

Original article

Molecular mechanism of icariin on rat asthmatic model

XU Chang-qing, LE Jing-jing, DUAN Xiao-hong, DU Wei-jing, LIU Bao-jun, WU Jing-feng, CAO Yu-xue and DONG Jing-cheng

Keywords: *icariin; Th1/Th2; T-bet/GATA-3; nuclear factor Kappa B; p65*

Background Effects of icariin on airway inflammation in asthmatic rats and the intervention of LPS induced inflammation are interfered with the mechanism of icariin. Our study aimed to observe the effect of icariin on ovalbumin-induced imbalance of Th1/Th2 cytokine expression and its mechanism.

Methods Sixty male SD rats were randomly divided into control group (PBS), asthma group (ovalbumin (OVA)-induced), dexamethasone group, and OVA+icariin low, medium and high dose groups (5, 10, 20 mg/kg, respectively). Each group had ten rats. The model of OVA sensitization was a rat asthma model. Enzyme-linked immunosorbent assay (ELISA) method was used to observe the effects of icariin on interleukin-4 (IL-4) and interferon γ (IFN- γ) in rats' lung tissue. Immunohistochemical staining was applied to detect the intervention effects of icariin on T cells (T-bet) and gatabinding protein 3 (GATA-3) in rat pulmonary tissue. Realtime RT-PCR was used to observe the intervention effects of icariin on T-bet and GATA-3 mRNA expression in rat pulmonary tissue and spleen lymphocytes. Western blotting was used to observe the icariin intervention effects on T-bet, GATA-3 and nuclear factor-Kappa B (NF- κ B) p65 protein expressions in rat pulmonary tissue.

Results The ELISA results from pulmonary tissue showed that IL-4 expression was significantly reduced ($P < 0.05$), while the IFN- γ expression increased but not significantly when we compared OVA+icariin medium and high dose groups with the asthma group. Immunohistochemical staining of pulmonary tissue showed that the GATA-3 decreased significantly while the T-bet staining did not change in the OVA+icariin high dose group. In pulmonary tissue and spleen lymphocytes T-bet and GATA-3 mRNA expressions were significantly reduced ($P < 0.05$) in icariin treatment groups compared with the asthma model group. GATA-3 and T-bet mRNA in rat spleen lymphocytes in the asthma group were higher than in the control group. GATA-3 mRNA expression in pulmonary tissue significantly decreased ($P < 0.05$) while T-bet mRNA expression decreased but not significantly in the icariin treatment group compared with the asthma group. T-bet and GATA-3 protein expressions in pulmonary tissue increased significantly compared with the asthma group, which meant that icariin could inhibit the increase of GATA-3 protein, but not of T-bet. The bronchus, blood vessels and periphery pulmonary tissue had infiltration of inflammatory cells in the OVA+icariin high dose group while NF- κ B p65 cells were reduced, and expression of NF- κ B p65 in this group was less than in the asthma group. The expression of total p65 protein decreased with icariin treatment while the expression of cytoplasmic p65 protein increased.

Conclusions Icariin could regulate the imbalance of Th1/Th2 cytokines in asthmatic rat pulmonary tissue. Icariin could regulate the imbalance of Th1/Th2 associated transcription factors T-bet and GATA-3 in asthmatic rat pulmonary tissue and spleen lymphocytes. Icariin could inhibit the activation of NF- κ B p65 protein in asthmatic rat pulmonary tissue.

Chin Med J 2011;124(18): 2899-2906

Immune responses polarized toward either T-helper 1 (Th1) or T-helper 2 (Th2) cells result in the activation of different inflammatory effector pathways and, consequently, different disease outcomes.¹ Many autoimmune diseases and tumors have been confirmed to be associated with either Th1- or Th2-polarized immune responses. Recent reports have suggested that an imbalance between the Th1 and Th2 cell levels might be associated with the development and progression of several forms of asthma.²

Although the regulation of the Th1/Th2 imbalance remains unclear, there is a growing body of evidence suggesting that two transcription factors, T-box expressed in T cells (T-bet) and gatabinding protein 3 (GATA-3), are determinants of Th cell differentiation.³ T-bet is a newly discovered Th1-specific transcription factor thought to

initiate Th1 development, whereas GATA-3 plays a pivotal role in the development of the Th2 phenotype. It

DOI: 10.3760/cma.j.issn.0366-6999.2011.18.019

Integrative Department, Huashan Hospital Affiliated to Fudan University, Shanghai 200040, China (Xu CQ, Le JJ, Duan XH, Du WJ, Liu BJ, Wu JF, Cao YX and Dong JC)

Correspondence to: Dr. DONG Jing-cheng, Integrative Department, Huashan Hospital Affiliated to Fudan University, Shanghai 200040, China (Tel: 86-21-5288801. Fax: 86-21-5288825. Email: jcdong2004@hotmail.com)

XU Chang-qing and LE Jing-jing contributed equally to the present study.

