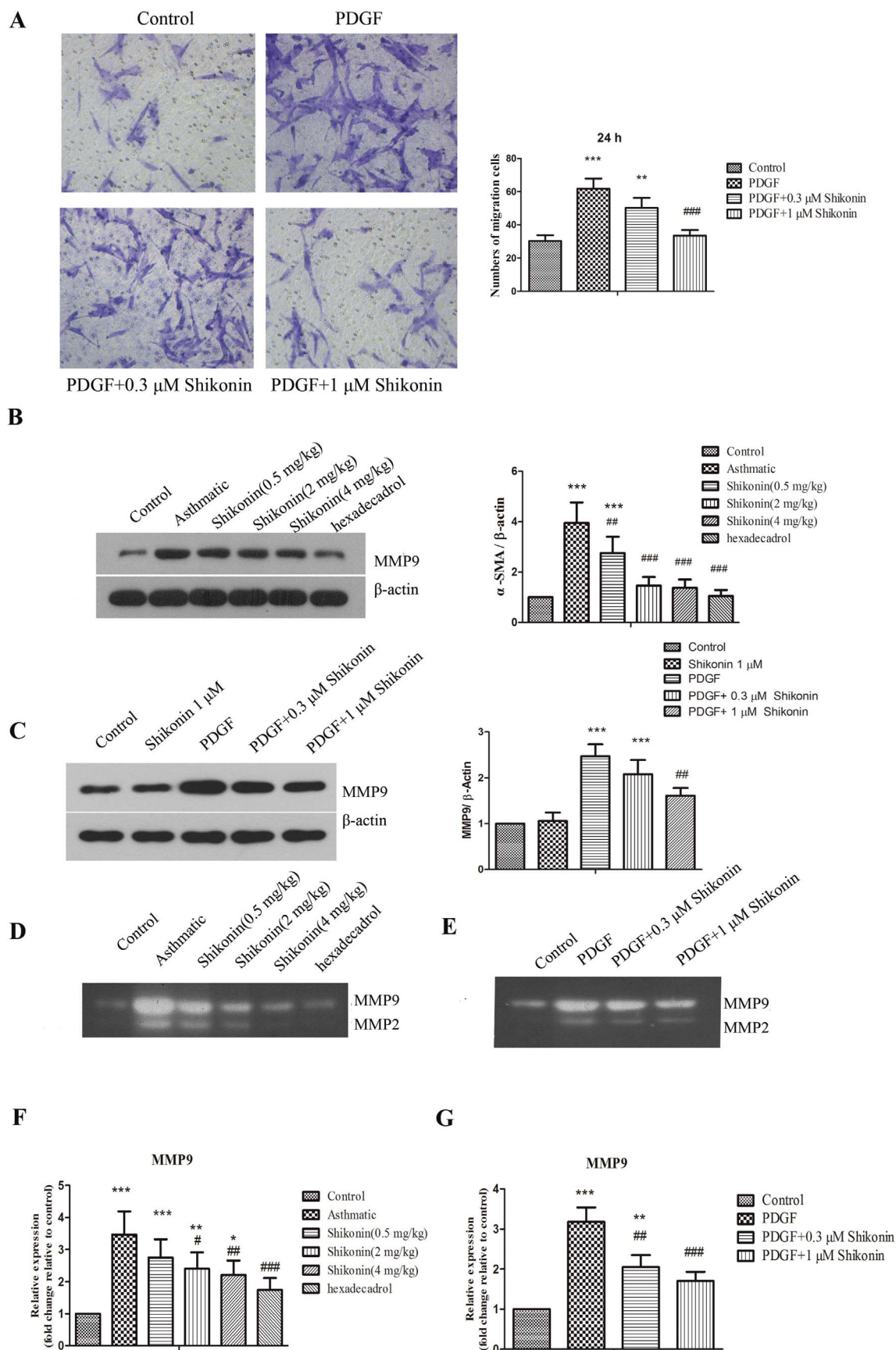


Fig. 6. Effect of shikonin on the migration potency in ASMCs and lung tissue. (A) ASMCs were treated with PDGF and shikonin, then transwell assay was used to detect the effect of shikonin on ASMC migration. (B) & (C) The protein expression of MMP-9 in lung tissue and ASMCs was detected by western blot. (D) & (E) The activities of MMP-9 and MMP2 in lung tissue and ASMCs were determined by gelatin zymography assay. (F) & (G) The mRNA expression of MMP-9 in lung tissue and ASMCs was detected by qPCR. The results shown are representative of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, versus the asthmatic or PDGF group.

