Tanshinone IIA attenuates ovalbumin-induced airway inflammation and hyperresponsiveness in a murine model of asthma

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Abstract

Objectives: Tanshinone IIA (T. IIA), one of the most pharmacologically active components extracted from Salviae miltiorrhiza, has anti-inflammatory and antioxidant features. The aim of the present study is to investigate the benefit of T. IIA on asthma using a murine model of asthma induced by ovalbumin (OVA).

Materials and Methods: Male BALB/c mice were used in the present study. The mice were sensitized by OVA intraperitoneal injection on days 0 and 14, and received aerosolized OVA challenge for 30 min daily on days 21-23. T. IIA (10 mg/kg twice daily) intraperitoneal injection was performed on days 18-23.

Results: Treatment of T. IIA reduced the levels of interleukin (IL)-4, IL-5, and IL-13 in bronchoalveolar lavage fluid (BALF) (<0.05 for all cases). The OVA-induced elevation of total white blood cells as well as differential white blood cells in BALF and blood were inhibited by T. IIA (<0.05 for all cases). The OVA-induced elevation of total white blood cells as well as differential white blood cells in BALF and blood were inhibited by T. IIA (<0.05 for all cases). Moreover, airway hyperresponsiveness was dampened in T. IIA-treated group (<0.05). T. IIA inhibited the activation of nuclear factor-κB in asthmatic mice (<0.05). The activity of nuclear factor erythroid-2-related factor 2 was enhanced in T. IIA-treated group (<0.05). T. IIA elevated the activities of heme oxygenase-1, glutathione peroxidase, and superoxide dismutase (<0.05 for all cases).

Conclusion: T. IIA inhibits OVA-induced airway inflammation and hyperresponsiveness. T. IIA is a potential therapeutic agent for asthma.

Introduction

Airway hyper-responsiveness and inflammation are the most common features of asthma (1). Cytokines from T helper 2 (Th2) cells is believed to play a vital effect in organizing the chronic inflammation of asthma (1). Inhibition of inflammation is an important strategy for pulmonary inflammatory disorders (2-4).

Nuclear factor erythroid-2-related factor 2 (Nrf2), a transcription factor, is known as a vital antioxidant defense mechanism. Scientific evidence has shown that Nrf2 is critical in protecting the lung against oxidative stress in asthma (5). Nuclear factor-κB (NF-κB), another important transcription factor, is believed to play a vital effect in organizing the expression of cytokines in pulmonary diseases (4). Inhibition of NF-κB has shown beneficial effect on asthma (6). Inhibition of NF-κB and activation of Nrf2 are associated with dampened airway inflammation and hyperresponsiveness in asthma (4, 5). Thus, induction of Nrf2 and inhibition of NF-κB are potential strategy for reduction of asthma.

Despite significant advances in the management of asthma, novel treatments for asthma are still required as the current strategies have their limitations (7-9). Recently, there are a growing interest on herbal medicines and natural products (10, 11). Tanshinone IIA (T.IIA) is a pharmacologically active component of Salviae miltiorrhiza, which is a traditional Chinese medicine and has antioxidant and anti-inflammation features (12-15). Oxidative stress-induced myocardial apoptosis was inhibited by T. IIA (16). T. IIA dampened lipopolysaccharide-induced pulmonary inflammation and edema in an animal model of acute lung injury (17). T. IIA is believed to play a beneficial role on chronic obstructive pulmonary disease (18). However, the effect of T. IIA on asthma remains unclear. The current study investigates the effect of T. IIA on ovalbumin (OVA)-induced airway inflammation and hyperresponsiveness using a murine model of asthma.

Materials and Methods

Animals

All experiments were conducted in accordance with the Helsinki convention for the use and care of animals. All experiment protocols were reviewed and approved by the Research Ethics Committee of Fujian Medical University.

Six-week old male BALB/c mice (obtained from the Experimental Animal Center of Fujian Medical University) were bred in a specific pathogen-free and temperature controlled (22±2 °C) animal facility. The mice were maintained on a 12 hr light/ 12 hr dark schedule and received standard laboratory rodent chow.

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and tap drinking water ad libitum.

**Sensitization and provocation protocols**

The sensitization and provocation protocols used in the present study were discussed previously (19). Mice were immunized using OVA (sigma-Aldrich, St Louis, MO, USA) intraperitoneal injection on days 0 and 14. OVA (50 μl) and an adjuvant, Al(OH)₃ (50 μl, Pierce, Rockford, IL, USA), were dissolved in normal saline (NS; 200 μl) before use. From the day 21 to 23, animals were exposed to aerosolized OVA (1% OVA) for 30 min once daily. Mice in control group received the equivalent Al(OH)₃, diluted in NS intraperitoneal injection, and exposed to a nebulized aerosol of NS at the same time points as the OVA challenged animals.