This work was supported by the Development Project of National Intensive Foundational Research (973 Project, Nos. 2009CB523000, 2009CB523001), National Basic Science Program of China (No. 2009CB523000), Shanghai Science and Technology Committee (No. 09XD1400700), Shanghai Health Bureau Scientific Research Foundation of Traditional Chinese Medicine (No. 2008L018), China Ministry of Education (No. 200802461053).

has been reported that the T-bet/GATA-3 ratio is indicative of the balance between Th1 and Th2 cells.^{4,5} It has also been reported that patients with active lupus nephritis exhibit increased T-bet and depressed GATA-3 expression in both urinary sediments and kidney tissue.⁶ The transcription factor nuclear factor-Kappa B (NF- κ B) plays a central role in the regulation of many genes responsible for the generation of inflammation mediators. Increased activation of NF- κ B has been found in peripheral blood mononuclear cells (PBMCs), neutrophils, and alveolar macrophages in patients with acute lung injury after LPS exposure.⁷ Recently, it has been shown that the PI3K/Akt signaling pathway plays an important role in negatively regulating LPS-induced acute inflammatory responses *in vitro* and *in vivo*.⁸ Inhibition of the PI3K/Akt signaling pathway can enhance the activation of NF- κ B, AP-1, and Egr-1 transcription factors and the expression of tumor necrosis factor- α (TNF- α), interleukin 6 (IL-6) and tissue factor by LPS in cultured human monocytic cells.⁹

Icariin, a major active component isolated from plants in the Epimedium family, has been previously confirmed to improve cardiovascular function, induce tumor cell differentiation and increase bone formation.¹⁰ Recently, studies have shown that icariin is a concentration-dependent chemopreventor in protecting DNA against radical-induced damage and also exerts anti-osteoporotic effects.¹¹

We used immunohistochemistry of Th1/Th2 transcription factors (T-bet/GATA-3), lung and spleen lymphocytes Th1/Th2 transcription factor expression of gene and protein, and using immunohistochemistry and Western blotting to observe icariin on NF- κ B p65 activation. The results showed that lung asthma model used icarrin displayed the imbalanced expression of Th1/Th2 cytokine. It was also found in lung and spleen lymphocyte expression of Th1/Th2 associated transcription factor. Further study found that icariin inhibits NF- κ B p65 protein activation.

METHODS

Main experimental material and apparatus

Experimental animals

Sixty male SD rats weighting (300 $\pm$ 10) g were supplied by Bill Experimental Animal Center Sichuan.

Main reagents

Icariin was purchased at Ronghe Medical and Scientific Development Co. Limited, Shanghai and the purity was 99.06 %. Ovalbumin (OVA) and aluminum hydroxide were from Sigma Company, USA. Dexamethasone injection was bought at Xianju Medicinel Co. Limited, Zhejiang. Enzyme-linked immunosorbent assay (ELISA) kits for IL-4, IL-13, TNF- α , and interferon γ (IFN- γ) were purchased at Xitang Biological Pharmaceutical Company, Shanghai, China.

Main apparatus

Refrigerated centrifuge (Eppendorf Centrifuge 5804R, Germany), Microscope (Leica DFX 320, Germany), Enzyme labeling meter (Eppendorf, Germany), Ultrasonic atomizer (The Medical and Instrumental Co. Limited, Shanghai, China), Electrophoresis System (BIO-RAD Company USA), Analytical system of gelatum imaging (GDS8000, UVP Company, UK), Medical ultrasonic atomizer of YS9801 type (Yisheng Technology Co. Limited Shanghai), SMV alternator of HetoSBD-50 type, Cryogenic box (HetoultraFreeZe, Denmark), Uncontaminated benchboard (Armamentarium Factory, Boxun Industry Co. Limited, Shanghai, China).

Empirical method

Replication of asthmatic model

Sixty male SD rats were randomly divided into control group (PBS), asthma group (OVA-induced), dexamethasone group, and OVA+icariin low, medium and high dose groups (5, 10, and 20 mg/kg, respectively). Each group had ten rats.

We put OVA into aluminium hydroxide solution, making a 10% OVA solution. Each rat was injected with 1 ml of OVA solution to sensitize. Only 10% PBS was injected into the control group. Asthma was provoked after 15 days by putting the sensitized rats into a closed container of 20 cm $\times$ 30 cm $\times$ 50 cm, where they inhaled atomized 1% OVA solution prepared in normal saline. The flow rate was 2 L/min and the exposure time was 30 minutes. Control group was injected normal saline instead, being induced continually for 28 days for experimental usage.

Experimental grouping and administration

There were six groups, including control group (PBS), asthma group (OVA), dexamethasone treatment group (OVA+dexamethasone/DXM), OVA+icariin low dosage group (5 mg/kg), OVA+icariin of medium dosage group (10 mg/kg) and OVA+icariin of high dosage group (20 mg/kg). PBS, dexamethasone tablets and icariin were given through intragastric administration. Because icariin was dissolved in 0.005% DMSO, PBS and dexamethasone tablet should be dissolved in DMSO of the same concentration and volume so as to make the experiment equal. The rats were dosed by intragastric administration each day after antigen exposure from the 15th day to the 28th day. At the 43rd day of the experiment, the materials were picked after 2-hour provocation.

Harvest of pulmonary tissue from each group

Two hours after the last inhalation, rats from each group were given an intraperitoneal injection of 2% pentobarbital sodium (40 mg/kg) to anesthetize them. Then the pulmonary lobes of the rats were sterilely dissociated. We excised the right lungs, placing them in freezing tubes which stored at -80°C for future RNA extraction. The left lungs were fixed in 10% neutral formalin for histology and immunohistochemistry

probing for cytokines.

ELISA

According to the manufacture's instructions, 0.1 ml of coating buffer with a known antigen diluted to 1–10 µg/ml was added to each well, 4°C overnight. They were washed 3 times the next day. Some dilution of the sample to be detected (unknown antibody) was added to the wells that had been coated with 0.1 ml of the above reaction, incubated for 1 hour at 37°C, washed, And do blank, negative and positive hole control in response to the hole, adding fresh enzyme diluted second antibody (anti-antibody) 0.1 ml, 37°C for 30–60 minutes, washing, and finally washed again with deuterium depleted water. Plus substrate Yexian color: holes in each reaction by adding the temporary prepared TMB substrate solution 0.1 ml, 37°C for 10–30 minutes. Termination reaction: in all wells by adding 2 mol/L sulfuric acid 0.05 ml. Results found: in the ELISA detector, and the 450 nm (if ABTS color, the 410 nm).

