

Inhaled budesonide protects against chronic asthma-induced neuroinflammation in mouse brain



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ARTICLE INFO

Article history:

Received 17 March 2014

Received in revised form 5 June 2014

Accepted 10 June 2014

Keywords:

Budesonide

Asthma

Neuroinflammation

Toll-like receptor 4

NF- κ B

ABSTRACT

Chronic asthma is one of the most common respiratory diseases, characterized by airway inflammation. However, little is known whether asthma-induced airway inflammation might influence the brain. We found that chronic asthma not only resulted in peripheral inflammation, but also induced neuroinflammation which was characterized by microglial activations and increased levels of TNF α and IL-1 β in the hippocampus and prefrontal cortex. Simultaneously, we found that there was significant neuronal loss in the asthmatic mouse brain. Inhaled budesonide, the classic therapeutic drug for chronic asthma, could inhibit asthma-induced microglial activation, down-regulate TNF α and IL-1 β but up-regulate TGF β and IL-10 of mouse brain, and thereby attenuate neuronal loss. Further study showed that chronic asthma increased the expressions of TLR4 and p65/NF- κ B in the brain, which could be reversed by budesonide treatment. Therefore, the present study reveals that inhaled budesonide protects against asthma-induced neuroinflammation in mouse brain, which might be contributed to attenuate neuronal loss.

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1. Introduction

Asthma is one of the most common chronic diseases in the world, affecting over 300 million people (O'Laughlen and Rance, 2012). It is characterized by bronchoconstriction and airway hyper-responsiveness, followed by inflammatory manifestations in the respiratory system (Wanner and Mendes, 2010; Schuijs et al., 2013). Inflammation of the airway wall is a central characteristic of asthma. The release of mediators from the inflammatory cells including eosinophils, lymphocytes, mast cells and macrophages, has been proposed to contribute directly or indirectly to changes in airway structure and function (Vig et al., 2006; Krishnamoorthy et al., 2012; Mikalsen et al., 2014). Therefore, anti-inflammatory medications are the most important treatment options for people with asthma. Anti-inflammatory therapy prevents asthma attacks and works by reducing swelling and mucus production in the airways (Levy et al., 2012). Inhaled corticosteroids are the most effective medications to reduce airway inflammation and mucus production (Braga et al., 2005; Walsh, 2006; Anderson-James et al., 2013). Glucocorticosteroids prevent airway inflammation via inhibiting expressions of pro-inflammatory cytokines and chemokines (Hahn, 2012; Shahid, 2013).

Notably, in individuals with asthma, potential changes in the brain could occur as a consequence of hypoxia or inflammation that resulted from asthma (Rosenkranz and Davidson, 2009). It was found that 13 of 21 people with asthma had brain magnetic resonance imaging abnormalities (Parker et al., 2011). The high rate of incidence suggests that brain abnormalities are relatively common in patients with asthma. Moreover, increasing evidence has revealed that stress and mood disorders adversely affect people with inflammatory diseases such as asthma (Rosenkranz and Davidson, 2009; Busse, 2012). Another study led by researchers from the University of Wisconsin found that certain brain areas could cause worsening of asthma symptoms when subjected to stress (Busse, 2012). So, the brain-body link between asthma and anxiety is being studied (Rosenkranz et al., 2005; Galluccio et al., 2008). But until now, little is known exactly on what or how asthma-induced airway inflammation might influence the brain. Therefore, the present study was to establish a mouse model of chronic asthma and to investigate the impacts of asthma in the brain.

2. Experimental procedures

2.1. Animals

Two-month-old female BALB/c mice, weighing 18 g to 22 g, were obtained from Experimental Animal Center of Jiangsu. All animal experiments were carried out in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were randomly assigned to three groups: control groups with saline treatment ($n = 12$); asthma groups with saline treatment ($n = 14$); and

Abbreviations: (BALF), bronchial alveolar lavage fluid; (PFC), prefrontal cortex; (TLR4), Toll-like receptor 4; (HRP), horseradish peroxidase; (TNF α), tumor necrosis factor α ; (IL-1 β), interleukin-1 beta; (TGF β), transforming growth factor beta; (IL-10), interleukin-10.

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asthma mice treated with budesonide ($n = 14$). Mice were treated as described in detail below. All animals were treated according to protocols approved by Nanjing Medical University.