**T. IIA administration**

T. IIA was obtained from Shanghai No. 1 Biochemical Pharmaceutical Co. Ltd. (Shanghai, China). The animals were randomly divided into various groups: control (sham+ NS), T. IIA control (sham+T. IIA), asthma+NS, and asthma+ T. IIA group. Briefly, the mice were treated with intraperitoneal injection of 10 mg/kg T. IIA twice daily on days 18-23. The dose of T. IIA selected in the present study was based on previously published articles (13, 16) and our preliminary study (data not shown). Equivalent NS was administrated for control group.

**Bronchoalveolar lavage fluid (BALF) analysis**

The mice were anesthetized (50 mg/kg thiopental intraperitoneal injection), and the trachea was cannulated with a 0.6 mm catheter and secured with a silk suture. Sterile NS (1 ml) was instilled through the catheter using a 1 ml syringe for 3 times. More than 90% of BALF was withdrawn. Then, BALF centrifugation was performed (1,200 rpm for 5 min at 4 °C) using a cytocentrifugation (cytospin 3, Shandon Instruments, Pittsburgh, PA). The sediment cells were washed and stained with Giemsa stain. Total and different subtypes of white blood cells were counted with a hemocytometer. The supernatant was analyzed for cytokines.

**Proinflammatory cytokines measurement**

Commercial enzyme-linked immunosorbent assay (ELISA) kits were used to measure the proinflammatory cytokine levels, including interleukin (IL)-4, IL-5, as well as IL-13, in BALF following the manufacturer’s protocol (eBioscience Co, San Diego, USA.).

**Airway responsiveness measurement**

The mice were anesthetized (50 mg/kg thiopental intraperitoneal injection) and mechanically ventilated with a rodent ventilator at 24 hr after the last aerosolized OVA or vehicle challenge. The mice were challenged with aerosolized methacholine (12.5, 25, 50 mg/ml; Sigma-Aldrich) or NS after being stabilized (19). Then, tissue resistance, tissue elastance, respiratory system elastance, respiratory system resistance, and airway resistance were measured (Buxco Research System).

**NF-κB and Nrf2 activity analysis**

A nuclear extract kit (Active Motif North America) was used to prepare nuclear extracts from lung tissues. NF-κB p65 binding activity was detected by using an ELISA assay kit (Active Motif, Carlsbad, CA, USA) following the manufacturer's protocol (20). Briefly, the nuclear extracts were incubated with the p65 subunit of NF-κB consensus site oligonucleotides (5’-GGGACCTTTCC-3’) immobilized to 96-well plates. The DNA binding activity of NF-κB p65 was detected with an antibody specific to the activated form of NF-κB p65 and visualized by colorimetric reaction catalyzed by horseradish peroxidase-conjugated secondary antibody, and absorbance was measured at 450 nm with a reference wavelength of 655 nm.

A TransAM Nrf2 assay kit (Active Motif, Carlsbad, CA, USA) was used to measure the Nrf2 binding activity as described previously (21). Briefly, the nuclear extracts were incubated in 96-well plates coated with immobilized oligonucleotide containing a consensus (5’-GTCACTGACTCGACAGATCTG-3’) binding site for antioxidant response element. The Nrf2 binding activity to the target oligonucleotide was detected by incubation with primary antibody specific for DNA-bound Nrf2, visualized by horseradish peroxidase conjugate and developing solution, and quantified at 405 nm.

**Heme oxygenase (HO)-1 activity assay**

The HO-1 activity in tissue homogenate was determined by measuring the generated bilirubin as previously described (22). Briefly, samples of lung tissue were added to a mixture containing glucose 6-phosphate, glucose 6-phosphate dehydrogenase, protomeron, and nicotinamide adenine dinucleotide phosphate. The reaction was continued for 1 hr at 37°C. The bilirubin level was determined by a detection reader at excitation and emission wavelengths of 464 and 530 nm, respectively.

**Antioxidant enzymes activities assay**

A glutathione peroxidase (GPx) activity detection kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) was used to measure the GPx activity in lung homogenate. The GPx activity was determined by measuring the level of oxidized glutathione (GSSG), which was converted from glutathione (GSH) by GPx. The sample of lung tissue was incubated with H₂O₂. The absorbance of GSSG was measured at 412 nm.

A superoxide dismutase (SOD) activity detection kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) was used to measure the SOD activity in lung homogenate. The level of formazan salt was used as an indicator of SOD activity. Briefly, sample of lung tissue was added to a mixture including tetrazolium salt and xantine oxidase enzyme at 37°C. The reaction was continued for 20 min. The absorbance of formazan salt was detected at 550 nm.

**Maleic dialdehyde (MDA) production measurement**

The MDA production was used to indicate reactive oxygen species (ROS) levels in pulmonary tissues. The sample of lung tissue was added to a mixture containing acetic acid, sodium dodecyl sulfate, aqueous solution of thio-barbituric acid, n-butanol, and pyridine. The mixture was shaken and centrifuged (4,000 rpm for 10 min). The MDA production was determined at 532 nm by a detection reader.
**White blood cell analysis**

Blood sample was collected by cardiac puncture under anesthesia (50 mg/kg ketamine intraperitoneal injection) with a heparinized syringe (5 ml) and analyzed as described previously (23).