Immunohistochemistry staining method

Deparaffinize and dry array slide as referred to in protocol of deparaffinization.⁸ Rinse array slide twice with PBS for 5 minutes each. The endogenous peroxidase activity was blocked at room temperature by a 5–10 minutes incubation in the final developmental 3% H₂O₂ in distilled water or PBS (pH 7.4). Rinse array slide in PBS for 5 minutes. Antigen retrieval. Rinse array slide in PBS for 5 minutes. Apply the blocking antibody (normal goat serum), incubate for 20 minutes at room temperature, and throw off residual fluid (not wash). Apply the primary antibody 60 minutes at room temperature or 4°C. Rinse array slide twice for 5 minutes each. Incubate array slide with a biotin-conjugated secondary antibody at 20°C–37°C for 20 minutes. Rinse array slide twice for 5 minutes each. Incubate array slide with SABC reagent at 37°C for 20 minutes. Rinse array slide 4 times for 5 minutes each. Proceed with chromogen of final developmental DAB or use DAB Kit (Control the degree of staining with regular microscopy). Wash array slide in distilled water. Stain and differentiate array slide in hematoxylin. Dehydration and transparency of array slide. Mount array slides.

PCR detection using real-time fluorescent quantitation

Take TNF-α as an example, after treatment, the RNeasy™ Plus mini kit was used to isolate the total RNA from the lung tissues, and the quality of RNA was subsequently evaluated by measuring the ratio of the absorbance at 260/280 nm. For reverse transcription, the SYBR Green 2-step qRT-PCR kit (DyNAmo™, Finnzymes, Finland) was used. For PCR amplification, the following mouse-specific sense and antisense primers were used: TNF-α, 5'-GCGACGTGGAAGTGGCAGAA-G-3' (forward) and 5'-TCCATGCCGTTGGCCAGGAG-G-3' (reverse). Real-time quantitative PCR was carried out in a 48-well plate using an Opticon MJ Research instrument (Bio-rad Inc., USA) and an optimized

standard SYBR Green 2-step qRT-PCR protocol.¹²

Western blotting evaluated the total NF-κB p65 in pulmonary tissue and sarcoplasm

Transfer the protein from polyacrylamide gel onto a nitrocellulose membrane. The membranes were quickly stained in amino black, then removed until the molecular weight standard was visible. We washed the membranes with 100 ml of water and used a destaining buffer if necessary at 37°C for one hour. Wash the membranes at room temperature in PBS-Tween buffer. Enclose the membrane in a plastic bag for probing with 100 µl NGS and 10 ml specific antibody in blotting buffer and shake at room temperature for two hours or overnight at 4°C, then wash four times with PBS-Tween buffer. Secondary antibody was goat-anti-rabbit IgG conjugated to bioepiderm (40 µl in 10 ml blotting buffer with 100 µl NGS). Membranes were shaken with secondary antibody for one hour at room temperature then washed. The membrane was developed with antibody protein-HRP (40 µl dissolved in 10 ml blotting buffer+NGS). Using the manufactures' instructions, we used ECL group agent BeyoECL, as a detection agent.

Statistical analysis

Experimental data was analyzed with the SPSS 12.0 package. The results are presented as mean ± standard deviation (SD). We did multiple statistical analyses to compare two groups. If there were more than two groups, we first used a normal testing method and homoscedasticity analyses via the Shapiro-Wilk method and the Levene method. If the data consisted of a normal distribution and homoscedasticity, we did one-factor analysis of variance (ONE-ANOVA). If the data were not consistent with a normal distribution, we did a rank test whose statistical significance was set at $P < 0.05$.

RESULTS

Effects of intervention with icariin on IL-4 and IFN-γ expression in rat pulmonary tissue

IL-4 expression in pulmonary tissue in the model group was significantly higher than in the control group. IL-4 expression in the DXM treatment group was significantly decreased compared to the model group ($P < 0.05$). IL-4 expression in pulmonary tissue in the icariin treatment groups was also significantly lower ($P < 0.05$) than in the asthma model group (Figure 1). IFN-γ expression in pulmonary tissue in the model group was significantly lower than in the control group. IFN-γ expression after DXM treatment was significantly ($P < 0.05$) increased compared to the model group. IFN-γ expression in pulmonary tissue in the icariin treatment group had a trend toward higher expression compared to the model group, but the increase was not statistically significant (Figure 2).

Influence of icariin on immunohistochemical staining (T-bet and GATA-3) in rat pulmonary tissue

GATA-3 staining of rat pulmonary tissue in the model group was greater than in the control group, especially in

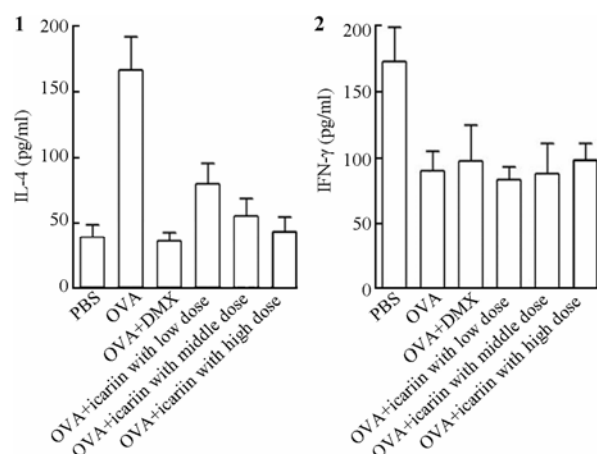


Figure 1. Intervention effect of icariin on IL-4 expression of rat pulmonary tissue in each group. $P < 0.05$ compared to OVA controls, $n=6$ per group.

