Original Article
Role of Toll-like receptor 2/NF-κB signaling pathway in the pathogenesis of allergic rhinitis

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Received January 30, 2019; Accepted May 9, 2019; Epub July 15, 2019; Published July 30, 2019

Abstract: We investigated the expression and role of the toll-like receptor 2 (TLR2)/nuclear factor (NF)-κB signaling pathway in the pathogenesis of allergic rhinitis (AR). One hundred rats were randomly divided into five groups: control (Group A), AR (Group B), AR + peptidoglycan (Group C), AR + peptidoglycan + pyrrolidine dithiocarbamate (PDTC; Group D), and AR + peptidoglycan + beclomethasone (Group E). Changes in nasal mucosa morphology, inflammatory cell infiltration, interleukin (IL)-12, IL-13, and immunoglobulin E (IgE) levels, TLR2 mRNA expression levels, and NF-κB protein expression levels were detected. Allergic injury was clearly observed in AR rat models. Rats stimulated with peptidoglycan demonstrated an increase in T helper 1 (Th1) cytokine IL-12 (interleukin-12) expression and a significant decrease in T helper 2 (Th2) cytokine IL-13 (interleukin-13) and IgE expression. Additionally, the quantity of neutrophils detected in Group C significantly increased in comparison to Group B; this was associated with enhanced TLR2 and NF-κB expression, indicating that the Th1/Th2 ratio changed after allergen stimulation, provoking cellular immunity. When the rats were injected with PDTC or beclomethasone, two inhibitors of NF-κB, the expression of NF-κB in Groups D and E was decreased compared to Group B. Increased expression of IL-12 and reduced expression of IL-13 and IgE were also detected; however, the mean neutrophil counts in groups D and E were significantly decreased in comparison to Group B, suggesting that lower expression of NF-κB inhibited excessive cellular immunity in nasal mucosa, and that allergic damage was alleviated accordingly. Our findings suggest that inhibition of the TLR2/NF-κB signaling pathway could correct the imbalance of Th1/Th2 cytokines in AR rats, which would provide a novel means to prevent and treat allergic diseases.

Keywords: NF-κB, Toll-like receptor, peptidoglycan, allergic rhinitis, signaling pathway

Introduction

In recent years, the prevalence of allergic rhinitis (AR) has increased significantly. Nearly one-third of AR patients develop concurrent asthma, which seriously affects quality of life and work efficiency [1, 2]. As an inflammatory disorder, AR causes inflammation in the nose and impacts the peripheral blood, bone marrow, and lungs. AR is reported to influence 10-25% of the world population [3]. The pathogenesis of AR is mainly a type I allergic reaction influenced by an imbalance in T helper 1 (Th1)/T helper 2 (Th2) immune response, cytokine release, and allergen-specific immunoglobulin production [4-6]. Rebalancing Th1/Th2 is, thus, useful for preventing and treating AR. For example, by decreasing the expression of Th2 cytokines and increasing the expression of Th1, the Th1/Th2 ratio could be rebalanced, reducing the pathogenesis of AR. The low morbidity of AR was reported to cause effective immune reactions caused by bacteria or virus infections during childhood development [7, 8].

The pathogenesis of AR is also impacted by toll-like receptors (TLRs), which can bind the pathogen-associated molecules to initiate intracellular signaling pathways and induce the activation of nuclear factor (NF)-κB [9, 10]. NF-κB is a ubiquitous transcription factor that regulates the expression of various proinflammatory genes and mediates responses to stimuli in the inflammatory process [11-14].

The role and mechanism of NF-κB in several diseases, such as esophageal carcinoma and viral encephalitis, have been explored [14, 15]. However, to the best of our knowledge, the effect of different NF-κB expression levels on
AR has not been reported. Thus, the role of the TLR/NF-κB pathway in the pathogenesis of AR is not fully understood. More than 10 known TLR family members exist, among which TLR2 is an important component that can recognize gram-positive bacteria, protozoan parasites, and microbial lipoproteins [16]. The ability of TLR to recognize and bind to foreign antigens are prerequisites for a body-specific immune response, and studies suggest that TLR may be a target for AR prevention and treatment; however, the detailed mechanism is unclear [17].