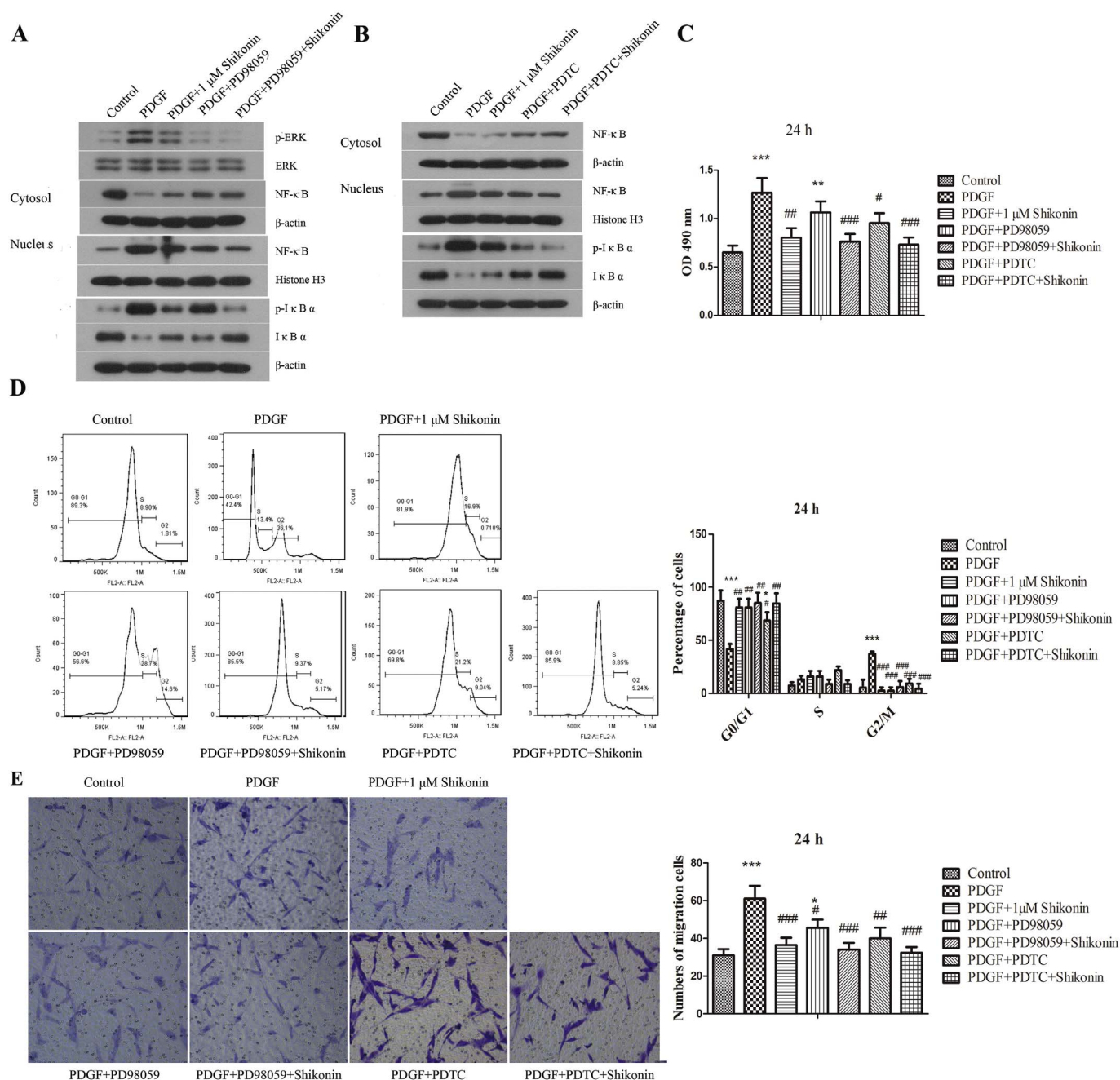


Fig. 7. PD-98059 and PDTC inhibited the proliferation and migration in ASMCs. (A) & (B) PD-98059 and PDTC had synergistic effect with shikonin on the protein expressions of ERK/NF- κ B signaling pathway. ASMCs from different treatment groups were prepared and western blot analysis was performed against p-I κ B α , I κ B α , NF- κ B, p-ERK, ERK, β -actin, Histone H3. β -Actin and Histone H3 were used as loading controls. (C) PD-98059 and PDTC inhibited the proliferation of ASMCs induced by PDGF by MTT assay at 24 h. (D) PD-98059 and PDTC affected the change of cell cycle progression induced by PDGF. The percentage of cells in the G1, S, and G2/M phases of the cell cycle was shown. (E) Transwell assay was used to detect the migration ability in ASMCs. The results shown are representative of at least three independent experiments. Each value represents the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, versus the control group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$, versus the PDGF group.

transwell assay was significantly promoted by PDGF. However, shikonin (0.3, 1 μ M) could inhibit PDGF-induced the migration remarkably.

Subsequently, the mRNA and protein expression of MMP-9 and activities of MMP-9 and MMP-2 were determined in vivo and in vitro. As shown in Fig. 6(B), (D) & (F), the expression of MMP-9 and activities of MMP-9 and MMP-2 in lung tissue of the asthmatic group were significantly increased compared to the control group, but this increase was significantly weakened by shikonin treatment. Similarly, the expression of MMP-9 and activities of MMP-9 and MMP-2 induced by PDGF in ASMCs were significantly increased compared to the control group, but this increase was significantly weakened by shikonin treatment (Fig. 6(C), (E) & (G)).

3.5. ERK1/2 MAPK and its downstream signaling pathway NF- κ B are involved in inhibition of proliferation and migration by shikonin in vitro

To further demonstrate the effect of ERK1/2 and NF- κ B signaling pathway, we selected the PD-98059 and PDTC which specifically inhibit the activation of ERK and NF- κ B respectively. As shown in Fig. 7(A) & (B), PD-98059 and PDTC could inhibit the increased expression of NF- κ B in nucleus, phosphorylated ERK1/2 (only PD-98059), and phosphorylated I κ B α protein induced by PDGF, which had synergistic effect with shikonin. Then we determined the effect of PD-98059 and PDTC on cell proliferation and cell cycle in ASMCs. The results showed that PD-98059 and PDTC could inhibit ASMC proliferation