Figure 2. Intervention effect of icariin on IFN- γ expression of rat pulmonary tissue in each group. Compared with the asthma group, IFN- γ expression in lung tissue of icariin treatment group tended to increase, but not statistically significant ($P > 0.05$, $n=8$ per group).

the cell nucleus. After treatment with OVA+DXM and OVA+icariin, GATA-3 expression decreased significantly (Figure 3). T-bet staining in pulmonary tissue in the OVA group was significantly increased compared to the PBS group, especially in the cell nucleus. After treatment with OVA+icariin, T-bet staining failed to show a significant decrease (Figure 4).

Intervention effect of icariin on T-bet and GATA-3 mRNA expression in pulmonary tissue and spleen lymphocytes

T-bet mRNA expression in rat pulmonary tissue in the OVA group was significantly higher than in the control

group. T-bet expression in the OVA+DXM group was significantly decreased compared to the model group ($P < 0.05$). GATA-3 mRNA expression in rat pulmonary tissue in the OVA+icariin treatment group decreased significantly ($P < 0.05$) compared to the model group (Figure 5). GATA-3 mRNA expression in rat pulmonary tissue in the DXM treatment group was higher than in the control group. GATA-3 mRNA expression in the DXM treatment group was significantly lower than in the model group ($P < 0.05$). GATA-3 mRNA expression in rat pulmonary tissue in the icariin treatment group was significantly decreased ($P < 0.05$) compared to the model group (Figure 6).

The effect of icariin on T-bet mRNA expression by rat spleen lymphocytes in the model group was to significantly increase the expression compared to the control group. T-bet expression in the OVA+DXM group was significantly decreased compared to the model group ($P < 0.05$). The T-bet mRNA expression by spleen lymphocytes in the OVA+icariin group was decreased compared to the model group, but the change was not significant (Figure 7). GATA-3 mRNA expression of rat spleen lymphocytes in the model group was significantly higher than in the control group. GATA-3 mRNA expression in the DXM treatment group was significantly decreased ($P < 0.05$) compared to the model group. GATA-3 mRNA expression by rat pulmonary tissue in the icariin treatment group decreased significantly ($P < 0.05$) compared to the model group (Figure 8).

Intervention effect of icariin on T-bet and GATA-3 protein expression in rat asthmatic pulmonary tissue

Two hours after the last OVA exposure, rats in each group were anesthetized, blood was taken from the heart then

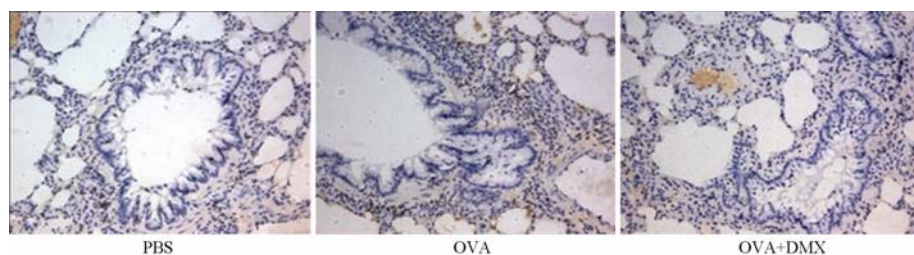


Figure 3. GATA-3 immunohistochemistry staining of rat pulmonary tissue in PBS, OVA and OVA+DXM groups (HE staining, original magnification $\times 200$). In the asthma model group (OVA group) GATA-3 in lung tissue staining is significantly increased compared with the control group (brown nuclear particles). In OVA + dexamethasone group and OVA +

icariin group, GATA-3 staining is significantly reduced.

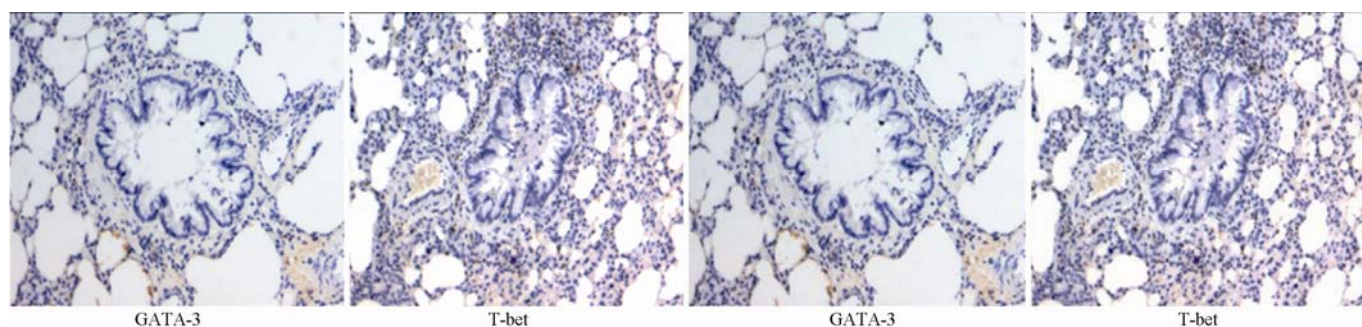


Figure 4. Intervention effect of icariin on GATA-3 and T-bet immunohistochemistry staining of rat pulmonary tissue in OVA+icariin group (HE staining, original magnification $\times 200$). In the asthma model group (OVA group) T-bet in lung tissue staining is significantly increased compared with the control group (brown nuclear particles). T-bet staining in OVA + icariin groups is not significantly reduced.