2.2. Sensitization and inhalational exposure

Allergic mouse models of asthma are generated as we previously reported (Guo et al., 2013). In general, the total schedule time is 12 weeks. After sensitized via 2 intraperitoneal injections of ovalbumin (10 μg) on days 0 and 14 of the experiment, starting on day 21, the mice were exposed to 1% aerosolized ovalbumin for 30 min a day, 3 days a week, for 9 weeks. Importantly, mice were exposed to 10% aerosolized ovalbumin for 30 min at the 9th, 18th and 24th times in order to induce aggravation.

2.3. Budesonide treatment

On day 21 after being sensitized, mice were treated with budesonide 1 h before OVA challenge or aggravation by intranasal administration (25 μl each time, 350 $\mu\text{g}/\text{kg}$), 3 days a week, for 9 weeks. Mice subjected to allergen challenge without budesonide treatment were treated with saline.

2.4. Preparation of bronchial alveolar lavage fluid (BALF)

The mice were anesthetized and placed in a supine position with the head tilted back, and then the trachea was cannulated. The lungs were lavaged three times with 0.3 mL of sterile PBS. The BALF was immediately centrifuged (5 min, 4 $^{\circ}\text{C}$, 3000 rpm) to separate the cells from the supernatant. The noncellular supernatants of BALF were stored and analyzed with ELISA kit.

2.5. Western blotting

The prefrontal cortex (PFC) and hippocampus tissues of mice were homogenized and solubilized in lysis buffer (Bio-Rad, Hercules, CA, USA). Proteins were separated on Tris–HCl polyacrylamide gels (Bio-Rad) and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). After blocking, the blots were incubated with anti-Toll-like receptor 4 (TLR4) (1:800, CST), anti-p65 (1:400, Santa Cruz) and anti-cd11b (1:1000, Abcam) antibodies in TBST overnight at 4 $^{\circ}\text{C}$, and then with horseradish peroxidase (HRP) conjugated secondary antibodies (Santa Cruz, CA, USA). Immunoreactive bands were detected by enhanced chemiluminescence plus detection reagent (Pierce, Rockford, IL, USA), and analyzed using an Omega 16ic Chemiluminescence Imaging System (Ultra-Lum, CA, USA).

2.6. Immunocytochemistry

After animals were perfused with 4% paraformaldehyde, brains were dissected out and maintained in 4% paraformaldehyde overnight. Brains were cryopreserved in 30% sucrose in phosphate buffered solution and then stored at -70°C until used. Parallel series of 30- μm -thick coronal sections were obtained in a freezing microtome. Sections were rinsed in phosphate buffer. Every sixth section was kept for immunohistochemistry. Tissue peroxidase was inactivated by incubating in 3% hydrogen peroxide in PBS for 30 min. After three washes in PBS sections were incubated for 2 h in blocking solution (bovine serum albumin in 0.3% Triton X-100 in PBS), the sections were incubated overnight with mouse monoclonal anti-cd11b (1:400, Abcam) and rabbit polyclonal anti-NeuN (1:200, Millipore). Then, the sections were incubated with HRP-conjugated secondary antibody (1:800, Chemicon) for 1 h. Positive cells were visualized by incubation in DAB. Control staining was performed without the primary antibodies. The number of NeuN-positive cells was assessed using Stereo Investigator 7, an unbiased stereological procedure with an optical fractionator (MicroBrightField Inc, Williston, VT). The sampling scheme was designed to have coefficient of error (CE) less than 10% in order to get reliable results. All stereological analyses were performed under the 200 $\times$ objective of an Olympus microscope.

2.7. Measurements of TNF α , IL-1 β , TGF β and IL-10 levels

The levels of tumor necrosis factor α (TNF α), interleukin-1 beta (IL-1 β), transforming growth factor beta (TGF β) and interleukin-10 (IL-10) were determined by ELISA according to the protocol of the manufacturer (Mouse Immunoassay Kits, R&D, USA). Absolute cytokine concentrations were determined by comparison to a standard curve.

