



Thymoquinone inhibits inflammation, neoangiogenesis and vascular remodeling in asthma mice

Xinming Su ^{*}, Yuan Ren, Na Yu, Lingfei Kong, Jian Kang

Department of Respiratory Medicine, Institute of Respiratory Diseases, The First Affiliated Hospital of China Medical University, Shenyang 110001, People's Republic of China

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ABSTRACT

Asthma is a chronic obstructive disease which is characterized by recurring airway inflammation, reversible airway obstruction, airway hyper responsiveness and vascular remodeling. Thymoquinone (TQ), an active ingredient isolated from *Nigella sativa*, was reported to exhibit anti-inflammation and anti-proliferation of in various cancer cells as well as epithelial cells. The aim of this study was to evaluate the effect of TQ on the inflammation, neoangiogenesis and vascular remodeling induced by Ovalbumin (OVA) in asthma mice *in vivo* and the anti-angiogenesis effects of TQ in VEGF-induced human umbilical vein endothelial cells (HUVECs) *in vitro*. Our results revealed that TQ inhibited the production of inflammatory factors interleukin-4/–5 (IL-4/–5) by enzyme-linked immunosorbent assay (ELISA). Immunohistochemistry analysis showed that the increase of platelet endothelial cell adhesion molecule-1, which is also known as CD31 and α -smooth muscle actin (SMA) expression in asthma mice challenged by OVA was suppressed by TQ. Moreover, TQ suppressed the activation of VEGFR2-PI3K-Akt pathway and up-regulated the expression of Slit glycoprotein-2 (Slit-2) both *in vivo* and *in vitro* with the inhibition of tube formation in HUVEC cells. Meanwhile immunofluorescence analysis showed that Slit-2 and Roundabout-4 (Robo-4) were co-expressing after TQ treatment in OVA-challenged asthma mice. Our study demonstrates that TQ attenuated the inflammatory reaction by antagonizing IL-4/–5 while the anti-neoangiogenesis effect of TQ is mediated by inhibition of vascular endothelial growth factor (VEGF) expression through VEGFR2/PI3K/Akt signaling pathway, which supports a potential role for TQ in ameliorating asthma.

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1. Introduction

Bronchial asthma is a chronic disorder characterized by recurring airway symptoms, inflammation, reversible airway obstruction [1], airway hyper responsiveness [2] and airway remodeling [3], which is accompanied by symptoms such as cough, wheezing, increased sputum production and sleep disturbance [4]. A variety of cells, such as mast cells, eosinophils, T lymphocytes, and neutrophils are involved in the process of airway inflammation of asthma [5]. Moreover, reports show that increase in size and number of blood vessels both inside and outside the smooth muscle layer as well as hyperemia of bronchial vasculature are contributing factors in airway wall remodeling in patients with bronchial asthma [6]. Due to the development of treatment complications, such as drug resistance and adverse effects, conventional medicine is still insufficient to provide a complete treatment of this disease; thus, it makes sense to investigate the potential mechanism and the regulatory factors of the variation of airway blood vessels in bronchial asthma in order to

provide alternative therapy, either to complement or replace existing conventional medicine.

Thymoquinone (TQ) is an active ingredient isolated from *Nigella sativa* and has been investigated for its anticancer, antioxidant and anti-inflammatory activities in both *in vitro* and *in vivo* models [7]. It is reported that TQ can inhibit the proliferation in various cancer cells such as human breast cancer MCF-7 cell line [8], colon cancer [9] and human epithelial ovarian cancer A2780 cell line [10]. El-Khouly et al. found that TQ can attenuate the severity of oxidative stress and inflammatory response during bleomycin-induced pulmonary fibrosis [11]. Notable, series of investigations revealed that TQ attenuates airway inflammation in a mouse model of allergic asthma by inhibiting the production of IL-4, IL-5 and IL-12 and some inflammation factors such as cyclooxygenase-2 (COX-2) and Prostaglandin D2 (PGD2) induced by ovalbumin (OVA) [12–14], which reflects that TQ has the anti-inflammatory and effect on bronchial asthma and allergic airway inflammation. However, the precise pathomechanism of TQ in the process of angiogenesis as well as vascular remodeling still remains unclear. Recently, Xu et al. reported that TQ relieves the angiogenesis through down-regulation of the expression of vascular endothelial growth factor (VEGF) as well as nuclear factor-kappa B (NF- κ B) signal pathways in human osteosarcoma both *in vitro* and *in vivo* [15]. VEGF, is a key factor

^{*} Corresponding author at: Department of Respiratory Medicine, Institute of Respiratory Diseases, The First Affiliated Hospital of China Medical University, 155 North Nanjing Street, Shenyang 110001, People's Republic of China.

E-mail address: xinming_med@126.com (X. Su).

in angiogenesis, high expression of which induces airway wall remodeling associated with angiogenesis in asthma [16]. Except for VEGF, little is known about the role of guidance cues such as Slit-family proteins (Slits) in directing blood vessel growth and organization during organ development [17]. Slit glycoprotein-2 (Slit2) inhibits vessel growth by down-regulating vascular endothelial growth factor receptor (VEGFR) signaling through Roundabout-4 (Robo-4) [15,17,18]. Based on the research above, we elucidate a role for TQ in the expression of vascular endothelial growth factor (VEGF) as well as Slit-2, which provide a basis for the potential development of TQ in relieving the angiogenesis and vascular remodeling in patients with bronchial asthma.

2. Materials and methods

2.1. Animals

Specific pathogen free Balb/c mice (6–8 weeks old; 20–22 g) were used for the experimental study. Mice were procured from Charles River Laboratories, Beijing, China and acclimatized for a week under standard laboratory conditions. All procedures were approved by the institutional animal care and use committee (IACUC).

2.2. Grouping of animals

Balb/c mice were divided into five groups (6 mice/group). Group 1: control group, treatment with TQ Vehicle i.p.; Group 2: OVA group, treatment with TQ Vehicle (DMSO) 1 h before every nebulization; Group 3: OVA + TQ group, treatment with 3 mg/kg TQ 1 h before every nebulization; Group 4: TQ group, treatment with 3 mg/kg TQ i.p.; and Group 5: OVA + dexamethasone (DEX) group, treatment with 1 mg/kg dexamethasone i.p. 1 h before every nebulization.

2.3. The establishment of sensitization and experimental protocol of asthma mice model

Briefly, all mice were sensitized with 10 µg OVA (A-5253, Biosharp, Hefei, China) adsorbed in 2 mg aluminum hydroxide given (i.p.) on days 0, 14 and 21 days. After two weeks, mice were exposed to 1% OVA through nebulizer for 30 min three times a week for 8 weeks. TQ (274,666, Sigma Aldrich, St. Louis, MO, USA) with the purity of 98.5% was first dissolved in 1 ml DMSO then diluted to 10 ml with distilled water and given mice from days 15 to 56 an hour before every nebulization (1% OVA). The non-sensitized mice were nebulized by saline in similar way. All mice were sacrificed on day 56 (24 h after the last OVA treatment). DEX (41021255, Tianjin Pharmaceutical Group Xinzhen CO., Tianjin, China) dissolved in phosphate buffer (PBS) was used as positive control.

2.4. Brochoalveolar lavage fluid (BALF)

Twenty-four hours after the last treatment all experimental mice were sacrificed. The chest cavities were carefully opened, tracheas were exposed and BALF was performed by delivering 0.8 ml cold PBS into the airway through a trachea cannula and gently aspirating the fluid. The lavage was repeated three times to recover a total volume of 2–3 ml. The cells were stained with Hematoxylin-Eosin (HE) or Glycogen dyeing liquid (PAS). BALF was centrifuged at 3000 rpm for 10 min and supernatants were collected and stored at –80 °C for further study and pellets were resuspended in PBS.

2.5. Isolation of lung cells

Lung cells were prepared after cannulating the trachea and perfusing the airways with cold PBS to collect BALF. Lungs were then sliced and incubated in 5 ml PBS containing 1.6 mg/ml collagenase type II (175 U/ml, Beyotime Co., Shanghai, China), for 15 min at 37 °C, with continuous

shaking slowly. The digested tissue was disrupted into a single cell suspension by passing through 20-gauge needle and filtered through a 45 µm nylon mesh. The resulting cell suspension was centrifuged at 1500 rpm for 5 min at 4 °C. Cells (whole lung cells) were washed in PBS, and used for IHC and Western blot.