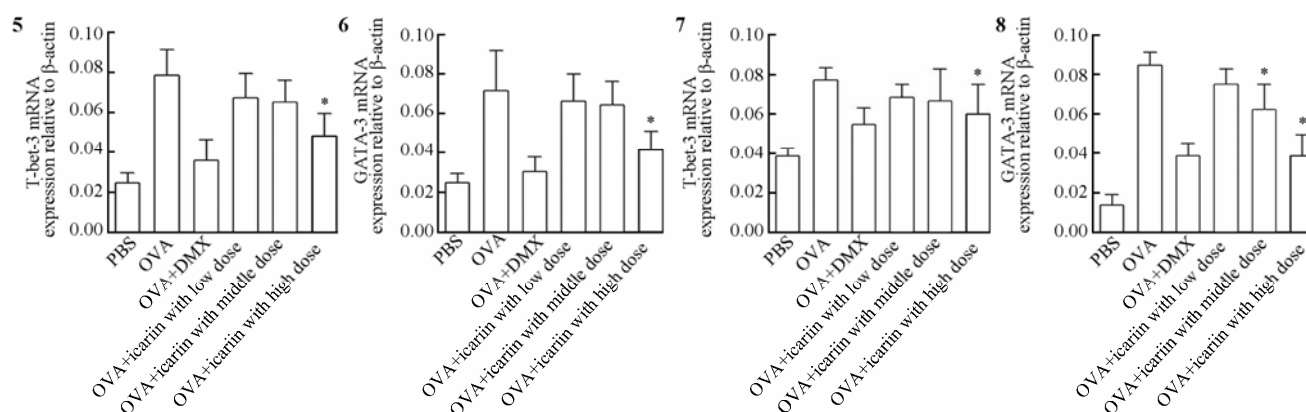


Figure 5. Intervention effect of icariin on T-bet mRNA expression in rat pulmonary tissue in each group. * $P < 0.05$ compared to OVA controls, $n=6$ per group.

Figure 6. Intervention effect of icariin on GATA-3 mRNA expression in rat pulmonary tissue in each group. * $P < 0.05$ compared to OVA controls, $n=6$ per group.

Figure 7. Effect of icariin on T-bet mRNA expression of rat spleen lymphocytes in each group. * $P > 0.05$ compared to OVA controls, $n=8$ per group.

Figure 8. Effect of icariin on GATA-3 mRNA expression of rat spleen lymphocytes in each group. * $P < 0.05$ compared to OVA controls, $n=6$ per group.

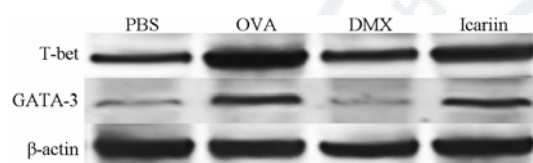


Figure 9. Changed expression of T-bet and GATA-3 protein in pulmonary tissue in each group after icariin intervention.

animals were sacrificed. We took fresh pulmonary tissue from four groups' of rats: the PBS group, OVA group, OVA+DXM group and 20 mg/kg icariin treatment group. Western blotting was used to detect T-bet and GATA-3 protein levels (Figure 9). Taking β -actin as a reference, we applied Image Lcd 3.2 software to calculate the Western blotting gray scale values. Taking the gray scale values in the OVA group as a reference, we undertook three independent experiments. The results showed that T-bet and GATA-3 expression in the OVA group increased significantly compared to the PBS group, and DXM and icariin inhibited the increase of GATA-3 protein while icariin had no obvious effect on T-bet.

The effect of icariin on immunohistochemical staining for NF- κ B p65 in rat asthmatic pulmonary tissue

We probed pulmonary tissue from four groups for NF- κ B

p65 expression by immunohistochemistry: PBS control group, OVA group, OVA+DXM group and OVA+high dose icariin. The lungs were normal in the PBS group and there were a small number of NF- κ B p65 expressing cells. In the lungs of the OVA group there was obvious allergic inflammation with vessel wall thickening of bronchiole and small vessels, inflammatory cell infiltration, an obvious increase in the number of eosinophiles and macrophages, damage to the Bronchial mucosa, edema in the peripheral tissue, and more cells labeling for NF- κ B p65, especially concentrated in the cell nucleus. In the OVA+DXM group, there was a slight inflammatory cell infiltration of bronchi, infra-vessel-mucosa and peripheral pulmonary tissue, and less NF- κ B p65 staining compared to the DXM treated lungs. Compared with the OVA group, there was less NF- κ B p65 expression after icariin treatment (Figure 10).

Icariin inhibited NF- κ B p65 protein expression of rat asthmatic pulmonary tissue, but was expressed in cell nucleus

Two hours after the last OVA exposure rats in each group were anesthetized, blood was taken from the heart then animals were sacrificed. We took fresh pulmonary tissue from four groups: the PBS group, OVA group,

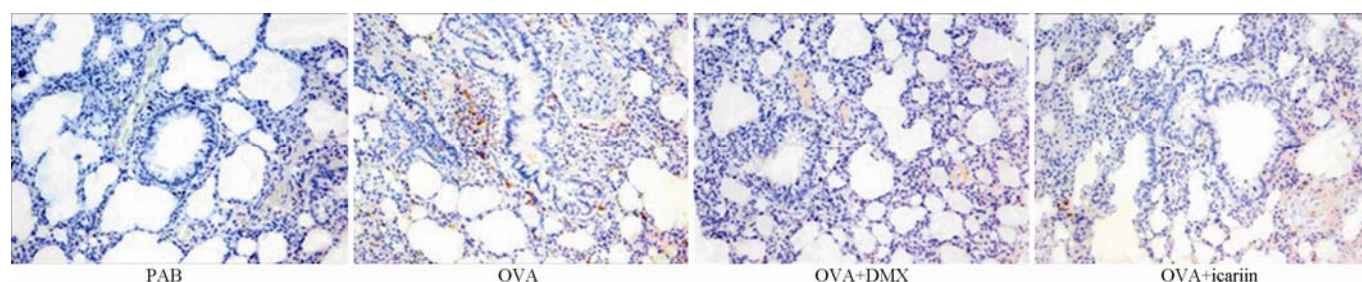


Figure 10. The NF- κ B p65 expression by immunohistochemical staining in pulmonary tissue (HE staining, original magnification $\times 200$). In the OVA + icariin treatment group (20 mg/kg) the bronchus, blood vessels and around submucosal inflammatory cells infiltrate in lung tissue.