2.8. Statistical analysis

All values were expressed as means $\pm$ SEM. The significance of the difference between control and samples treated with various drugs was determined by one-way ANOVA, followed by the post hoc least significant difference test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Chronic asthma induces inflammatory response in mouse airway

TNF α and IL-1 β levels were determined from the BALF samples by ELISA. In asthmatic mice, the mean concentration of TNF α and IL-1 β was significantly increased to 634 ± 114 pg/mg protein, and 184 ± 48 pg/mg protein, respectively. Intranasal administration of budesonide

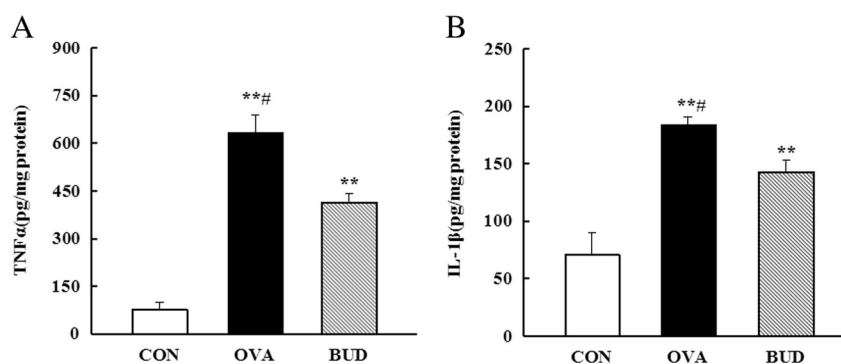


Fig. 1. Effects of budesonide on chronic asthma-induced increases of pro-inflammatory factors in BALF. A. The levels of TNF α in BALF of three groups; B. The levels of IL-1 β in BALF of three groups. $n = 12$ –14. Data represent the mean $\pm$ SEM. ** $p < 0.01$ vs. CON group; # $p < 0.05$ vs. BUD group. CON: control group; OVA: asthma model group; BUD: budesonide-treated group.

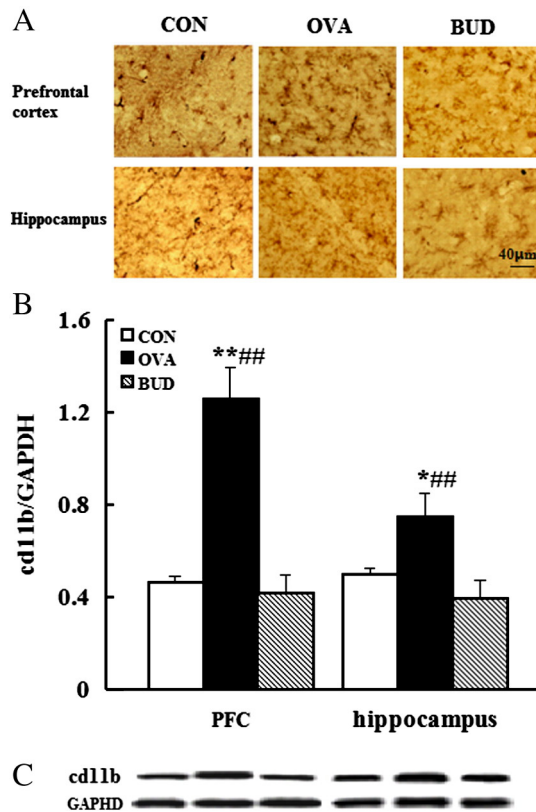


Fig. 2. Effects of budesonide on chronic asthma-induced microglial activations. A. Representative micrographs of cd11b-positive cells in the different groups; B. The expressions of cd11b protein in three groups ($n = 4$). Data represent the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. CON group; ### $p < 0.01$ vs. BUD group. CON: control group; OVA: asthma model group; BUD: budesonide-treated group; PFC: prefrontal cortex.

could decrease the two pro-inflammatory factor levels (TNF α : 412 ± 96 pg/mg protein; IL-1 β : 142 ± 32 pg/mg protein), confirming that budesonide could attenuate airway inflammation (Fig. 1).

3.2. Budesonide reverses chronic asthma-induced microglial activations in mouse brain

Microglial activation in inflammatory response is characterized by a robust increase of number and volume. In our study, cd11b was to be utilized as a marker of activated microglia. As shown in Fig. 2A, the cell body of cd11b positive cells was much larger in PFC and the hippocampus of asthmatic group. In the mouse PFC and hippocampus of asthmatic mice, the cd11b expressions were robustly increased. Treatment with budesonide could reverse the increases of cd11b expression induced by asthma. Similarly, budesonide treatment inhibited asthma-induced microglial activations (Fig. 2B).