2.6. ELISA

Cells treated with or without OVA and/or TQ were harvested and washed in cold PBS two times. The concentrations of interleukin-4 (IL-4) and interleukin-5 (IL-5) in BALF and the whole lung cells were determined with the Mouse Interleukin-4/–5 detection kits (WanLei Life sciences, Shenyang, China) according to the manufacturer's protocol. The concentration level of OVA-IgE in serum was determined by Mice ovalbumin IgE (OVA-IgE) detection kit assay kit (WanLei Life sciences, Shenyang, China).

2.7. Hematoxylin-eosin/glycogen dyeing liquid staining

Samples from the lung were isolated, fixed with 10% paraformaldehyde (Sinopharm Group Ltd., Shanghai, China) and embedded in paraffin wax. Sections were cut at 5 µm using a microtome and deparaffinized tissue sections were subjected to staining with hematoxylin (Solarbio, Beijing, China) and eosin (Sinopharmgroup.LTD, Shanghai, China)/Glycogen dyeing liquid (PAS, Baso Co., Zhuhai, China) for histological examination (CM69001, Cleica, Germany). The slides were examined by light microscopy and photographed. All complete airways cut in cross section (max/min diameter ratio < 1.5) were sized by measuring the airway basement membrane perimeter and the number of blood vessels within the associated airway wall were counted. Results were showed as vessel number normalized to airway perimeter² (perimeter × perimeter), which is proportional to airway area [19]. Airways without bronchial vessels were not included. All images were taken at 400× magnification.

2.8. Cell culture

Human umbilical vein endothelial cells (HUVECs; obtained from ATCC) were cultured in gelatin-coated plates with Dulbecco Modified Eagle Medium (DMEM, Gibco®, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U of penicillin and 100 mg/ml of streptomycin (Gibco®, Grand Island, NY, USA), and incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

2.9. Tube formation assay

Matrigel (10 mg/ml, BD Biosciences, San Diego, CA) was dissolved at 4 °C overnight, and each well of pre-chilled 96-well plates was coated with 50 µl Matrigel and incubated at 37 °C for 2 h. HUVECs were seeded onto the Matrigel in 100 µl DMEM supplemented with 10% FBS and incubated with or without 100 nmol/l TQ in the presence or absence of 10 ng/ml VEGF for stimulation at 37 °C for 24 h in a humidified 5% CO₂ atmosphere. Morphological changes of cells were observed under a microscope (Motic, Xiamen, China) and photographed at 100× magnification. Tubular structures were quantified by manual counting and percent inhibition was expressed using untreated wells as 100%.

2.10. Western blot analysis

Cells were harvested and lysed in ice-cold radioimmuno-precipitation (RIPA) buffer (Beyotime Co., Shanghai, China) plus PMSF (Beyotime Co., Shanghai, China), and total protein concentrations in the supernatant were determined using the BCA Protein Assay Kit (Beyotime Co., Shanghai, China) following manufacturer's instructions.

Western blot analysis was performed using a standard protocol. The primary antibodies used in this study were as follows: anti-VEGF, VEGF-2, PI3K P85 and AKT (1:1000, Boster Bio Co., Wuhan, China);

anti-p-VEGF2, p-PI3K P85, p-AKT and Slit-2 (1:1000, Bioss Bio Co., Beijing, China); and anti- β -actin (1:1000, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA). The second antibodies were goat anti-rabbit or goat anti-mouse IgG horseradish peroxidase (HRP) (Beyotime Co., Shanghai, China). Briefly, equal amounts of total proteins were fractionated on sodium dodecyl sulfate polyacrylamide gels and transferred electrophoretically onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% (w/v) skim milk in TBS-T buffer (10 mM Tris-HCl, 150 mM NaCl, and 1% Tween 20) for 30 min. The filters were hybridized with the appropriate primary antibodies overnight at 4 °C. Subsequently, the membranes were incubated with secondary antibodies at room temperature for 1 h, and protein bands were developed with an enhanced chemiluminescence detection kit ECL detection reagent (7 Sea Biotech, Shanghai, China) according to the manufacturer's instructions. Quantitative analysis for Western blot was made by Gel-Pro-Analyzer software (GEL-PRO ANALYZER, Media Cybernetics, Rockville, MD, USA).

2.11. RT-PCR analysis

Cells were harvested and extracted using RNA simple Total RNA Kit (DP419, TIANGEN Co, Beijing, China) according to the manufacturer's protocol. Briefly, 1 μ g total RNA was reverse transcribed in a volume of 20 μ l for the synthesis of complementary (cDNA) using 2 $\times$ Power Taq PCR MasterMix (PR1702, BioTeke Co., Beijing, China). The Reverse Transcription (RT) products were then amplified for PCR. Primers used in RT-PCR were as follows: Slit-2, TTAGTGAAGCGGTGGGTAC (sense) and TTGGGAAGTGTGTAAGG (antisense); β -actin, CTGTGCCATCTACGAGGGCTAT (sense) and TTTGATGTCACGACGATTTC (antisense). For each PCR reaction, a master mix that includes SYBR GREEN Master Mix (Solarbio Co., Beijing, China), forward primer, reverse primer, and 10 ng template DNA was prepared; the reaction condition for the RT was as follows: 25 °C for 10 min, 42 °C for 50 min, and 95 °C 5 min. The PCR amplification conditions were: 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s, then 4 °C for 5 min. The PCR results were verified by varying the number of PCR cycles for each cDNA and set of primers. PCR reaction was performed using Exicycler™ 96 RT-PCR machine (Bioneer, Daejeon, Korea) with β -actin as a control. RT-PCR was performed at least in quadruplicate.

2.12. Immunofluorescence

Immunofluorescent staining was performed on frozen sections of lung. Samples from the lung were isolated, fixed with 10% paraformaldehyde (Sinopharm Group Ltd., Shanghai, China) and embedded in paraffin wax. Microwave antigen retrieval was carried out in citrate buffer for 10 min followed by 15 min of cooling at room temperature. Sections were blocked and permeabilized in 2% goat serum (ZSGB-BIO, Beijing, China) for 30 min and then incubated with anti-CD31, Slit2, robo (1) and robo (2) (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) antibody at 4 °C overnight. The secondary antibody (Beyotime Co., Shanghai, China) in blocking reagent was incubated at room temperature for 90 min. Both antibodies were diluted in 1% goat serum albumin in PBS. Samples were stained with DAPI (Beyotime Co., Shanghai, China) and observed in an inverted fluorescence microscope (Olympus, Tokyo, Japan); all images were taken at 400 $\times$ magnification. For negative control, samples were processed omitting the primary antibody.

2.13. Immunohistochemistry

Paraffin embedded tissue sections of 5 μ m thickness were rehydrated first in xylene and then in graded ethanol solutions. The slides were then blocked with 5% goat serum for 15 min. The sections were then immunostained with primary antibody CD31 (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA), VEGF (Boster Bio Co., Wuhan, China) and α -SMA (abcam, Cambridge, MA, USA) and incubated overnight at 4 °C. After washing the slides with PBS for 3 times, the sections

were incubated with secondary antibody in room temperature for 30 min. Sections were then washed with PBS and incubated for 5–10 min in DAB Horseradish Peroxidase Color Development Kit (0.02% diaminobenzidine containing 0.01% hydrogen peroxide, Beyotime Co., Shanghai, China). Counter staining was performed using hematoxylin (Solarbio, Beijing, China) and the slides were visualized under a light microscope (Olympus, Tokyo, Japan); all images were taken at 400 $\times$ magnification. Quantitative analysis for Immunohistochemistry was made by Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA) software and shown as average optical density (AOD) value.

2.14. Statistical analysis

Data were presented as the mean values $\pm$ standard deviation (SD) from at least three experiments. Statistical comparisons were analyzed by Bonferroni's multiple comparison tests or analysis of variance. $P < 0.05$ was considered as a statistically significant difference.