OVA+DXM group and OVA+ high dose icariin. Western blotting was used to probe for T-bet and NF- κ B p65 protein expression (Figure 11). Taking β -actin as a reference, we used Image Lcd 3.2 software to determine the Western blotting gray scale values. Taking gray scale value of OVA group as a reference, we undertook three independent experiments. The results were as follows: The total p65 expression in the OVA group was increased compared to the PBS control group. DXM and icariin treatment inhibited the increase of NF- κ B p65 protein, but could increase p65 protein expression in the sarcoplasm (Figure 11).

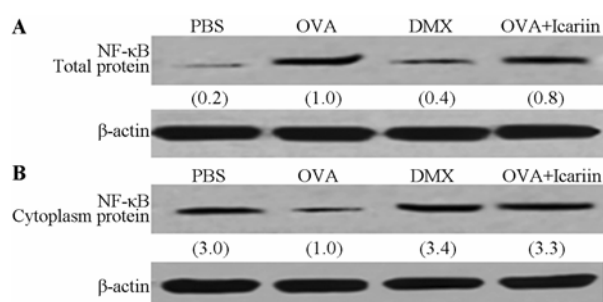


Figure 11. The change in NF- κ B p65 protein expression in pulmonary tissue after intervention with DXM and icariin.

DISCUSSION

Dr. SHEN Zi-ying of our Department has applied epimedium as the principal agent for “warming and invigorating kidney yang” to treat asthma patients by homotherapy for heteropathy since the 1950s. Our research team followed the TCM theories of asthma; such as “it is sthenia when in lung while it is asthenia when in kidney”, “to treat lung acutely while to treat kidney chronically”, “chronic disease involving kidney”, developing the theory of asthmatic intervention by the formula of invigorating the kidney and tonifying the qi. The results show the formula of invigorating the kidney and tonifying the qi have significant effects on preventing seasonal asthmatic attacks, proving the effect of invigorating the kidney may improve the function of the HPA axis so as to explain the main principle of treating bronchial asthma by invigorating the kidney. The early research showed that epimedium could reduce the airway inflammation of an asthma attack, and could inhibit the RANTES and MCP-3 expression in pulmonary tissue. The main effective ingredient of epimedium is icariin, which has an anti-inflammatory function. Researches show that icariin has a function similar to adrenal cortex hormone. The first part of this study investigated the influence of icariin on asthmatic-related cytokines. The results show that icariin can inhibit the serum IL-4 expression in the asthmatic rat while increasing the IFN- γ . This part of the experiment further explored the inhibition mechanism of icariin on airway inflammation in a rat OVA-asthma model. This study described some of the anti-inflammatory functions of icariin when used to treat airway inflammation. It

provided information to help define further mechanisms of inhibition of asthmatic airway inflammation by icariin.

Bronchial asthma is a disease with chronic airway inflammation as the main pathologic change, whose pathogenicity is closely related to the imbalance of Th1/Th2-type immune response. The main immune manifestation is the decrease of the Th1-type immune response and the increase of the Th2-type immune response.¹² Th1 cells mainly excrete cytokines like IL-2, IFN- γ and TNF- β , participating in immunologic responses mediated by cytotoxicity T-cells (CTL), and having an important effect on intracellular pathogens like viral infection and mycobacterial infections.^{13,14} Th2 cells mainly excrete cytokines IL-4, IL-5, IL-6, IL-9 and IL-13, whose main functions are to stimulate the proliferation of B cells, to generate of IgG and IgE antibodies and to participate in the humoral immune response.^{15,16} These two form a cytokine network by the way of self-enhancement and mutual antagonism, having different physiologic functions and serving to help regulate the immune response.¹⁷ The purpose of adjusting the balance between Th1 and Th2 is to inhibit the asthmatic inflammatory reaction, which has clinical applications.^{18,19} We observed that icariin had an effect on the Th1/Th2 balance. First, we observed the effect of icariin on IL-4 and IFN- γ expression of rat asthmatic pulmonary tissue which showed that IL-4 expression in icariin treatment groups was significantly lower than in the asthmatic rats ($P < 0.05$). Figure 1 shows that IFN- γ expression in pulmonary tissue in the asthma group was significantly lower than in the normal control group. IFN- γ expression in pulmonary tissue after icariin treatment was elevated compared to the asthma group, but not significantly (Figure 2). According to the experimental results, icariin could inhibit the IL-4 expression in serum of asthmatic rats and in pulmonary tissue. Icariin also increased the IFN- γ level in serum of asthmatic rats, although the change was not statistically significant. Icariin still could adjust the imbalance of Th1/Th2 cytokines. This might be one of the mechanisms by which icariin inhibits airway inflammation in asthma.

After observing the effects of icariin on Th1/Th2 related cytokines, we further investigated the regulating effects of icariin on Th1/Th2 related transcription factors. T-bet and GATA-3 are two cell specific transcription factors, which are expressed by Th1 cells and Th2 cells, respectively, and control the Th1 and Th2 cell differentiation.^{20,21} T-bet is the key transcription factor of Th1 cell differentiation while GATA-3 is one of the most important transcription regulation factor in Th2 cell development,¹¹ and can facilitate cell differentiation from Th0 cell to Th2 while inhibiting the cell differentiation from Th0 cell to Th1. It can also reverse the development of Th1 cells to a Th2 phenotype.^{22,23} GATA-3 is expressed by Th2 cells and it is now the only defined Th2 specificity transcription factor composed of key Th2-type cytokines, which could adjust IL-5, IL-4

and IL-13.^{24,25} In this experiment, the effect of icariin on expression of T-bet and GATA-3 in pulmonary tissue in the asthmatic model was to increase GATA-3 compared to the controls, especially in the cell nucleus. After treatment with DXM and icariin, GATA-3 staining decreased significantly, especially in the icariin group (Figure 3). T-bet staining in pulmonary tissue in the asthma model group was significantly higher than in the control group, especially in the nucleus. But T-bet staining did not decrease significantly after the treatment with OVA+icariin (Figure 4).

