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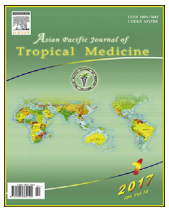

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Effect of PPARγ agonist (rosiglitazone) on the secretion of Th2 cytokine in asthma mice

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ABSTRACT

Objective: To explore the effect of PPARγ agonist (rosiglitazone) on the secretion of Th2 cytokines and the proportion of immune cell subsets in asthma mice.
Methods: Ovalbumin (OVA)-sensitized mice were used to build asthma models. Those mice were divided into the normal control group, model group and rosiglitazone group. Differences of the changes in lung histopathology of mice in the three groups were observed through hematoxylin and eosin (HE) strain, and the numbers of the total cells, eosinophils and neutrophils in BALF of mice in the three groups were compared. ELISA and real-time PCR were employed to detect the protein levels of interleukin (IL)-5, IL-13, IL-4 and IL-10 and mRNA level, respectively. Flow cytometry number was implied to analyze the proportion of immune cell subsets in peripheral blood of mice.
Results: Compared with the mice in the control group, and mice of the model group, the infiltration of inflammatory cells in BALF increased, bronchial smooth muscle became thickened, a large amount of collagen deposited, the secretion of Th2 cytokine increased significantly, the ratio of regulatory T cells (Treg) decreased, the ratio of T17 cells rose distinctly; while in mice of the rosiglitazone group, the changes of their lung histopathology were improved obviously, the number of infiltration of inflammatory cells declined, the thickened smooth muscle relieved, the deposition of collagen decreased, the secretion of Th2 cytokine was inhibited, the ratio of Treg went up, and the increased of the ratio of T17 cells was inhibited but still not return to normal level.
Conclusions: Rosiglitazone can regulate the proportion of Treg and Th17 cells and inhibit the secretion of Th2 cytokines, which inhibit the airway inflammatory response for asthma mice effectively.

1. Introduction

Bronchial asthma is an allergic disease in respiratory system characterized by chronic inflammation with an increasing incidence worldwide, which severely threatens human health. The pathogenesis of the disease has not been illuminated completely yet [1,2]. More and more researches have pointed out that bronchial asthma is accompanied by the activation of a large number of immune cells including eosinophils, neutrophils, macrophages, mast cells and T cells. The activation of immune cells induces the synthesis and release of multiple inflammatory factors. The interaction of these inflammatory cells and cytokines causes pathology changes such as bronchiolar inflammation, the hyperplasia of mucous epithelium and smooth muscle, collagen deposition and so on [3,4]. Hence, it is of great significance to exploit drug to inhibit airway inflammatory response and explore its mode of action for clinical treatment and research of bronchial asthma. Peroxisome proliferator activated receptor-γ (PPARγ) is an important number of the inflammatory signaling pathway. Its special agonist (rosiglitazone) plays a potential role in multiple inflammation-related injury models [5–7]. This study aims to explore the effect of PPARγ agonist (rosiglitazone) on asthma mice and its influence on the secretion of Th2 cytokine and the proportion of immune cell subsets.

2. Materials and methods

2.1. Major reagents

Ovalbumin (OVA) (Sigma, USA), rosiglitazone (batch number: 040511, GSK, China), ELISA kits for interleukin (IL)-5, IL-13, IL-4 and IL-10 (eBioscience) were used in this study.

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Trizol reagent was purchased from Life (USA), reverse transcription kits and SYBR Green Real-time PCR reagent was bought from Takara. The primer was designed and compounded by Sangon Biotech (Shanghai) Co. Ltd. 10× mice hemocyte lysate was from BD. Cytokine staining reagents, cellular nuclei staining reagents, Fc Block antibody, stream antibodies Foxp3-APC, CD25-FITC, CD4-PE, IL-17-Percific blue and CD3-PE Cy7 were all bought from eBioscience.