3. Results

3.1. TQ inhibited the lung inflammation in asthma mice

Treatment with OVA increased the concentration of OVA-IgE in serum of mice, and TQ inhibited the increase effect of OVA-IgE with the same effect as Dexamethasone (DEX), the anti-inflammation and anti-immune disease drug (Fig. 1A). Then production levels of IL-4 and IL-5 in both BALF (Fig. 1B) as well as lung tissue (Fig. 1C) after challenged with OVA were determined by ELISA analysis. Firstly, ELISA analysis showed that the concentration of IgE in mice serum was increased from 49.89 ± 9.68 in Con group to 1258.95 ± 309.88 in OVA group ($^{##}P < 0.01$), while it was decreased to 769.93 ± 139.24 by treatment with TQ in OVA + TQ group ($^{**}P < 0.01$) with the same trend as in OVA + DEX group (555.83 ± 125.12). Then BALF was collected 24 h after the last OVA challenge. OVA produced significant amounts of these cytokines in BALF of OVA group compared to Con group. The concentration of IL-4 in Con group versus OVA group was 17.30 ± 4.86 versus 61.88 ± 9.68 ($^{##}P < 0.01$, Fig. 1B, left). And the concentration of IL-5 in BALF of Con group versus OVA group was 19.14 ± 4.77 versus 143.99 ± 38.18 ($^{##}P < 0.01$, Fig. 1B, right). Intraperitoneal administration of TQ before OVA challenge normalized the elevated cytokine levels (OVA + TQ group), with greater effect on IL-4 and IL-5. Data showed that IL-4 in OVA group versus OVA + TQ group was 61.88 ± 9.68 versus 37.91 ± 9.61 ; IL-5 in OVA group versus OVA + TQ group was 143.99 ± 38.18 versus 74.90 ± 19.64 , ($^{**}P < 0.01$, Fig. 1B). In addition, the levels of IL-4 and IL-5 in OVA-sensitized mice appeared to be the same trends as that in BALF. Data showed that IL-4 in OVA group versus OVA + TQ group was 38.54 ± 8.32 versus 97.23 ± 16.50 ; IL-5 in OVA group versus OVA + TQ group was 58.20 ± 11.22 versus 216.59 ± 47.51 ($^{**}P < 0.01$, Fig. 1C). However, administration of TQ inhibited the increase of these cytokine levels (IL-4: from 97.23 ± 19.50 to 67.16 ± 16.35 , and IL-5: from 216.59 ± 47.51 to 127.54 ± 30.00 , $^{**}P < 0.01$), while administration of TQ alone (TQ group) had no effect on the cytokine response in both BALF and lung tissue. In addition, the effect of TQ was compared with that of DEX, data indicating no significant differences between two groups (data not shown).

3.2. TQ suppresses lung inflammation and angiogenesis in histology and morphometry

Representative PAS stained histologic sections from OVA and/or TQ treated mice 24 h after the last OVA challenge are shown in Fig. 2A. Data showed that there was a marked increase in the number of PAS-positive cells in the airway mucosa indicating mucous metaplasia as well as inflammation infiltration in airway epithelial cells in OVA group compared to Con group (from 0.00 ± 0.00 in Con group to 44.06 ± 10.33 in OVA group, $^{##}P < 0.01$), and the number of PAS-positive cells

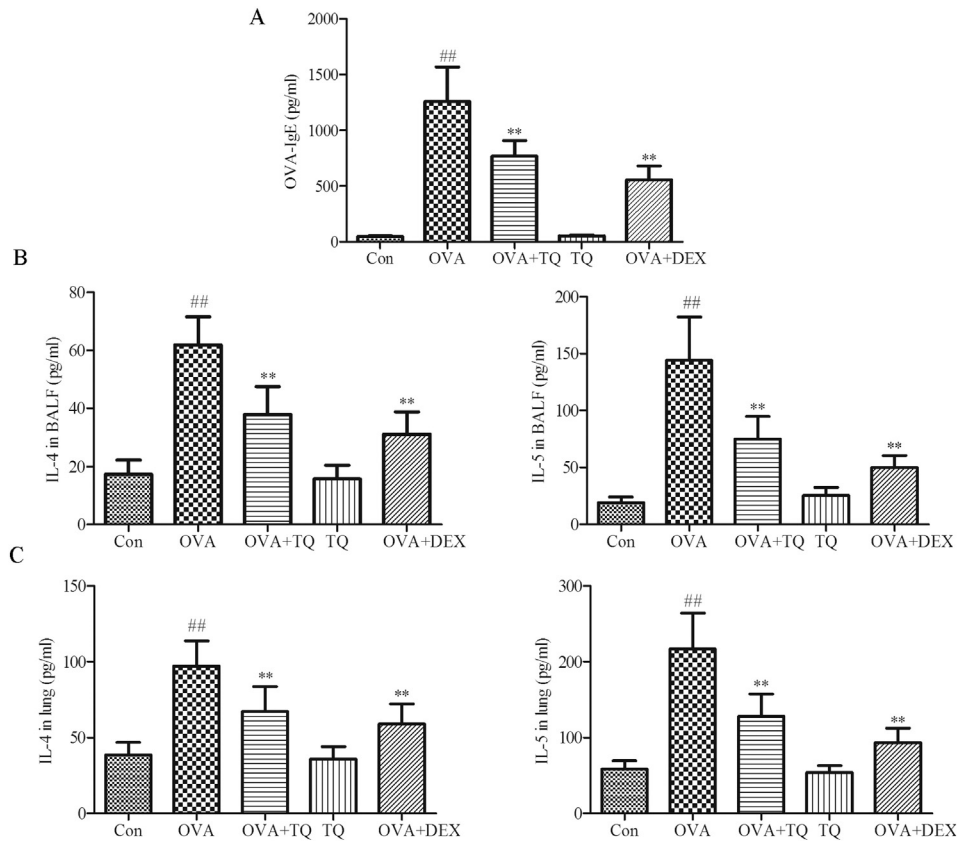


Fig. 1. TQ inhibited the lung inflammation in asthma mice. (A) TQ inhibited the level of OVA-IgE in serum. Level of OVA-IgE in serum after treatment with or without OVA and/or TQ was tested by ELISA. (B and C) TQ inhibited the expression of IL-4 and IL-5 in both BALF (B) as well as lung tissue (C) after sensitized and challenged with OVA. Levels of IL-4 and IL-5 in BALF or lung tissue were measured by ELISA. DEX was used as the positive control. Data are expressed as mean $\pm$ S.E.M. ** $P < 0.01$ versus OVA group; ## $P < 0.01$ versus Con group. Con, control; OVA, ovalbumin; TQ, thymoquinone; DEX, dexamethasone.

was decreased by treatment with TQ (Fig. 2A, from 44.06 ± 10.33 in OVA group to 20.76 ± 7.15 in OVA + TQ group, ** $P < 0.01$). Thus, TQ inhibited the inflammation infiltration as well as metaplasia in OVA-challenged asthma mice.

Also H&E staining in histological examination of lung tissues collected 24 h after OVA challenge showed the abundance of blood vessels observed in OVA group compared to Con group (from 77.80 ± 15.11 in Con group to 135.43 ± 27.33 in OVA group, ## $P < 0.01$), while TQ inhibited the increase of blood vessels induced by OVA indicating the anti-neoangiogenesis effect in lung of asthma mice (Fig. 2B, from 135.43 ± 27.33 in OVA group to 104.77 ± 24.70 in OVA + TQ group, ** $P < 0.01$). Images also displayed a striking difference between the Con group and OVA group with regard to an associated influx of inflammatory cells, airway wall thickening, and smooth muscle contraction, which was significantly suppressed by treatment of TQ (Fig. 2B). In addition, treatment with TQ alone did not affect lung inflammation as well as neoangiogenesis. These results demonstrated the anti-inflammatory and anti-neoangiogenesis effects of TQ on OVA-induced lungs of asthma mice.

3.3. TQ attenuated the neoangiogenesis and vascular remodeling in asthma mice

Vascular remodeling and neoangiogenesis may participate in the asthma through facilitating the hyperplasia and the differentiation of airway smooth muscle cells. To investigate the anti-vascular remodeling effect of TQ on OVA-challenged in asthma mice, the expression of both platelet endothelial cell adhesion molecule (CD31) and α -smooth muscle actin (α -SMA) were tested in the samples of normal lung tissue, lung tissue challenged with OVA with or without treated with TQ (Fig.