Immunohistochemical analysis showed that icariin could inhibit GATA-3 expression in pulmonary tissue in the asthma model. Therefore, we applied real-time PCR to further investigate the influence of icariin on T-bet and GATA-3 mRNA expression. The effect of icariin on T-bet and GATA-3 mRNA expression in the asthmatic rats showed that T-bet mRNA expression in pulmonary tissue after icariin treatment decreased significantly compared to the asthma group (Figure 5). GATA-3 expression also decreased after icariin treatment (Figure 6). The effect of icariin on T-bet and GATA-3 mRNA expression in spleen lymphocytes in asthmatic rats found T-bet mRNA expression decreased compared to the asthma group, but not significantly (Figure 7). GATA-3 mRNA expression was also decreased, but the difference was significant (Figure 8). From the results above, icariin inhibits GATA-3 expression in pulmonary tissue and in spleen lymphocytes in asthmatic rats and it also inhibits T-bet mRNA expression in pulmonary tissue. Although it did not achieve statistical significance, icariin also reduced T-bet mRNA expression in the spleen lymphocytes. These data contribute to our hypothesis that icariin can adjust the Th1/Th2 imbalance in asthma.

Icariin had inhibitory effects on T-bet and GATA-3 mRNA expression of rat pulmonary tissue and spleen lymphocytes in the asthmatic model, so did it also have the same inhibitory effect on T-bet and GATA-3 protein expression? We took fresh pulmonary tissue from four groups of rats: the control, model group, OVA+DXM group and OVA+ high dose icariin group, and used Western blotting to determine protein expression (Figure 9). Expressions of T-bet and GATA-3 were significantly increased in the model group compared to the control group. DXM and icariin both inhibited the increase of GATA-3 protein while icariin did not have obvious inhibitory effect on T-bet. The experiment above is consistent with the immunohistochemical results. Regulation of GATA-3 may be one of mechanisms by which icariin can modify immune function in asthmatics.²⁶

In previous studies we found that the anti-inflammatory function of icariin was related to NF- κ B.²⁷ NF- κ B is one of the important nuclear factor that can adjust the expression of multiple genes; including proinflammatory factors, chemotatic factors, adhesion molecules and their

receptors. NF- κ B also plays a very important role in inflammatory damage, tumor growth, apoptosis and cytothsis.^{28,29} We found that NF- κ B had a considerable effect on the mechanisms and development of bronchial asthma.^{30,31} The immunohistochemical analysis of pulmonary tissue from these four groups showed that icariin could inhibit NF- κ B p65 expression (Figure 10). The results showed that the total NF- κ B p65 protein induced by OVA increased in asthmatic pulmonary tissue while plasma NF- κ B p65 decreased, which indicated that NF- κ B p65 was activated and mediated the airway inflammatory reaction (Figure 11). The results also showed that icariin could inhibit the increase of total NF- κ B p65 protein induced by OVA in rats' asthmatic pulmonary tissue (Figure 11A). Meanwhile, icariin could increase NF- κ B p65 level in endochylema (Figure 11B).

In conclusion, icariin could reduce the inflammatory cell infiltration in rat asthmatic bronchi, under the vessel mucosa and in peripheral tissue, which eases the tracheal epithelium and circumvascular inflammation. It could inhibit the cell differentiation and chemotactic response of eosinophile granulocyte and macrophage² in the asthmatic rats' peripheral blood, inhibiting the expression of inflammatory factor and mediators of asthma, and inhibit NF- κ B p65 activation. The way that icariin inhibits NF- κ B p65 is different from the classical inhibition pathway. Icariin could inhibit p65 protein entering nucleus from the endochylema, which may be the target of icariin action.

REFERENCES

1. Park HJ, Lee CM, Jung ID, Lee JS, Jeong YI, Chang JH, et al. Quercetin regulates Th1/Th2 balance in a murine model of asthma. *Int Immunopharmacol* 2009; 9: 261-267.
2. Xates A, Callard R, Stark J. Combining cytokine signalling with T-bet and GATA-3 regulation in Th1 and Th2 differentiation: a model for cellular decision-making. *J Theor Biol* 2004; 231: 181-196.
3. Yu HR, Chang JC, Chen RF, Chuang H, Hong KC, Wang L, et al. Different antigens trigger different Th1/Th2 reactions in neonatal mononuclear cells (MNCs) relating to T-bet/GATA-3 expression. *J Leukoc Biol* 2003; 74: 952-958.
4. Chakir H, Wang H, Lefebvre DE, Webb J, Scott FW. T-bet/GATA-3 ratio as a measure of the Th1/Th2 cytokine profile in mixed cell populations: predominant role of GATA-3. *J Immunol Methods* 2003; 278: 157-169.
5. Chan RW, Lai FM, Li EK, Tam LS, Chow KM, Li PK, et al. Imbalance of Th1/Th2 transcription factors in patients with lupus nephritis. *Rheumatology* 2006; 45: 951-957.
6. Shao DZ, Lin M. Platonin inhibits LPS-induced NF-kappaB by preventing activation of Akt and IKKbeta in human PBMC. *Inflamm Res* 2008; 57: 601-606.
7. Andersen JN, Sathyanarayanan S, Di Bacco A, Chi A, Zhang T, Chen AH, et al. Pathway-based identification of biomarkers for targeted therapeutics: personalized oncology with PI3K pathway inhibitors. *Sci Transl Med* 2010; 2: 43-55.
8. Park SY, Park GY, Ko WS, Kim Y. Dichroa febrifuga Lour.