In order to explore the impact of NF-κB expression levels on the development of AR, one hundred rats were divided into five groups for rat nasal control experiments with different reagents, and the results of the different groups were compared and discussed in terms of the variations in nasal mucosa morphology, inflammatory cell infiltration, interleukin (IL)-12, IL-13, and immunoglobulin E (IgE) levels, TLR2 mRNA expression levels, and NF-κB protein expression levels. The findings of this study can be used to enhance the understanding of the role of the TLR2/NF-κB signaling pathway in treating allergic diseases.

Materials and methods

Methods

First, peptidoglycan from gram-positive bacterial cell walls was applied to rat nostrils to stimulate the nasal mucosa. Then, the stimulated mucosal epithelial cells and TLR2 on the surface of local inflammatory cells activated NF-κB and induced the release of related Th cytokines. Two chemical compounds, pyrroline dithiocarbamate (PDTC) and beclomethasone, were used to inhibit the activation of NF-κB. One hundred rats divided into five groups were tested with different reagents (peptidoglycan, PDTC, and beclomethasone) to promote or inhibit the expression of NF-κB. Changes in the morphology of the nasal mucosa, the quantity of inflammatory cell infiltration, the levels of interleukin (IL)-12, IL-13, and immunoglobulin E (IgE), as well as the expression of TLR2 and NF-κB between the different groups were compared to explore the impact of NF-κB expression levels on the development of AR.

Experimental animals

A total of 100 healthy Wistar rats (250-300 g) of both sexes provided by the Laboratory Animal Center of Fujian Medical University were used. All experiments were approved by the Ethics Committee of Fujian Medical University.

Rat model establishment and grouping

Rats were randomly divided into five groups with 20 rats in each group and were placed on ad libitum feeding for one week. Except for Group A, the rats were sensitized every other day seven times by intraperitoneal injection of 1 mL of normal saline containing 0.3 mg of ovalbumin (Sigma-Aldrich) and 330 mg of Al(OH)₃. Afterwards, enhanced sensitization of the nasal cavity was induced for seven consecutive days by instilling 50 μL of ovalbumin (10 μg/100 μL) into each nostril to produce the AR models.

Rats were grouped as follows: Group A (normal control group): Intraperitoneal injection was performed using 1 mL of normal saline and 50 μL nasal drops; Group B (AR group): Rats were sensitized with ovalbumin (0.3 mg/1 mL) to establish an AR model. After the model was established, 50 μL nasal drops of ovalbumin (10 mg/100 μL) were administered every other day to maintain the allergic state; Group C (AR + peptidoglycan group): AR models were created as in Group B. While the allergic state was maintained, 50 μl nasal drops of peptidoglycan (10 μg/100 μL) were applied daily for seven consecutive days; Group D (AR + peptidoglycan + PDTC group): Rats were given the same treatment as Group C. While peptidoglycan was applied, 1 mL PDTC (1 mg/kg) was intramuscularly injected into the hind limb every day for seven consecutive days; Group E (AR + peptidoglycan + beclomethasone): Rats were given the same treatment as Group C. While peptidoglycan was applied, 50 μL beclomethasone (0.8 mg/2 mL) nasal drops were applied daily for seven consecutive days.