3). As the expression of CD31 and α -SMA were used to assess the angiogenesis and the thickened subepithelial smooth muscle in airway of bronchial asthma mice, respectively [20,21], lung tissue challenged with OVA showed positive expression of CD31 (Fig. 3A) and α -SMA (Fig. 3B) using immunohistochemistry (IHC) analysis; however, TQ treatment inhibited the expression of CD31 and α -SMA, which showed decreased expression of both CD31 and α -SMA in OVA + TQ group. And junior staining of CD31 and α -SMA was noted in normal lung tissue (Con group). The quantitative value of average optical density (AOD) analysis of CD31 and α -SMA between Con group and OVA group was 1.70 ± 0.39 versus 3.18 ± 0.64 (10^{-2}) and 3.22 ± 0.71 versus 9.73 ± 2.23 (10^{-3}), respectively, (## $P < 0.01$). And the AOD values of CD31 and α -SMA in OVA group versus OVA + TQ group was 3.18 ± 0.64 versus 2.20 ± 0.49 (10^{-2}) and 9.73 ± 2.23 versus 5.02 ± 0.84 (10^{-3}), respectively, (Fig. 2A&B). Data showed that TQ attenuated the neoangiogenesis and vascular remodeling through suppressing the expression of CD31 and α -SMA in asthma mice challenged with OVA.

3.4. TQ inhibited the PI3K-Akt-VEGF pathway through down-regulation of VEGF, p-VEGFR2, p-PI3K and p-AKT in vivo

To further study the mechanism of how TQ inhibited the neoangiogenesis and vascular remodeling in asthma mice, immunohistochemistry (IHC) staining was used to document the change of VEGF in lung tissue challenged with OVA with or without treated with TQ (Fig. 4A). Data showed that VEGF expression was positive in OVA-challenged lung tissue, which was decreased by TQ treatment. The quantitative value of AOD analysis of VEGF between Con group and OVA group was 1.12 ± 0.28 versus 2.76 ± 0.53 (10^{-2} , ## $P < 0.01$).

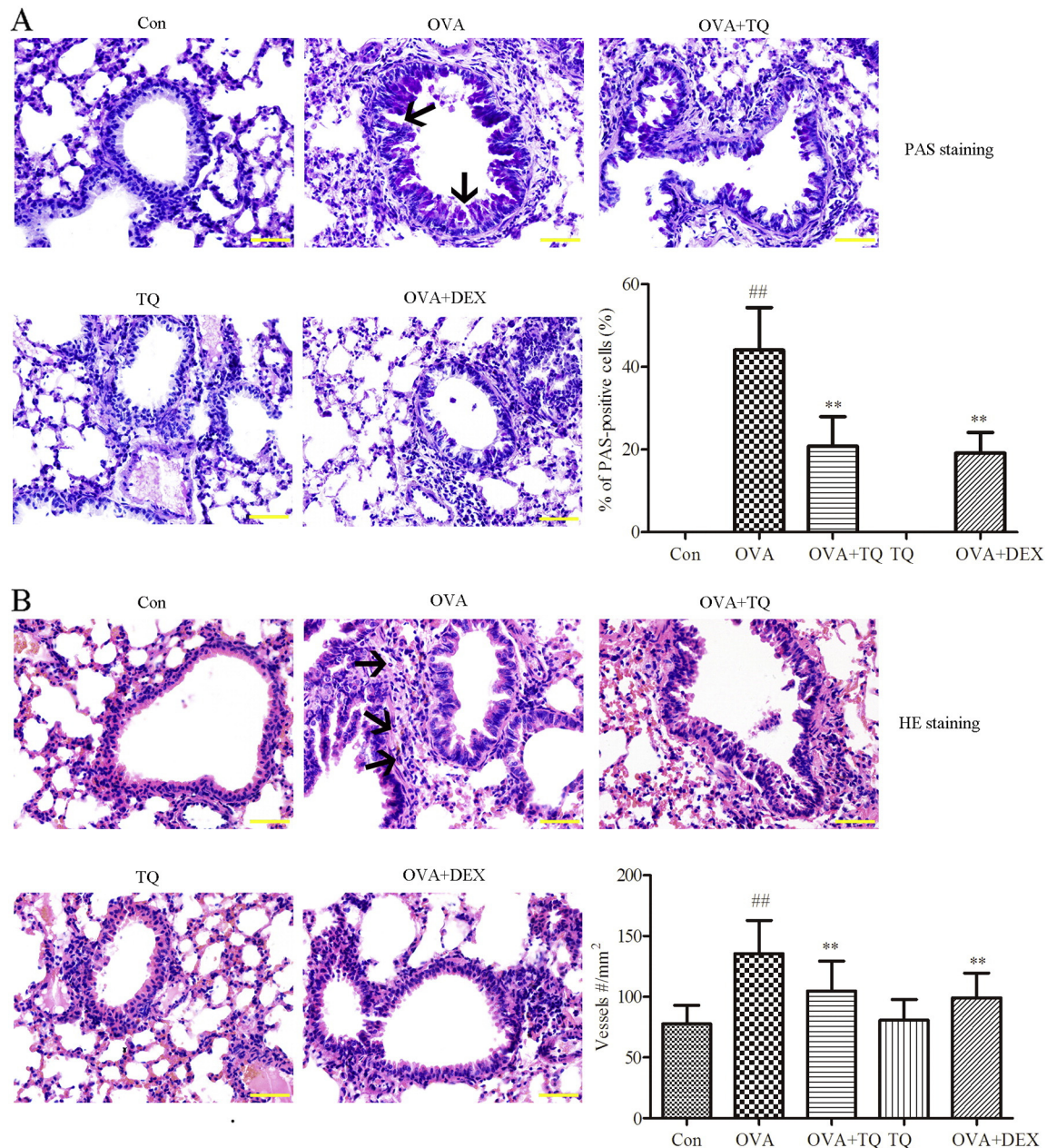


Fig. 2. TQ abrogates lung inflammation and angiogenesis in histology and morphometry. Lung tissue was collected from each group. Tissue sections were stained with PAS, to examine lung mucous metaplasia (A). The number of PAS-positive cells is counted and expressed in (A). (B) H&E staining, to detect lung angiogenesis, average number of bronchial blood vessels normalized to perimeter² (mm²; proportional to airway area) in lung (400 $\times$ magnification). Arrows indicate some of the bronchial vessels as well as PAS-positive cells within the airway wall, respectively. DEX was used as the positive control. Data are expressed as mean $\pm$ S.E.M. ^{**} $P < 0.01$ versus OVA group; ^{##} $P < 0.01$ versus Con group. Con, control; OVA, ovalbumin; TQ, thymoquinone; DEX, dexamethasone.

And the AOD value of VEGF in OVA group versus OVA + TQ group was 2.76 ± 0.53 versus 2.08 ± 0.42 (10^{-2} , ^{**} $P < 0.01$, Fig. 4A, below).

For the expression levels of VEGF in lung tissue, statistically significant differences were found between OVA-challenged lung tissue and OVA-challenged lung tissue pre-treatment with TQ by Western blot analysis (Fig. 4B). It was found that the level of VEGF expression was up-regulated by OVA-challenged lung tissue, whereas treatment with TQ decreased the level by half (Fig. 4B below, from 2.11 ± 0.35 in OVA group to 1.51 ± 0.24 in OVA + TQ group). To further confirm whether VEGFR-2 participates in the regulation of OVA-challenged asthma in mice, we next examined possible signaling pathways that could regulate the expression change of VEGFR by treatment of TQ. One was PI3K-Akt signal pathway, which is important for regulation of VEGF, and it is also well documented that the phosphatidylinositol

3-kinase/protein kinase B (PI3K/Akt) pathway played an important role in angiogenesis and vascular remodeling [22]. Therefore, we investigated the changing levels of the PI3K-Akt pathway in OVA-challenged lung tissue with or without TQ. When the lung tissue samples were submitted to Western blot analysis, the expression levels of VEGFR2, p-VEGFR2, PI3KP85, p-PI3K P85, Akt and p-Akt in TQ-challenged lung tissue with or without TQ treatment were also detected (Fig. 4B). As shown in Fig. 4B, comparing to the control group, the expression levels of VEGFR2, PI3KP85 and Akt were not changed, whereas levels of p-VEGFR2, p-PI3K P85 and p-Akt were up-regulated in lung tissue challenged by OVA. Conversely, levels of p-VEGFR2, p-PI3K P85 and p-Akt protein were significantly reduced by TQ treatment. The quantitative value of gray intensity analysis of p-VEGFR2, p-PI3K P85 and p-Akt between OVA group and OVA + TQ group was 3.39 ± 0.75 versus