2.2. Animal models and drug administration

In this study, OVA-sensitized mice were used to build asthma models. Twenty-four female mice of clean grade (6–8 weeks, 18–22 g) were provided by the Laboratory Animal Unit of Congqing Medical University. All mice were divided into the control group, model (OVA) group and rosiglitazone (OVA + ROSI) group in accordance with the randomized digital table with eight mice in each group. Mice in the OVA and OVA + ROSI groups were given 0.1 mL sensitization liquid OVA containing 100 μg of OVA and 31 mg of AL(OH) by intraperitoneal injection at day 0 and day 7. Fourteen days later, those mice were placed in transparent container, and then 2% OVA normal saline solution was sprayed by using ultrasonic fogging tester to trigger asthma for 30 min once per day. Besides, mice in the rosiglitazone group were also treated with 5×10^{-5} mol/L of rosiglitazone simultaneously for continuous stimulation for 7 days. In the normal control group, normal saline was used to replace OVA sensitization and atomization motivation. After the final stimulation, the middle lobe of the lung tissue in mice was taken out for routine paraffin section and hematoxylin and eosin (HE) strain to observe the changes of their lung histopathology. Experiments involved animals in this study was approved by the Biomedical Ethics Committee of The First Affiliated Hospital of Chongqing Medical University.

2.3. The collection and management of BALF

After mice in the three groups were anaesthetized by 0.75% pentobarbital sodium, a transverse cutting was conducted under the cricoid cartilage of mice in which the irrigating tube was placed. One milliliter sterile normal saline were injected slowly and recycled three times. The recycled liquid was centrifuged at 15000 r/min for 10 min at 4 °C. The supernatant was collected and stored at −80 °C for detecting cytokines. Cell sediments were used to counting cell numbers and RNA extraction.

2.4. BALF cell counts and classification

After the centrifugation of BALF, 200 μL of normal saline was used to suspend cell sediments and mix. Out of them, 20 μL of the cell suspension liquid was mixed with 380 μL of acetic acid. Blood cell counting board was used to count the total cells. Besides, two pieces of cell rejection slice were prepared and stained by Wright–Giemsa stain, respectively. Then, the numbers of eosinophils and neutrophils were recorded. Every specimen was responsible for at least 400 cells.

2.5. Analysis of lung histopathology in mice

Routine HE stain was conducted in sections of lung tissues of those mice to observe their lung histopathology. Image analysis software Un-Scan-It 6.0 was used to test the thickness of bronchial smooth muscle and collagen deposition in mice.

2.6. ELISA

Ebioscience ELISA kits were used to determine the contents of IL-5, IL-13, IL-4 and IL-10 in BALF of mice, which was conducted in accordance with the construction strictly. Half an hour before the procedure, the reagent was place at room temperature for 30 min. The specimen and gradient concentration standards (100 μL/well) were added into a 96-well plate and then closed the plate for incubation at 37 °C for 30 min. A washer was used 4 times. Then, 100 μL of biotinylated antibody was added into every well and the plate was sealed for incubation at 37 °C for 60 min. After washing for 4 times, 100 μL color-developing agent was added into every well. Ten minutes later, stop buffer was added and mixed to detect OD_{450}. The washer and enzyme-labeled liquid were all purchased from Biotech.

2.7. Real-time PCR

Routine Trizol method was applied to extract the total RNA of cells. Every sample was dissolved in 10 μL RNase free water. PrimeScript™ RT Reagent Kit with gDNA Eraser kits (Tarara) were employed to remove genomic DNA and conduct reverse transcription. Realtime PCR was used to detect the levels of IL-5, IL-13, IL-4 and IL-10 and RNA levels. The expression level of RNA was calculated by $2^{-\Delta\Delta}$Cq method with β-actin serving as the reference. Realtime PCR 7500 instrument was from ABI.

2.8. Flow cytometry

A total of 500 μL of peripheral blood of mice were collected and 50 μL/tube of heparin was added into it for anticoagulation. Then, 3 mL 1 × hemocyte lysate was used for broken management for 3 min. After that, 10 mL pre-cooling PBS was added to terminate dissociation. The device was centrifuged at low temperature of 4 °C with centrifuged speed of 500 g for 5 min. Cells were collected and closed for 10 min and then stained with diolame. Antibodies CD3, CD4 and CD25 were added and incubated in dark at 4 °C for 30 min. After washed by PBS, it was fixed for 0.5 h by fixer. After nucleus-cross management, Foxp3 and IL-17 antibody were added and also incubated in dark at 4 °C for 30 min. After washed by cleaning mixture twice, BD cantoII flow cytometer was implied for detection and Flow Jo6.0 software was employed for analysis and statistics.