The behavioral score of the rats

Ten minutes after the first nasal irrigation with ovalbumin, peptidoglycan, beclomethasone, or normal saline, the rats were observed individually for the frequency of nose rubbing, sneezing, and rhinorrhea for 30 minutes, and a score for each behavior was given accordingly. Then, the accumulative score was obtained by adding the score of each behavior for each rat (Table 1). Rats with a total score over 5 were considered successful AR models.
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Isolation and histology observation of nasal mucosa

After the last nasal drop treatment, the rats were intraperitoneally injected with 1 mL chloral hydrate (10 g/L) for anesthetization. The chest was cut open to expose the heart and then the left ventricle was perfused with normal saline. The nasal cavities were dissected from the middle nasal concha (middle turbinal), and nasal mucosa was removed. Nasal mucosa from one nasal cavity was fixed in 10 g/L neutral paraformaldehyde at 4°C for 8 hours and the specimens were serially sliced. One slice was chosen from every four sections, and four slices in total were acquired from each specimen for hematoxylin and eosin staining and immunohistochemical staining. The structure change of the nasal mucosa was observed with an electron microscope (Leica XS-T10). Eosinophils and neutrophils were counted with a 40× objective lens.

Detection of TLR2 mRNA expression levels in nasal mucosa

Total RNA was extracted using the Trizol single-step method. The primer sequences used for TLR2 were: F5-GGA AGC AGG TGA CAA CCA TT-3 and R5-AAT CCT GCT CGC TGT AGG AA-3. Polymerase chain reaction (PCR) was performed on a fluorescence quantitative PCR system (StepOne™ Real-Time PCR System, Applied Biosystems). A two-step PCR amplification standard procedure was used, and real-time quantitative PCR reaction was performed using SYBR Green I fluorescent dye technology to obtain amplification curves of each group of samples. We used the 2^(-ΔΔCt) method to calculate the relative mRNA expression levels of the target gene.

Detection of NF-κB protein expression levels

Following the steps described in the Protein Extraction Kit, the NF-κB protein was denatur-
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ed and quantified. The protein samples and protein markers were applied on top of a 4% stacking gel and 10% separating gel. The electrophoresis apparatus was operated at 80 V for 30 minutes. After the dye front transferred to the stacking gel, the voltage was increased to 120 V. The marker strip bands showed up clearly. The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer unit at 160 mV for 70 min-
utes. After the transfer, one membrane was incubated with the NF-kB primary antibody (1:200) and another membrane was incubated with the internal reference protein, GAPDH, primary antibody (1:4000) at 4°C overnight. The membranes were then incubated with the secondary antibody (1:10000) at 4°C overnight. Reagents were then applied on the incubated membranes, and light green fluorescent strips showed up after five minutes. The membranes were dried using filter paper and covered by film. Then, these membranes were exposed to light and rinsed with water. The markers were calibrated for analysis and scanning. Quantity One image analysis software was used to determine the average gray value of NF-kB protein and GAPDH protein in each group, and the relative value of NF-kB protein was obtained by dividing the two average gray values.

**Statistical analysis**

The experimental data were expressed as the mean ± standard deviation (SD). Statistical analysis was conducted using the SPSS13.0 statistical software package. The significance test for the differences between the experimental groups was performed using an analysis of variance (ANOVA). When the variance was homogeneous, the Fisher’s least significant difference (LSD) procedure was used to perform pairwise comparisons. When the variance was not homogeneous, the Tamhane’s T2 method was used to perform pairwise comparisons. \( P < 0.05 \) was considered statistically significant.

**Results**

**Symptom assessment scores**

The symptom assessment scores after different days of nasal drop treatment are shown in Table 2 (mean ± SD). After 14 days of sensitization, as expected, Group B showed obvious symptoms of AR on the first day of nasal drip.