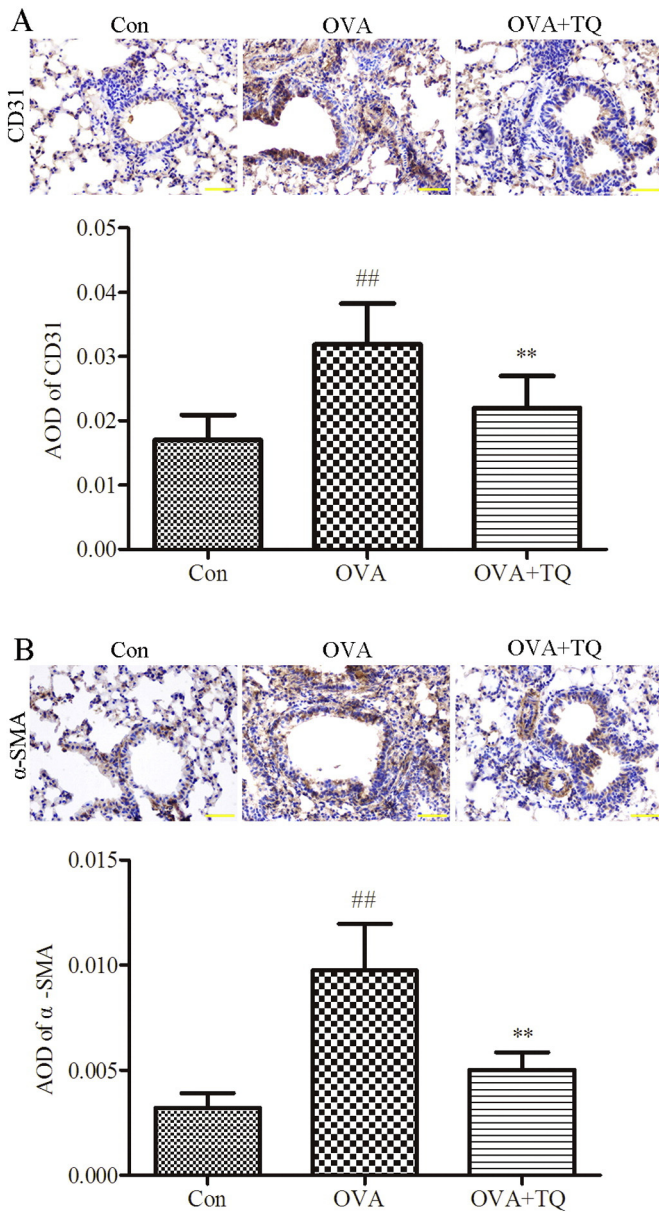


Fig. 3. TQ attenuated the neoangiogenesis and vascular remodeling in asthma mice. (A) TQ inhibited the increase of CD31 in asthma mice. Lung tissue was collected from each group. Tissue sections of each group incubated with anti-CD31 antibody were tested by immunohistochemistry analysis. (B) TQ inhibited the expression increase of α -SMA in asthma mice caused by OVA. Lung tissue sections incubated with anti- α -SMA antibody were tested by immunohistochemistry analysis (400 $\times$ magnification). The quantitative value of average optical density (AOD) analysis of CD31 and α -SMA was calculated and shown in (A&B, below). Data are expressed as mean $\pm$ S.E.M. ** $P < 0.01$ versus OVA group; ## $P < 0.01$ versus Con group. α -SMA, alpha-smooth muscle actin.

1.54 ± 0.75 , 2.00 ± 0.40 versus 1.35 ± 0.32 and 3.15 ± 0.64 versus 1.92 ± 0.43 respectively, $P < 0.01$ (Fig. 4B, below). Thus, treatment with TQ decreased the phosphorylation of VEGFR2, PI3K and Akt. Therefore we conclude that TQ inhibited the neoangiogenesis and vascular remodeling of asthma mice through VEGFR2-PI3K-Akt pathway.

3.5. TQ recovered the expression of Slit-2 down-regulated by OVA and mediated the co-expression of Slit-2 and Robo-4 in asthma mice

In nontransformed epithelial cells and cancerous cells, the Slit-2/Robo signaling system limits outward migration in response to motogenic attractants and remains positionally confined within their primitive location [23]. To study the role of Slit-2 in OVA-challenged in asthma mice,

first, the mRNA level of Slit-2 in lung tissue of asthma mice was tested by RT-PCR analysis (Fig. 5A). Data showed that mRNA down-regulated by OVA (from 1.00 ± 0.00 to 0.35 ± 0.11 , ** $P < 0.01$) was increased by TQ (from 0.35 ± 0.11 to 0.59 ± 0.18 , ** $P < 0.01$). The expression level of Slit-2 was also examined by Western blot analysis (Fig. 5B). Similarly, the expression of Slit-2 reduced by challenge of OVA (from 1.00 ± 0.00 to 0.45 ± 0.09 , ** $P < 0.01$) was up-regulated by treatment with TQ (from 0.45 ± 0.09 to 0.72 ± 0.16 , ** $P < 0.01$). Thus, TQ could limit the reduction of Slit-2 challenged by OVA in epithelial cells of asthma mice.

Slit2 is reported to express in cells surrounding the vasculature [24, 25], suggesting the presence of a stromal source for SLITs, at least in some tissues. To test the expression of Slit2 and its transmembrane receptor Robo-4 in vascular endothelial cells of asthma mice, immunofluorescent double staining analysis was performed in OVA-challenged vascular endothelial cells with antibodies directed against Slit2, CD31 and Robo-4. Data showed that comparing to Con group, the fluorescence of Slit2 (red fluorescence) was weakened whereas the fluorescence of CD31 (green fluorescence) was enhanced by challenge of OVA. TQ could increase the fluorescent expression of Slit2 and decrease the fluorescence of CD31 (Fig. 5C). After double staining with Robo-4 (red fluorescence) and CD31 (green fluorescence), we observed that unlike the change of CD31, the fluorescent expression of Robo-4 was not affected by either challenge of OVA or the treatment of TQ (Fig. 5D). Finally, in double staining with Slit2 (red fluorescence) and Robo-4 (green fluorescence) group, the fluorescence of Robo-4 was still not changed by OVA or TQ, but in the OVA + TQ group, the co-fluorescent expression of Slit2 and Robo-4 was observed in Fig. 5E. Thus, we can conclude that the expression of Slit2 was decreased by challenge of OVA whereas TQ could increase it and lead to the co-fluorescent expression of Slit2 and its transmembrane receptor Robo-4 in asthma mice.

3.6. TQ inhibits angiogenesis induced by VEGF in vitro

Since we have known that TQ inhibited angiogenesis in asthma mice, to further investigate the anti-neoangiogenesis effect of TQ, human umbilical vein endothelial cells (HUVEC) were used in this study. Tube formation assay was performed to investigate the effect of TQ on the capillary-like structure formation of HUVECs. HUVECs were seeded onto Matrigel and stimulated to form capillary networks in the presence of VEGF (10 ng/ml). Data showed that robust and complete tube network formation was observed in VEGF-stimulated HUVECs comparing with that in control. However, treatment with TQ resulted in significant inhibition of tube formation of VEGF-stimulated HUVECs on Matrigel. (Fig. 6A). In addition, incomplete sprouting or broken network between tubes of HUVEC was observed in VEGF + TQ group.