- inhibits the production of IL-1 β and IL-6 through blocking NF- κ B, MAPK and Akt activation in macrophages. *J Ethnopharmacol* 2009; 125: 246-251.
9. He W, Sun H, Yang B, Zhang D, Kabelitz D. Immunoregulatory effects of the herba *Epimedium* glycoside icariin. *Arzneimittelforschung* 1995; 45: 910-913.
 10. Zhao F, Tang YZ, Liu ZQ. Protective effect of icariin on DNA against radical-induced oxidative damage. *J Pharm Pharmacol* 2007; 59: 1729-1732.
 11. Vale-Pereira S, Todo-Bom A, Geraldine L, Schmidt-Weber C, Akdis CA, Mota-Pinto A. FoxP3, GATA-3 and T-bet expression in elderly asthma. *Clin Exp Allergy* 2011; 41: 490-496.
 12. Neurath MF, Finotto S, Glimcher LH. Th1/Th2 lymphocyte polarization in asthma. *Allergy Nat Med* 2002; 8: 567-573.
 13. Colavita AM, Reinach AJ, Peters SP. Contributing factors to the pathobiology of asthma. The Th1/Th2 paradigm. *Clin Chest Med* 2000; 21:263-277.
 14. Nakanishi K. Pathological role of super Th1 cells in induction of atopic dermatitis and bronchial asthma. *Alerugi* 2008; 57: 989-994.
 15. McGee HS, Agrawal DK. TH2 cells in the pathogenesis of airway remodeling: regulatory T cells a plausible panacea for asthma. *Immunol Res* 2006; 35: 219-232.
 16. Nakamura Y, Hoshino M. TH2 cytokines and associated transcription factors as therapeutic targets in asthma. *Curr Drug Targets Inflamm Allergy* 2005; 4: 267-270.
 17. Park HJ, Lee CM, Jung ID, Lee JS, Jeong YI, Chang JH, et al. Quercetin regulates Th1/Th2 balance in a murine model of asthma. *Int Immunopharmacol* 2009; 9: 261-267.
 18. Fietta P, Delsante G. The effector T helper cell triade. *Riv Biol* 2009; 102: 61-74.
 19. Wegmann M. Th2 cells as targets for therapeutic intervention in allergic bronchial asthma. *Expert Rev Mol Diagn* 2009; 9: 85-100.
 20. Robinson DS, Lloyd CM. Asthma: T-bet a master controller? *Curr Biol* 2002 30; 12: R322-R324.
 21. Wang SY, Yang M, Xu XP, Qiu GF, Ma J, Wang SJ, et al. Intranasal delivery of T-bet modulates the profile of helper T cell immune responses in experimental asthma. *J Investig Allergol Clin Immunol* 2008; 18: 357-365.
 22. Raby BA, Hwang ES, Van Steen K, Tantisira K, Peng S, Litonjua A, et al. T-bet polymorphisms are associated with asthma and airway hyperresponsiveness. *Am J Respir Crit Care Med* 2006; 173: 64-70.
 23. Zhu J, Yamane H, Cote-Sierra J, Guo L, Paul WE. GATA-3 promotes Th2 responses through three different mechanisms: induction of Th2 cytokine production, selective growth of Th2 cells and inhibition of Th1 cell-specific factors. *Cell Res* 2006; 16: 3-10.
 24. Barnes PJ. Role of GATA-3 in allergic diseases. *Curr Mol Med* 2008; 8: 330-334.
 25. Sel S, Wegmann M, Dicke T, Sel S, Henke W, Yildirim AO, et al. Effective prevention and therapy of experimental allergic asthma using a GATA-3-specific DNase. *J Allergy Clin Immunol* 2008; 121: 910-916.
 26. Erpenbeck VJ, Hohlfeld JM, Discher M, Krentel H, Hagenberg A, Braun A, et al. Increased messenger RNA expression of c-maf and GATA-3 after segmental allergen challenge in allergic asthmatics. *Chest* 2003; 123(3 Suppl): s370-s371.
 27. Wu JF, Dong JC, Xu CQ. Effects of icariin on inflammation model stimulated by lipopolysaccharide *in vitro* and *in vivo*. *Chin J Integ Tradit Western Med (Chin)* 2009; 29: 330-334.
 28. Charokopos N, Apostolopoulos N, Kalapodi M, Leotsinidis M, Karamanos N, Mouzaki A. Bronchial asthma, chronic obstructive pulmonary disease and NF- κ B. *Curr Med Chem* 2009; 6: 867-883.
 29. Edwards MR, Bartlett NW, Clarke D, Birrell M, Belvisi M, Johnston SL. Targeting the NF- κ B pathway in asthma and chronic obstructive pulmonary disease. *Pharmacol Ther* 2009; 121: 1-13.
 30. Bamborough P, Callahan JF, Christopher JA, Kerns JK, Liddle J, Miller DD, et al. Progress towards the development of anti-inflammatory inhibitors of IKK β . *Curr Top Med Chem* 2009; 9: 623-639.
 31. Xu CQ, Liu BJ, Wu JF, Xu YC, Duan XH, Cao YX, et al. Icariin attenuates LPS-induced acute inflammatory responses: involvement of PI3K/Akt and NF- κ B signaling pathway. *Eur J Pharmacol* 2010; 642: 146-153.

(Received October 20, 2010)

Edited by WANG De