<table>
<thead>
<tr>
<th>Group</th>
<th>Average Eosinophil Count</th>
<th>Average Neutrophil Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.81 ± 0.33*</td>
<td>1.33 ± 0.41*</td>
</tr>
<tr>
<td>B</td>
<td>13.41 ± 2.37*</td>
<td>5.32 ± 2.25*</td>
</tr>
<tr>
<td>C</td>
<td>7.42 ± 2.35*</td>
<td>12.37 ± 2.58*</td>
</tr>
<tr>
<td>D</td>
<td>9.24 ± 1.39*</td>
<td>4.38 ± 2.34*</td>
</tr>
<tr>
<td>E</td>
<td>8.54 ± 1.47*</td>
<td>4.11 ± 2.36*</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation (SD), \( n = 20 \). Group A: normal control; Group B: allergic rhinitis (AR); Group C: AR + peptidoglycan; Group D: AR + peptidoglycan + pyrrolidine dithiocarbamate (PDTC); Group E: AR + peptidoglycan + beclomethasone. “*” indicates significant differences compared to Group A, and “#” indicates significant differences compared to Group B (\( P < 0.05 \)).
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stimulation, i.e., the rats showed clear symptoms of nose rubbing, sneezing, and rhinorrhea. Two days after nasal drip, the behavioral scores of Group B were also significantly higher than those of the other groups. Groups C, D, and E also showed nasal symptoms of AR during the stimulation phase, but the nasal symptom score was significantly lower than that of Group B. These results show that peptidoglycan, PDTD, and beclomethasone reduced the degree of AR symptoms.

Values are mean ± standard deviation (SD), n = 20. Group A: normal control; Group B: allergic rhinitis (AR); Group C: AR + peptidoglycan; Group D: AR + peptidoglycan + pyrrolidine dithiocarbamate (PDTC); Group E: AR + peptidoglycan + beclomethasone.

Changes in histomorphology

The nasal mucosa of Group A was normal pseudostratified ciliated columnar epithelium. The dense fibrous connective tissue of the submu-

cosa was thin. The mucosal epithelial structure was intact with neat arrangement, and the cilia thickness was consistent without observed inflammatory cell infiltration (Figure 1). The epithelium of the nasal mucosa in Group B was shedding and incomplete. Many lymphocytes and eosinophils infiltrated the lamina propria, indicating an obvious allergic injury. The nasal mucosa of Groups C, D, and E were thickened. The mucosal epithelium was exfoliated and incomplete after treating the AR rats with peptidoglycan and PDTC or beclomethasone; however, the damage to the mucosal epithelium was less than that of Group B. Moreover, the lamina propria of the mucous membrane showed a large amount of inflammatory cell infiltration, with an obvious expanded gland in Groups C, D, and E. The allergic damage was then alleviated.

Eosinophil and neutrophil counts

The mean eosinophil and neutrophil counts in Group B were significantly higher (16.6 times) than in Group A (P < 0.05) (Table 3). The quantities of eosinophils were significantly reduced after treating rats with peptidoglycan and PDTC or beclomethasone in comparison to Group B. In contrast, in comparison to Group B, the neutrophil count was significantly increased after allergen stimulation in Group C, while it was decreased in Groups D and E (P > 0.05).

Distribution and level of IL-12, IL-13, and IgE in nasal mucosa

The results showed that IL-12, IL-13, and IgE were mainly distributed in the pseudostratified ciliated columnar epithelium and glandular cells of the lamina propria of the nasal mucosa (Figure 2). The expression (200×) of IL-12, IL-13, and IgE in Group B was significantly higher than in Group A (P < 0.05). The expression of IL-12 in Group C was further elevated than that
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The expression of TLR2 in nasal mucosa

The expression of TLR2 in Group B was 2.2 times higher than in Group A (P < 0.05). The expression of TLR2 was further elevated in Groups C, D, and E in comparison to Group B (P < 0.05) (Figure 3).

Expression level of NF-κB in nasal mucosa

The NF-κB protein expression level in the nasal mucosa of AR rats was higher than in the control group (P < 0.05). The expression level of NF-κB was further increased after peptidoglycan stimulation but decreased after injecting rats with PDTC or beclomethasone (P < 0.05; Figure 4).

Discussion

The behavioral scores of AR rats were higher than those of the rats in the normal control group. The mucosal structure of rats in Group B was also seriously damaged. These results clearly confirm that the allergic reaction appeared in the AR rats with increased quantities of eosinophils and neutrophils, as well as increased expression of inflammatory factors [18].