tion (Fig. 7(C)) and cell cycle change (Fig. 7(D)) induced by PDGF.

Finally we determined the effect of PD-98059 and PDTC on cell migration in ASMCs. The results showed that PD-98059 and PDTC could inhibit the ASMCs migration (Fig. 7(E)) induced by PDGF.

4. Discussion

Our study finds some new interesting information about shikonin treatment inhibiting the ERK-NF- κ B signaling pathway in vitro and in vivo, which is one of the potential mechanisms of shikonin alleviating allergic airway inflammation and remodeling.

Firstly, we found that shikonin may play an important role in alleviating allergic airway inflammation and remodeling in vivo. Asthma is a chronic inflammatory response disease accompanied by immune function disorder, which involves of a variety of cell infiltration, inflammatory mediators, cytokines and adhesion molecules. IgE antibody plays an important role in its pathogenesis, which may make the eosinophils infiltrate into the airway. According to the present findings, a large number of infiltrating inflammatory cells, increased mucus formation and tubular structure damage were found in asthmatic group by histological examination of lung sections. Moreover the number of total leucocytes, eosinophils and concentration of IgE in BALF were evidently higher than those in the control group. Shikonin attenuated airway inflammation by diminishing the infiltrating inflammatory cells and IgE concentration. Airway remodeling is attributed to chronic repeated stimulation by inflammation mediators, which can induce the collagen deposition in airway epithelial basement membrane. α -SMA, a characteristic marker of smooth muscle, can reflect the quantity and contraction ability of smooth muscle, which is considered to be one of the important indexes of early airway remodeling [15]. HYP is one of the main components of collagen protein and its concentration is an important index of collagen tissue metabolism. According to the current study, shikonin attenuated the airway remodeling by reducing the increased collagen depositing, α -SMA expression and HYP concentration in lung tissue induced by OVA challenge.