2.9. Statistic management

Measurement data in this study were expressed by mean ± SD and analyzed by SPSS software. Comparisons between groups were illustrated by One-way ANOVA and pairwise comparisons among groups were analyzed by LSD method. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Administration of PPARγ agonist (rosiglitazone) improving airway inflammation response for asthma mice

Compared with the control group, the numbers of the total cells, eosinophils and neutrophils in mice of the OVA group all

increased (Table 1, $P < 0.05$), which indicated the success of model construction. The airway inflammation response in mice of the rosiglitazone group was relieved and the their numbers of the total cells, eosinophils and neutrophils were all lower than those of the OVA group (Table 1, $P < 0.05$), but did not return to the normal levels, since the numbers of both eosinophils and neutrophils were all significantly higher than those of the control group (Table 1, $P < 0.05$).

in mice of the OVA group increased significantly (Tables 2 and 3, $P < 0.05$); while the protein levels of Th2 cytokines IL-5, IL-13, IL-4 and IL-10 and mRNA level in mice of the rosiglitazone group decreased as compared to those of the OVA group, which indicated that the inflammatory response in mice was relieved. However, only the level of IL-4 was back to its normal level, the expressions of other Th2 cytokines were still much higher than those of the control group (Tables 2 and 3, $P < 0.05$).

Table 1

Numbers of the total cell count, eosinophil cells and neutrophil cells in BALF of mice in each group as well as airway reconstruction status of mice ($n = 8$).

Group	Total cell count (10^4/mL)	Eosinophil cell (10^4/mL)	Neutrophil cell (10^4/mL)	Smooth muscle layer thickness (μm)	Collagen content (AU)
Control	11.45 ± 1.68	0.18 ± 0.01	0.13 ± 0.02	8.20 ± 1.43	28.23 ± 4.03
OVA	24.57 ± 2.85*	7.93 ± 0.56*	3.45 ± 0.40*	36.72 ± 5.04*	89.44 ± 11.07*
OVA + ROSI	13.07 ± 2.82#	3.22 ± 0.41*#	1.28 ± 0.11*#	20.39 ± 6.92*#	33.65 ± 8.79#

*Compared with the control group, $P < 0.05$; #Compared with the OVA group, $P < 0.05$.

Table 2

Levels of the cytokines in BALF in mice ($n = 8$).

Group	IL-5 (pg/mL)	IL-13 (pg/mL)	IL-4 (pg/mL)	IL-10 (pg/mL)
Control	34.71 ± 5.05	94.73 ± 8.09	38.37 ± 5.61	7.88 ± 2.05
OVA	94.72 ± 11.26*	277.13 ± 33.12*	79.07 ± 9.41*	44.82 ± 6.13*
OVA + ROSI	47.22 ± 5.67*#	121.64 ± 33.71*#	43.76 ± 5.82#	20.37 ± 4.22*#

*Compared with the control group, $P < 0.05$; #Compared with the OVA group, $P < 0.05$.

Table 3

The relative expression level of mRNA in cytokines and changes in Treg and Th17 cell ratio.

Group	IL-5	IL-13	IL-4	IFN-γ	Treg (%/CD4)	Th17 (%/CD4)
Control	1.02 ± 0.08	1.10 ± 0.11	0.95 ± 0.09	1.04 ± 0.21	4.72 ± 0.87	0.75 ± 0.12
OVA	86.73 ± 5.23*	114.05 ± 8.91*	44.11 ± 6.20*	15.94 ± 2.61*	2.31 ± 0.75*	4.22 ± 1.08*
OVA + ROSI	12.77 ± 3.08*#	26.71 ± 4.94*#	4.11 ± 1.02*#	3.55 ± 0.59*#	4.09 ± 0.91#	1.27 ± 0.89#

*Compared with the control group, $P < 0.05$; #Compared with the OVA group, $P < 0.05$.

3.2. Influence on airway remodeling in mice administered with rosiglitazone

The results of HE stain showed that mice in the normal control group had normal bronchus and vascular structure without inflammatory cell infiltration. In mice of the OVA group, airway inflammatory response took place obviously, airway smooth muscle became thickened significantly (Table 1, $P < 0.05$), mucous epithelium proliferated, a large amount of collagen deposited in the bronchus, vascular alveolar walls and pulmonary mesenchyme accompanied by mass infiltration of inflammatory cells (Table 1, $P < 0.05$). Symptoms of mice in the rosiglitazone group were improved, their thickened airway smooth muscle was relieved and the deposition of collagen reduced (Table 1, $P < 0.05$).