**Statistical analysis**

All data were presented as mean±SEM. Differences in values were measured by one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls method, and were considered statistically significant if P value less than 0.05. SPSS 19.0 software (IBM, Armonk, USA) was used for all statistical analysis.

**Results**

**T. IIA reduces airway inflammation**

The total and differential white blood cells counts were shown in Figure 1A and Table 1. As indicated in Figure 1A, compared with control animals, the number of total inflammatory cells in BALF was increased in asthmatic mice ($P<0.05$). Meanwhile, the number of eosinophils in BALF was elevated in asthmatic mice ($P<0.05$). In blood, the number of total inflammatory cells was increased by 1.74-fold in vehicle-treated asthmatic mice compared to control ($P<0.05$), (Table 1). T. IIA inhibited the total inflammatory cells and eosinophils in blood and infiltration in BALF ($P<0.05$ for all cases), (Table 1; Figure 1A). The IL-4 level in BALF was increased by 6-fold in asthmatic mice ($P<0.05$), (Figure 1B). The OVA-induced elevating of IL-4 was inhibited by T. IIA treatment ($P<0.05$), (Figure 1B). Moreover, T. IIA treatment decreased IL-5 and IL-13 levels by 38% and 45%, respectively ($P<0.05$ for both cases), (Figure 1C and D).

**Effect of T. IIA on airway hyperresponsiveness**

We performed a methacholine dose–response curve to evaluate if T. IIA protects lung against airway hyperresponsiveness. As shown in Figure 2, tissue resistance, tissue elastance, respiratory system elastance, respiratory system resistance, and airway resistance were increased in OVA-sensitized mice challenged with methacholine ($P<0.05$ for all cases). Our results showed that T. IIA markedly inhibited airway hyperresponsiveness compared to vehicle-treated asthmatic mice ($P<0.05$ for all cases), (Figure 2A, B, C, D, and E).

**T. IIA inhibits NF-κB activation and elevates Nrf2 activity**

The activity of NF-κB was increased in OVA-treated mice ($P<0.05$), (Figure 3A). T. IIA treatment decreased the activity of NF-κB by 52% compared to the vehicle-treated asthmatic mice ($P<0.05$), (Figure 3A). Moreover, T. IIA enhanced Nrf2 activity in asthmatic mice compared to vehicle-treated animals ($P<0.05$), (Figure 3B).

**Effect of T. IIA on antioxidant enzymes activities and ROS production**

The activities of GPx, SOD, and HO-1 were enhanced in T. IIA-treated asthmatic animals ($P<0.05$ for all cases), (Figure 3C, D, and E). Moreover, T. IIA treatment inhibited the OVA-induced ROS generation compared to vehicle-treated asthmatic animals ($P<0.05$), (Figure 3F).

**Table 1.** Effect of Tanshinone IIA on total blood and differential white blood cells counts ($\times$10^3 cells/ml)

|                | sham+NS | sham+T. IIA | asthma+NS | asthma+T. IIA |
|----------------|---------|-------------|-----------|--------------|
| Total cells    | 6.23±1.57 | 6.01±1.35   | 10.81±2.13* | 7.83±1.53*   |
| Lymphocytes    | 4.23±0.78 | 4.02±0.91   | 6.26±1.46* | 4.96±0.74*   |
| Neutrophils    | 1.55±0.17 | 1.56±0.22   | 3.45±0.66* | 2.23±0.35*   |
| Eosinophils    | 0.21±0.09 | 0.18±0.10   | 0.50±0.23* | 0.29±0.18*   |
| Monocytes      | 0.20±0.12 | 0.21±0.07   | 0.46±0.37* | 0.32±0.15*   |

Values are as mean±SEM. *P<0.05, compared to Sham+ normal saline (NS) group; #P<0.05, compared to asthma+NS group. T. IIA, Tanshinone IIA
IL-4, IL-5, and IL-13 belong to Th2 cytokines, which play a fundamental role in asthma (31-33). Evidences have shown that IL-4 exacerbates asthma via induction of Th17 cell (46, 47). Consistent with our study, evidences have shown that induction of HO-1 reduces airway inflammation induced by OVA (48).

MDA is a commonly used indicator of oxidative stress (49). Increased MDA levels were found in adult and children patients with asthma (50, 51). Our results showed that the MDA levels were elevated in asthmatic mice. Moreover, our results showed that the OVA-induced elevation of MDA was inhibited in T. IIA-treated asthmatic mice. This result suggests a benefit of T. IIA in regulating the equilibrium of oxidant-antioxidant in asthma.

Our findings, combined with previous data, suggest that T. IIA has antioxidant effect (16, 44). Nrf2 plays a vital role in reduction of oxidative stress (39). Our results showed that the MDA levels were elevated in asthmatic mice. Moreover, our results showed that the OVA-induced elevation of MDA was inhibited in T. IIA-treated asthmatic mice. This result suggests a benefit of T. IIA in regulating the equilibrium of oxidant-antioxidant in asthma.

The current results suggest that T. IIA inhibits OVA-induced airway inflammation and hyperresponsiveness. T. IIA is a potential therapeutic agent for asthma.

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No conflict of interest was involved in this research.

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