3.3. Budesonide inhibits inflammatory responses caused by chronic asthma in mouse brain

To further investigate the impacts of budesonide in mouse brain, we determined the levels of TNF α , IL-1 β , TGF β and IL-10 in the mouse PFC and hippocampus. In the mouse PFC, the levels of TNF α , IL-1 β , TGF β and IL-10 were up-regulated in asthmatic group, budesonide treatment could down-regulate TNF α , IL-1 β and TGF β , however, up-regulate IL-10. In the mouse hippocampus, asthma increased the levels of TNF α and IL-1 β , and decreased IL-10, but failed to affect TGF β . Budesonide treatment also could significantly inhibit the asthma-induced up-regulations of TNF α and IL-1 β , and remarkably elevate the levels of the anti-inflammatory factors, TGF β and IL-10 (See Fig. 3).

3.4. Budesonide inhibits TLR4-NF κ B signal pathway in mouse brain

Activation of the Toll-like receptor 4 (TLR4)-nuclear factor κ B (NF κ B) signaling axis is associated with inflammation. Therefore, we analyzed the effects of budesonide on TLR4-NF κ B signaling transduction. As shown in Fig. 4, in mouse PFC, asthma resulted in up-regulations of TLR4 to 275%, and p65/NF κ B to 143%, compared with control mice. Intranasal administration of budesonide significantly reduced the expressions of TLR4 and p65/NF κ B. In the mouse hippocampus, we also found that budesonide treatment reversed asthma-induced increases of TLR4 and p65/NF κ B.

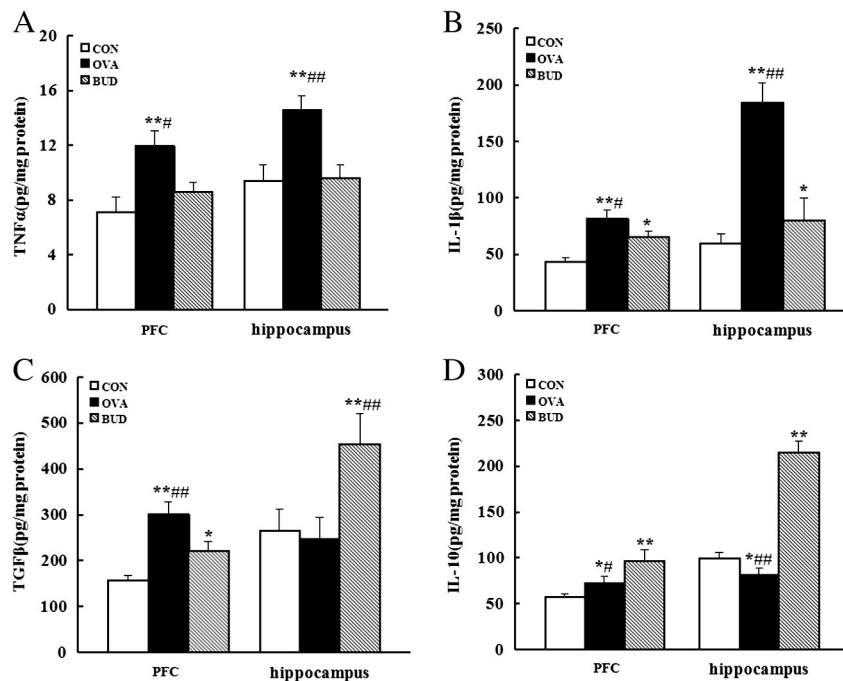


Fig. 3. Effects of budesonide on the levels of TNF α (A), IL-1 β (B), TGF β (C) and IL-10 (D). Data represents the mean $\pm$ SEM. $n = 12-14$, * $p < 0.05$, ** $p < 0.01$ vs. CON group; # $p < 0.05$, ## $p < 0.01$ vs. BUD group. CON: control group; OVA: asthma model group; BUD: budesonide-treated group; PFC: prefrontal cortex.