To further dissect the molecular mechanisms underlying the effect of shikonin on anti-allergic airway inflammation and remodeling, we focused on the NF- κ B pathway. NF- κ B is a protein complex, which is also a kind of important transcription activating factor, widely existing in all eukaryotic cells [16]. NF- κ B plays important roles in regulating the genes related to immune response and inflammatory reactions. So far the dysregulation of NF- κ B is reported to link to inflammation, cancer, autoimmune diseases, viral infection, septic shock, and improper immune development [17–20]. A large number of inducers can activate the NF- κ B, such as reactive oxygen species, tumor necrosis factor alpha, interleukin 1-beta, bacterial lipopolysaccharides, isoproterenol, cocaine and ionizing radiation [21–25]. In un-stimulated cells NF- κ B is most sequestered by I κ B α in the cytoplasm, while upon stimulation with inducers, I κ B α is rapidly phosphorylated and degraded [26] to activate NF- κ B. Then the activated NF- κ B translocates to the nucleus, where it binds to promoters of inflammatory genes and regulates gene transcription. Many researches have reported that the allergic airway inflammation and remodeling had close relationship with the activation of NF- κ B [27–29]. In our study, we found that NF- κ B was activated in the lung tissue of OVA challenged mice and ASMCs induced by PDGF, while the activation of NF- κ B could be inhibited by shikonin, at least in part via suppressing I κ B α activation. Moreover, suppression of NF- κ B activity by PDTC, a specific inhibitor of NF- κ B, had synergistic effect with shikonin on inhibiting ASMCs proliferation, cell cycle change and migration induced by PDGF. These results suggest that the mechanism underlying the anti-asthmatic effects of shikonin may be attributed to the inhibition of NF- κ B transcriptional activity.

ERK is activated by various stimuli such as growth factors, cytokines, virus infection, transforming agents, and carcinogens, which is characterized by phosphorylation. ERK is known to activate many

transcription factors, such as c-myc, c-fos, c-jun, ATF, NF- κ B and ELK1 [30,31], which is closely related to cell proliferation and differentiation. Our study demonstrated that shikonin exerted inhibitory effect on the phosphorylation of ERK in the lung tissue of OVA challenged mice and ASMCs induced by PDGF. Moreover, PD-98059, a specific inhibitor of ERK, prominently repressed NF- κ B activity and had synergistic effect with shikonin on inhibiting proliferation, cell cycle change and migration in ASMCs induced by PDGF, suggesting that ERK acting as an upstream regulator of NF- κ B in allergic airway disease.

MMP9 has various functions in normal physiological processes, such as embryonic development, reproduction, angiogenesis, bone development, wound healing, cell migration, as well as in pathological processes, such as arthritis, intracerebral hemorrhage, and metastasis. NF- κ B has been found to be a key transcription factor in the regulation of MMP9 expression [32,33]. In the present study, we found that the mRNA and protein expression of MMP9 and enzyme activities of MMP9 and MMP2 were increased in the lung tissue of OVA challenged mice and ASMCs induced by PDGF, which could be weakened by shikonin treatment. In addition, cellular migration ability was significantly promoted by PDGF, while shikonin, PD-98059 and PDTC could inhibit the migration remarkably.

Taken together, our results contributed that shikonin may ameliorate asthmatic inflammation and remodeling by inhibition of the ERK-NF- κ B signaling pathway. These findings provide solid evidence that shikonin is a potential anti-inflammatory agent in asthma treatment.

Competing interests

The authors have no conflicting financial interests.

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