The allergen, peptidoglycan, was used to stimulate the nasal mucosa in our study. The study carried out by Fu showed that TLR and NF-κB can be potential targets for the treatment of allergic diseases [19]. As a pattern recognition receptor, TLR is an important factor in the body’s activation of innate immunity and induction of adaptive immunity against pathogenic microorganisms through stimulation of NF-κB expression [20]. Our results show that

Table 4. Average optical density values of IL-12, IL-13, and IgE in nasal mucosa of groups A-E

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-12</th>
<th>IL-13</th>
<th>IgE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.09 ± 0.009015*</td>
<td>0.10171 ± 0.006957*</td>
<td>0.06217 ± 0.00401*</td>
</tr>
<tr>
<td>B</td>
<td>0.12378 ± 0.007633*</td>
<td>0.44242 ± 0.009588*</td>
<td>0.387 ± 0.005948*</td>
</tr>
<tr>
<td>C</td>
<td>0.19186 ± 0.007904*</td>
<td>0.14556 ± 0.005906*</td>
<td>0.217841 ± 0.006673*</td>
</tr>
<tr>
<td>D</td>
<td>0.14567 ± 0.006995*</td>
<td>0.2441 ± 0.006134*</td>
<td>0.24317 ± 0.00739*</td>
</tr>
<tr>
<td>E</td>
<td>0.15722 ± 0.008546*</td>
<td>0.26056 ± 0.009719*</td>
<td>0.2858 ± 0.00579*</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation (SD), n = 20. Group A: normal control; Group B: allergic rhinitis (AR); Group C: AR + peptidoglycan; Group D: AR + peptidoglycan + pyrrolidine dithiocarbamate (PDTC); Group E: AR + peptidoglycan + beclomethasone. * indicates significant differences compared to Group B, and # indicates significant differences compared to Group B (P < 0.05).
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expression of TLR2 and NF-κB increased significantly in the peptidoglycan group (Group C) compared to Group B, without peptidoglycan treatment, suggesting that peptidoglycan induces the expression of TLR2 and NF-κB and affects T cell differentiation and cytokine production by activating the TLR2/NF-κB signaling pathway.

The balance of Th1/Th2 cytokines plays a vital role in the pathogenesis of AR [21]. Th1 cytokine, IL-12, has been reported to inhibit IgE synthesis and mediate cellular immunity [22, 23]. This agrees with our results as IL-12 had a higher expression level in Group C than in Group B, but the level of IgE was lower in Group C. Th2 cytokine, IL-13, plays an important role in promoting the synthesis of IgE in B lymphocytes [24]. Increased secretion of IgE by B lymphocytes is associated with humoral immunity. These results indicated that humoral immunity was converted to cellular immunity after peptidoglycan stimulation, producing many neutrophils and reducing nasal mucosal damage in comparison to AR rats.

Lower expression levels of TLR2 and NF-κB were observed in rats treated with PDTC (Group D) and beclomethasone (Group E) than in Group C, indicating that PDTC and beclomethasone inhibit over-expression of TLR2 and NFκB, which resulted in reduced numbers of neutrophils, thus, preventing excessive increased cellular immunity in the nasal mucosa of AR rats.

Our experiments have shown that peptidoglycan acts on TLR receptors on the surface of effector cells and activates NF-κB through the TLR2/NF-κB signaling pathway, which further activates NF-κB transcription-related genes and stimulates the release of many Th1-related cytokines, such as IL-12, and inhibits the synthesis of Th2-related cytokines, such as IL-13. Increasing the Th1/Th2 ratio can correct the Th1/Th2 imbalance in the pathogenesis of AR and can convert humoral immunity into cellular immunity. NF-κB inhibitors and glucocorticoids can effectively reduce the expression of NF-κB and inhibit the excessive enhanced cellular immunity. Thus, the expression of NF-κB plays an important regulatory role in the pathogenesis of allergic diseases and in the transformation from humoral immunity to cellular immunity in the development of allergic diseases. The application of NF-κB inhibitors could provide a new way to prevent and treat allergic diseases.

Acknowledgements

We would like to acknowledge the funding from Yumiao Funding of Putian University (Project No. 2014058).

Disclosure of conflict of interest

None.

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