3.3. Influence of rosiglitazone on cytokines in asthma mice

Compared with the control group, the protein levels of Th2 cytokines IL-5, IL-13, IL-4 and IL-10 and mRNA level in BALF

3.4. Changes of the ratio of peripheral blood regulatory T cells (Treg) and Th17 cells

The results of flow cytometer demonstrated that Treg accounted for 4.72% of $CD4^+$ T cells in normal mice, while Th17 cells accounted for less than 1%. In asthma mice of the OVA group, the ratio of Treg declined significantly, while the ratio of Th17 cells rose markedly. Compared with the OVA group, the changes of the ratio of Treg and Th17 cells in mice of the rosiglitazone group relieved significantly, but not returned to the normal levels (Table 3, $P < 0.05$).

4. Discussion

PPARγ is the transcription factor activated by ligand of the nuclear hormone receptor superfamily, which expresses in multiple tissues and cells. Early researches have found that PPARγ could regulate the differentiation of adipocytes and lipid metabolism and also be closely related to the insulin resistance in diabetes patients. Its special agonist rosiglitazone is also approved to be used to treat diabetes mellitus type 2 [8,9]. Recent

researches have confirmed that PPARγ could also influence multiple signaling pathways of inflammatory responses, participate in the production and secretion of cytokines and play a key role in the cardiovascular system and the development of tumors as well [10–12]. There have been researches [13] reporting that PPARγ was closely related to the airway inflammatory response in mice. The results of this study also indicated that the special agonist of PPARγ, rosiglitazone, could regulate the ratio of peripheral blood Treg and Th17, reduce the inflammatory cells infiltration of lung tissue, inhibit the secretion of Th2 cytokines and inhibit the airway inflammatory response of asthma mice effectively.

Inflammatory cells infiltration and airway remodeling are two important features of bronchial asthma. OVA-sensitized mice were used to build asthma models. The results showed that the numbers of the total inflammatory cell count, eosinophil cells and neutrophil cells in BALF of mice all increased, mucous epithelium proliferated and a large amount of collagen deposited in the bronchus, vascular alveolar walls and pulmonary mesenchyme, which indicated the success of model construction. In addition, we found that in mice administered with rosiglitazone the inflammatory cell infiltration declined, their thickened bronchial smooth muscle relieved significantly and the collagen deposition in the vascular alveolar walls and pulmonary mesenchyme reduced markedly, which manifested that rosiglitazone could inhibit the airway inflammatory response of asthma mice effectively showing certain effect on the treatment of asthma, which showed consistency with the early researches focusing on lung injury [14,15].

Under physiological condition, the ratio of *in vivo* Th1/Th2 cells is in equilibrium so as to maintain normal humoral immunity and cell mediated immunity for the body. However, in asthma patients, the balance of the ratio of *in vivo* Th1/Th2 cells is broken with the inhibited Th1 cell function and hyperfunction of Th2 cell function. Therefore, there are researches pointing out that the mass production of Th2 cytokines (IL-5, IL-13, IL-4 IL-10, etc.) plays an important role in the course of asthma [16–18]. Results of this study showed that the protein levels of Th2 cytokines IL-5, IL-13, IL-4 and IL-10 and mRNA level in BALF in asthma mice increased significantly, while rosiglitazone could markedly decrease the protein levels of IL-5, IL-13, IL-4 and IL-10 and mRNA level, which indicated that rosiglitazone may relieve asthma symptoms for mice by inhibiting the synthesis and secretion of Th2 cytokines.

Recently, there are researches reporting that Treg and Th17 cells play important roles in the development of asthma. These two cells demonstrate antagonistic effects mutually, which has important effect on keeping the immune state for the body [19–21]. It was also found in our study that compared with those of the normal mice, the proportion of Foxp3+ Treg in asthma mice decreased significantly, while the Th17 cells increased markedly, which indicated that the unbalance of Treg/Th17 participated in the course of airway inflammation in asthma mice. Moreover, compared with the OVA group, the ratio of Treg in peripheral blood in mice of the rosiglitazone group returned back and the ratio of Th17 cells declined significantly, which implied that rosiglitazone might further inhibit the inflammation in the airways mediated by Th2 cells by increasing the ratio of Treg in peripheral blood.

In conclusion, the results of this study suggested that the special agonist of PPARγ, rosiglitazone, could relieve the symptoms of asthma by increasing the ratio of Treg cells, inhibiting the number of Th17 cells and suppressing the secretion of Th2 cytokines.

Conflict of interest statement

We declare that we have no conflict of interests.

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