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Thymoquinone inhibits inflammation, neoangiogenesis and vascular remodeling in asthma mice

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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 immunesorbent 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 actinalpha (α-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 information 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.

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 fiborsis [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 cyclooxyge-nase-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-κB) signal pathways in human osteosarcoma both in vitro and in vivo [15]. VEGF, is a key factor

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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 TQi.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 OVAtreatment). DEX (41021255, Tianjin Pharmaceutical Group Xinzheng CO., Tianjin, China) dissolved in phosphate buffer (PBS) was used as positive control.

2.4. Brochoalveoler 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 for10 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 m 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 perimeter^2 (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% CO_2.

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% CO_2 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 radioimmunoprecipitation (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);