To study whether TQ inhibited neoangiogenesis through VEGFR/PI3K/Akt signal pathway in VEGF-stimulated HUVECs, the expression levels of VEGFR, VEGFR2, p-VEGFR2, PI3KP85, p-PI3K P85, Akt and p-Akt in VEGF-stimulated HUVECs with or without TQ treatment were also detected by Western blot analysis. Identical to the trend in asthmatic mice, data showed that TQ suppressed the expression level of VEGF in HUVECs (from 2.18 ± 0.32 in VEGF group to 1.47 ± 0.16 in VEGF + TQ group, ** $P < 0.01$). Also, Stimulation with VEGF increased the phosphorylation of VEGFR2 (from 1.00 ± 0.00 in Con group to 2.34 ± 0.24 in VEGF group, ** $P < 0.01$), which was significantly decreased by TQ treatment (from 2.34 ± 0.24 in VEGF group to 1.55 ± 0.20 in VEGF + TQ group, ** $P < 0.01$) (Fig. 6B). As shown in Fig. 6B, comparing to the control group, the expression levels of VEGFR2, PI3KP85 and Akt were not changed, whereas levels of p-VEGFR2, p-PI3K P85 and p-Akt were up-regulated in HUVECs stimulated by VEGF. Conversely, levels of p-PI3K P85 and p-Akt protein were significantly reduced by TQ treatment. The quantitative values of gray intensity analysis of p-PI3K P85 and p-Akt between VEGF group and VEGF + TQ group were 1.80 ± 0.36 versus 1.20 ± 0.18 and 2.49 ± 0.37 versus 1.25 ± 0.12 respectively, ** $P < 0.01$ (Fig. 6B). Thus, treatment with TQ decreased the phosphorylation of

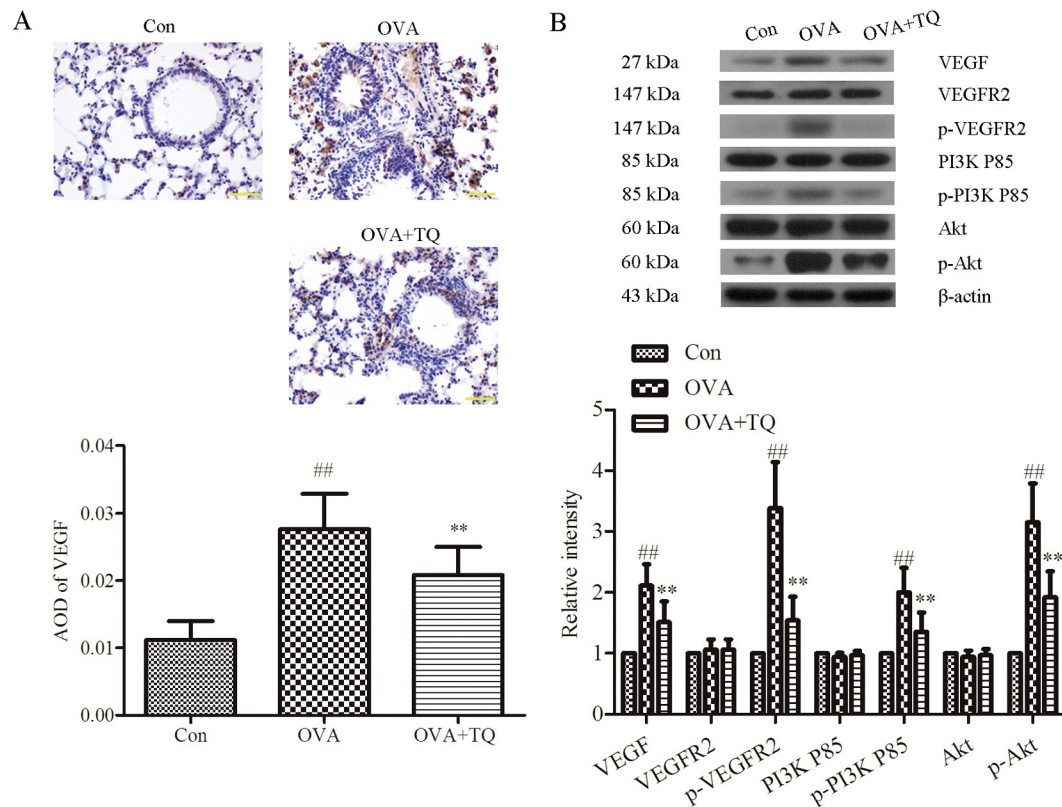


Fig. 4. TQ inhibited the PI3K-Akt-VEGF pathway through down-regulation of VEGF, p-VEGFR2, p-PI3K and p-AKT. (A) TQ inhibited the expression increase of VEGF in asthma mice blood vessels. Lung tissue sections of each group incubated with anti-VEGF antibody were tested by immunohistochemistry analysis (400× magnification). The quantitative value of AOD analysis of VEGF was calculated and shown in (A, below). (B) TQ down-regulated the expression of VEGF, p-VEGFR2, p-PI3K and p-AKT in lung tissue by Western blot analysis, and the quantitative analysis of gray intensity was calculated and showed in (B, below). ^{**}*P* < 0.01 versus OVA group; ^{##}*P* < 0.01 versus Con group. VEGF, vascular endothelial growth factor.

VEGFR2, PI3K and Akt in VEGF-stimulated HUVECs. Therefore we conclude that TQ inhibited the neoangiogenesis through VEGFR2-PI3K-Akt pathway in VEGF-stimulated HUVECs.

To study the role of Slit-2 in VEGF-stimulated HUVECs, the mRNA and protein levels of Slit-2 were tested by RT-PCR and Western blot analysis, respectively (Fig. 6C&D). Data showed that mRNA reduced by VEGF (from 1.00 ± 0.00 to 0.41 ± 0.07 , ^{##}*P* < 0.01) was increased by TQ treatment (from 0.41 ± 0.07 to 1.25 ± 0.12 , ^{**}*P* < 0.01) using RT-PCR analysis. Similarly, the expression of Slit-2 reduced by stimulation of VEGF (from 1.00 ± 0.00 to 0.25 ± 0.03 , ^{##}*P* < 0.01) was up-regulated by treatment with TQ in HUVECs (from 0.25 ± 0.03 to 0.59 ± 0.07 , ^{**}*P* < 0.01) with Western blot analysis. Thus, TQ could limit the reduction of Slit-2 in VEGF-stimulated HUVECs. These data suggest that TQ inhibits neoangiogenesis through VEGFR2-PI3K-Akt pathway by up-regulation of Slit-2 in VEGF-stimulated HUVECs.

4. Discussion

It is likely that inflammation contributes to vascular remodeling and generation of ILs and eosinophils in asthma. The exact role of inflammatory factors or inflammatory cells in asthma remains needs to be elucidated. It is now well established that the airway smooth muscle cells interact with infiltrating cells such as eosinophils, macrophages, mast cells, neutrophils as well as with resident cells such as epithelial cells, endothelial cells and fibroblasts, secreting chemokines CCL5 and CCL17, IL-8, IL-4 and IL-5 [26,27]. In addition to secreting chemokines that recruit leukocytes into the airways, airway smooth muscle cells promote leukocyte retention and activation through the expression of cell adhesion molecules [28]. Thus, the intertwined relationship between the infiltrating cells and airway smooth muscle cells helps to perpetuate the inflammatory processes. Increasing evidence suggests that Slit2 signaling through Robo-4 is required for neoangiogenesis and

vascular remodeling of airway wall in the pathogenesis of severe asthma [17,29]. Therefore, decrease of inflammatory factors and action of Slit2/Robo-4 signaling pathway are considered to be important for controlling asthma especially in patients with asthma symptoms [5]. In this study, we studied the inhibition effect of TQ in inflammation, neoangiogenesis and airway wall remodeling of OVA-challenged asthma mice. It is reported that TQ attenuates airway inflammation in a mouse model of allergic asthma [13]. Our results revealed that TQ inhibited the production of inflammatory factors IL-4/–5 (Fig. 1), suppressed the increase of neovascularization as well as vascular remodeling (Fig. 2) through down-regulating the expression of CD31 and α -SMA in asthma mice challenged by OVA (Fig. 3). Moreover, our study also showed that VEGFR2-PI3K-Akt pathway was down-regulated by TQ both in vivo and in vitro (Figs. 4&6B) while Slit-2 and Robo-4 were co-expressing after TQ treatment in OVA-challenged asthma mice (Fig. 5) with the inhibition of tube formation by TQ in HUVEC cells (Fig. 6A). Although studies have previously discussed the impact of TQ on OVA-induced asthma [30–33], including suppressing the production of inflammatory factors such as IL-4, IL-5 and IL-10 [34,35] and attenuating the lung inflammation [36] in OVA-induced asthma, our study was the first to illuminate the potential mechanism between inflammation and neoangiogenesis as well as vascular remodeling in asthma. Our findings demonstrated that TQ inhibited OVA-challenged inflammation, neoangiogenesis and vascular remodeling in asthma mice by blocking the activation of VEGFR2-PI3K-Akt pathway and signaling at least, partial through co-expressing of Slit-2 and Robo-4.

Inflammation is believed to play an important role in the pathophysiology of asthma through the release of a variety of inflammatory mediators, including major basic protein (MBP), cysteinyl leukotrienes (CysLTs), radical oxygen species, and cytokines [37,38]. This cytokines such as IL-4/–5 could be involved in the inflammation in asthma [39]. It was reported that the striking decrease in blood and sputum

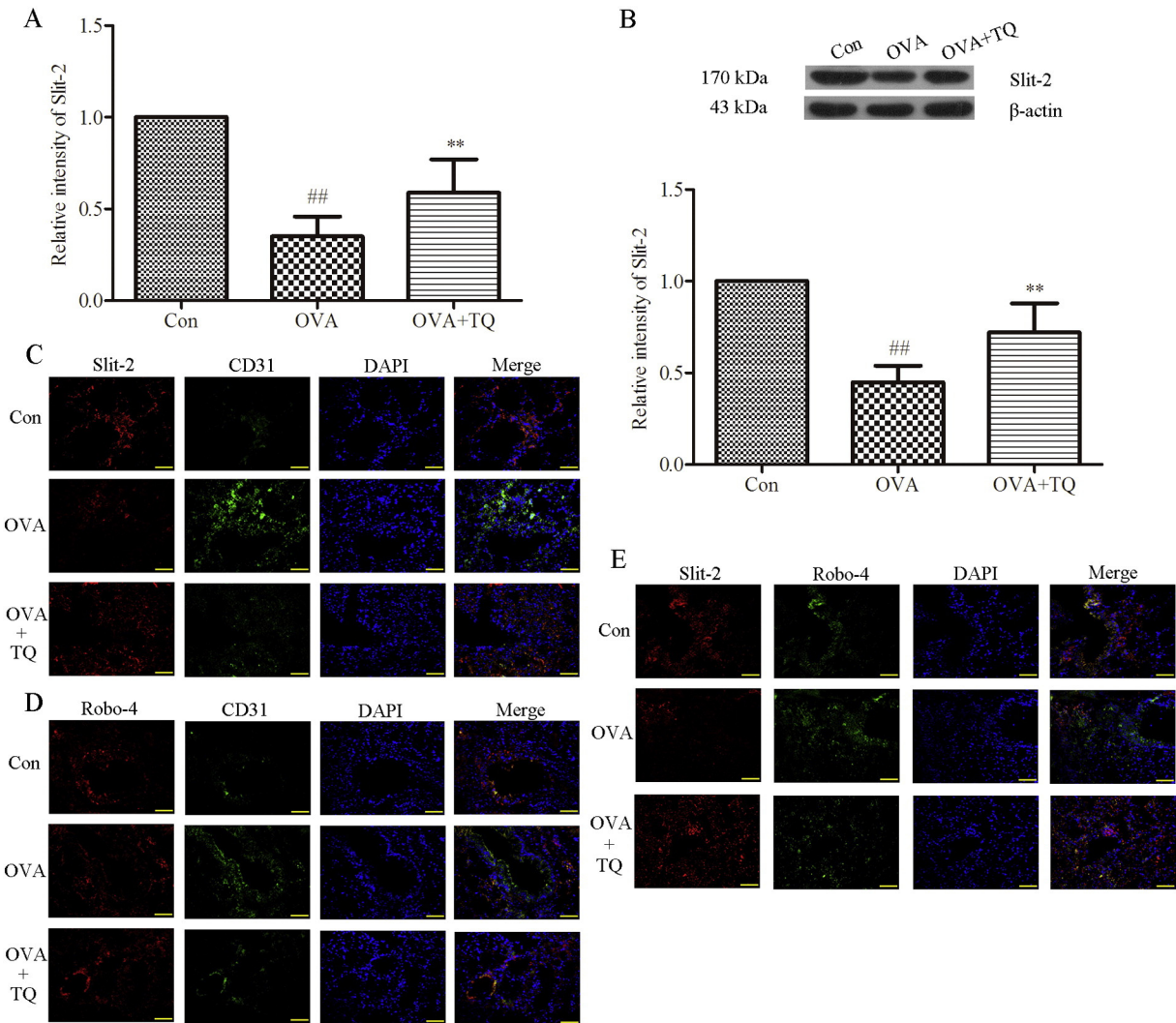


Fig. 5. TQ recovered the expression of Slit-2 down-regulated by OVA and mediated the co-expression of Slit-2 and Robo-4. (A) TQ recovered the mRNA expression of Slit-2 in mice lung tissue. The relative expression level of Slit-2 mRNA was tested by RT-PCR. (B) The expression level of Slit-2 protein. The expression level of Slit-2 protein in mice lung tissue was compared by Western blot analysis, and the quantitative analysis of gray intensity was calculated and showed in (B, below). (C) The expression level of Slit-2 and CD31 in airway endothelial cells. Representative images of sections immunostained for Slit-2 (red) and CD31 (green) were observed by immunofluorescence analysis. (D) The expression level of Robo-4 and CD31 in airway endothelial cells. Representative images of sections immunostained for Robo-4 (red) and CD31 (green) were observed by immunofluorescence analysis. (E) The expression level of Slit-2 and Robo-4 in airway endothelial cells. Representative images of sections immunostained for Slit-2 (red) and Robo-4 (green) were observed by immunofluorescence analysis. Scale bar = 100 μ m. ^{##} $P < 0.01$ versus Con group, ^{**} $P < 0.01$ versus OVA group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

eosinophi numbers after monoclonal antibody to IL-5 has potential for the treatment of asthma. Monoclonal antibody (mAb) versus IL-5 might be especially effective for the treatment of eosinophilic childhood asthma, and when used early, it could inhibit the structural changes of airways remodeling [40]. We found that TQ inhibited the production of IL-4 as well as IL-5 in both BALF and lung tissue of OVA-challenged asthma mice (Fig. 1B&C). Moreover, accumulating evidence established that eosinophils largely contribute to the development of vascular remodeling of asthma [41–43]. For example, Flood-Page et al. reported that anti-IL-5 mAb significantly reduced eosinophils expressing mRNA for transforming growth factor (TGF)- β and the concentration of TGF- β protein in BALF [43]. Humbles et al. reported that mice with selective ablation of the eosinophil lineage do not develop collagen deposition or increases in airway smooth muscle cells in response to allergen, indicating a critical role of eosinophils in the development of airway remodeling [42]. Similar findings were also reported by Lee et al. [41]. In our study, we demonstrated that after the decrease of IL-5, eosinophilia and goblet cell hyperplasia of lung tissue challenged by OVA was effectively blocked by TQ using histological examination (Fig. 1D&E). Data

showed that TQ could control the refractory eosinophilia and goblet cell hyperplasia by antagonizing IL-4/–5 in OVA-challenged asthma mice.

In the inhibition of TQ in neoangiogenesis challenged by OVA, it is reported that TQ could attenuated pyrogallol-induced vascular endothelial differentiation in a dose-dependent manner [44]. Angiogenesis and vascular remodeling are the critical process in chronic airway diseases including asthma, and as the maker of the expression of endothelial differentiation, CD31 is widely used in quantifying neoangiogenesis. We tested the expression of CD31 and α -SMA in OVA-challenged endothelial cells in asthma mice by immunohistochemistry analysis and found that TQ attenuated the expression of CD31 as well as α -SMA, which reflects the wall thickness, indicating that TQ attenuated the neoangiogenesis and vascular remodeling of asthmatic mice through down-regulation of CD31 and α -SMA (Fig. 3A&B).

In vascular remodeling, the VEGF family of proteins plays a critical role. VEGF promotes a variety of responses in the airway wall, including hyperpermeability, endothelial cell proliferation and angiogenesis with new vessel formation in vivo [45–47]. VEGF receptors VEGFR-1 and -2

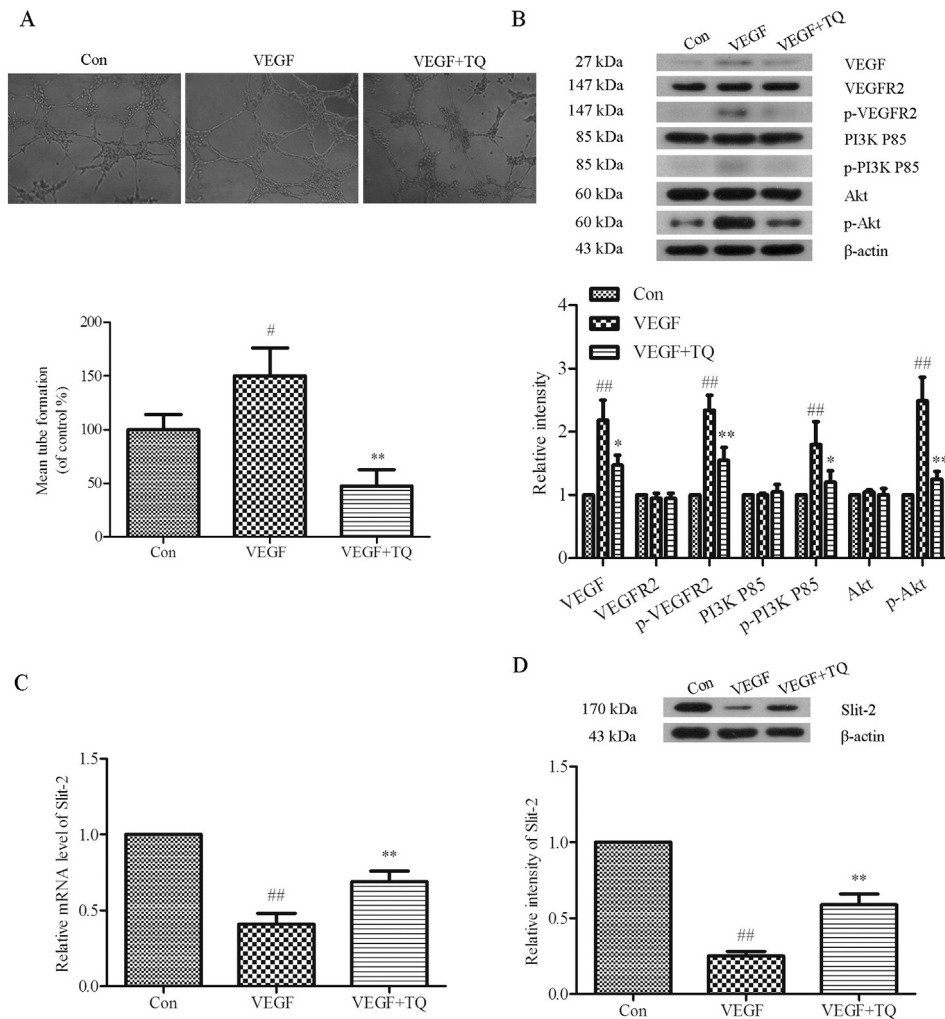


Fig. 6. TQ inhibits angiogenesis induced by VEGF in vitro. (A) TQ inhibits tube formation in VEGF-stimulated HUVECs. 100 nmol/l TQ was treated with or without VEGF (10 ng/ml) for 24 h in HUVECs, and the angiogenesis was tested by tube formation assay. (B) TQ down-regulated the expression of VEGF, p-VEGFR2, p-PI3K and p-AKT in VEGF-stimulated HUVECs by Western blot analysis, and the quantitative analysis of gray intensity was calculated. (C&D) TQ increased the level of Slit-2. The mRNA and protein levels of Slit-2 after treatment with TQ were tested by RT-PCR (C) and Western blot analysis (D), respectively, in VEGF-stimulated HUVECs. Scale bar = 400 μ m. [#]P < 0.05 and ^{##}P < 0.01 versus Con group; ^{*}P < 0.01 versus VEGF group.

are present on endothelial, epithelial and smooth muscle cells as well as inflammatory cells, including macrophages and T cells [48], in which, VEGFR-2 is the primary receptor interacting with its ligand VEGF. VEGFR-2 is reported to regulate vascular endothelial proliferation, differentiation, migration, capillary like formation and vascular permeability. Therefore, inhibition of the VEGFR-2-mediated signaling pathway represents an excellent approach in antiangiogenic intervention. We have found that in OVA-challenged lung tissue of asthma mice, the expression of VEGF and its receptor VEGFR-2 was up-regulated whereas TQ significantly decreased the high expression levels of VEGF as well as VEGFR-2 (Fig. 4A&B) with the same trend in VEGF-stimulated HUVECs (Fig. 6B). It has been documented that the expression of VEGF is regulated via the PI3K/Akt signaling pathway. The PI3K/Akt signaling pathway has been reported involving in the neoangiogenesis and air wall remodeling [49] and its activation stimulates VEGF expression [50]. Being an important component of the PI3K/Akt signaling pathway, Akt mediates a variety of cellular responses in regulating angiogenic growth factors by activating VEGF expression [51]. Studies have demonstrated a link between PI3K/Akt pathway activation and increased VEGF expression and several upstream factors have been shown to regulate VEGF expression [51]. Also, Yu et al. reported that TQ-induced reactive oxygen species causes apoptosis of chondrocytes via PI3K/Akt and p38 kinase pathway [52]. Besides, Shashi et al. reported that TQ promotes G1 arrest through targeting of Akt translation inhibition of cyclin D1

and induces apoptosis in breast cancer cells [53]. TQ can be employed as an endogenous Akt suppressor that inhibits breast cancer cell survival, and preventing Akt induced therapeutic resistance as an adjuvant for combination therapies in combating breast cancer. In addition, the inhibition of Akt phosphorylation was associated with PTEN upregulation and ROS generation [54,55], as well as the down-regulation of VEGF [56]. The present study demonstrated that the expression of p-VEGFR2, p-PI3K and p-Akt were up-regulated, which showed AKT was activated by OVA challenge in asthma mice or by VEGF in HUVECs (Fig. 6B), whereas TQ significantly suppressed the activation of these proteins (Fig. 4B&C), suggesting that TQ inhibits neoangiogenesis and vascular remodeling by regulation of VEGF expression possibly directly or indirectly through the VEGFR2/PI3K/Akt signaling pathway *in vivo* and *in vitro*. The precise mechanism of it requires further investigations.

Accumulating evidence indicates that Slit-2 signaling through Robo1 and Robo2 is required for neoangiogenesis [29]. Slit-2 treatment of endothelial cells and epithelial origin inhibited their migration and proliferation *in vitro* [57]. Our study revealed that OVA-challenged down-regulation of Slit-2 in asthma mice was inhibited by the treatment of TQ both in protein level and the mRNA level (Fig. 5A&B). Also, TQ increased both protein and mRNA level in VEGF-stimulated HUVECs (Fig. 5C&D). It has been reported that SLIT signals act through a Robo4-mediated pathway to counter VEGF/VEGFR signaling and restrain angiogenesis [15]. At the molecular level, Slit-2 activation, as

the downstream signal of PI3K/AKT signal pathway, was abolished by the inactivation of Robo, indicating that decreased activity of these signaling proteins may contribute to the angiogenesis and migration [29]. The mechanistic details of these effects remain to be elucidated, but the data suggest that signaling between Slit-2 and Robo-4 is a critical environmental signal for endothelial cell neoangiogenesis and migration. Also Cai et al. reported that Robo4 suppressed glioma-induced endothelial cell proliferation, migration and tube formation *in vitro* by inhibiting VEGFR2-mediated activation of PI3K/AKT signaling pathway [58]. In our study, we found the fluorescence of CD31 was significantly enhanced whereas the fluorescence of Slit-2 was weakened by challenge of OVA in the endothelial cell of asthma mice (Fig. 5C). TQ could reverse these changes induced by OVA. Meanwhile, the expression of Robo-4 was not affected by OVA challenge (Fig. 5D). Moreover, we have found the fluorescence of Slit-2 was increased by TQ and the co-expression of Slit-2 and Robo-4 was observed by immunofluorescent double staining analysis (Fig. 5E). The mode of the interaction between Slit-2 and Robo-4 remains to be clarified. Thus, we can conclude that TQ inhibited the neoangiogenesis and vascular remodeling by up-regulation of Slit-2 through VEGFR2/PI3K/AKT signaling pathway in asthma mice.

Our study demonstrates the anti-inflammation and anti-neoangiogenesis effect of TQ in OVA-challenged asthma mice. TQ attenuated the inflammatory reaction by antagonizing IL-4/–5 while the anti-neoangiogenesis effect of TQ is mediated, at least in part, by inhibition of VEGF expression through VEGFR2/PI3K/Akt signaling pathway, which was accompanied by the up-regulation of Slit-2 and probably through the heterodimeric binding with Slit-2 and Robo-4. Our study supports a potential role for TQ in ameliorating airway inflammation, neoangiogenesis and vascular remodeling in asthma mice.

Author contribution

Xinming Su and Yuan Ren conceived and designed the experiments; Yuan Ren, Na Yu and Lingfei Kong performed the experiments; Lingfei Kong and JianKang analyzed the data; Jian Kang contributed reagents/materials/analysis tools; Xinming Su wrote the paper. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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