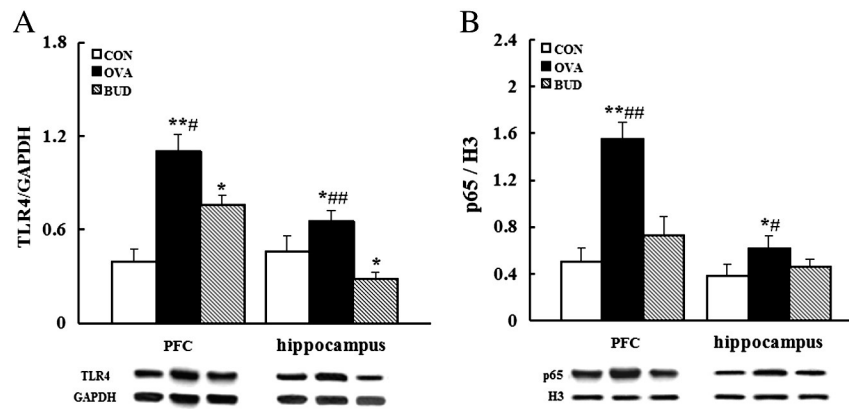


Fig. 4. Effects of budesonide on the expressions of TLR4 (A) and p65/NF- κ B (B) in the mouse PFC and hippocampus. Data represents the mean $\pm$ SEM, $n = 4$, * $p < 0.05$, ** $p < 0.01$ vs. CON group; # $p < 0.05$, ## $p < 0.01$ vs. BUD group. CON: control group; OVA: asthma model group; BUD: budesonide-treated group; PFC: prefrontal cortex.

3.5. Budesonide attenuates chronic asthma-induced neuronal damages in mice

Finally, we observed the changes of NeuN-positive cell in the mouse PFC and hippocampus, in order to evaluate the impacts of chronic asthma and budesonide treatment in neuronal damage. As shown in Fig. 5, we found that chronic asthma decreased the number of NeuN-positive neuronal cells both in the hippocampus and PFC. Intranasal administration of budesonide could rescue the NeuN-positive cells in both brain regions.

4. Discussion

Chronic airway inflammation is one of the main features of asthma (Nakagome and Nagata, 2011). The pro-inflammatory mediators can induce airway hyper-reactivity and airway wall remodeling (Naik and Wala, 2013; van den Berge et al., 2013). Additionally, these mediators may further help in the recruitment and activation of other inflammatory cells, thus augmenting the inflammatory cascade (Spinazzi et al., 2008). In the present study, we found that the levels of TNF α and IL-1 β from BALF were significantly increased in asthmatic mice, and treatment with budesonide could attenuate airway inflammation.

Many reports implicated hypoxia-elicited inflammation during hypoxic conditions in the outcomes of various human diseases such as ischemia and peripheral and autonomic dysfunctions (Eltzschig et al., 2013). On the one hand, inflammatory diseases are characterized by tissue hypoxia, and stabilization of hypoxia-dependent transcription factors, such as hypoxia-inducible factor (HIF) (Rosenberger et al., 2009). For example, intestinal inflammation is characterized by severe mucosal

hypoxia and concomitant HIF stabilization (Grenz et al., 2012). Similarly, airway inflammation that occurs during asthma results in the stabilization of HIF1 α . On the other hand, diseases primarily caused by oxygen deficiency are characterized by secondary inflammation (Mamlouk and Wielockx, 2013). For example, brain ischemia is characterized by inflammatory responses that subsequently lead to brain dysfunction (Kraft et al., 2013). It is known that inflammation alters neurovascular functions, leading to increased blood–brain barrier permeability, decreased nutrient supply, and faulty clearance of toxic molecules. Increased pro-inflammatory factors in blood such as IL- β and TNF α , directly or indirectly transported to the brain, are contributed to increased brain damage (Kraft et al., 2013). Moreover, recruitment of peripheral monocytes can directly increase inflammatory response in the brain, resulting in impaired blood flow recovery and leading to augmented neuronal loss. Coexisting diseases such as atherosclerosis, hypertension and diabetes all involve peripheral inflammation, which is likely to influence stroke outcome (Shay and Celeste, 2012). Also, peripheral inflammation may be associated with altered brain excitability and increased sensitivity to seizures (Vezzani et al., 2011), and enhance the damage to dopaminergic neurons in the Parkinson's disease animal model (Engler et al., 2009). Therefore, peripheral inflammation produces a synergistic aggravation in damage induced by any insult in the brain, resulting in a greater loss of neurons. Microglia is a type of glial cells that are the resident macrophages in the brain, and thus act as the first and main form of active immune defense in the central nervous system (Harry, 2012; Cunningham, 2013; Suzumura, 2013). However, activated microglia would release inflammatory cytokines and increase neuronal injury (Skaper et al., 2012; Das Sarma, 2014). In the present study, we found that the cell body of cd11b positive cells was much

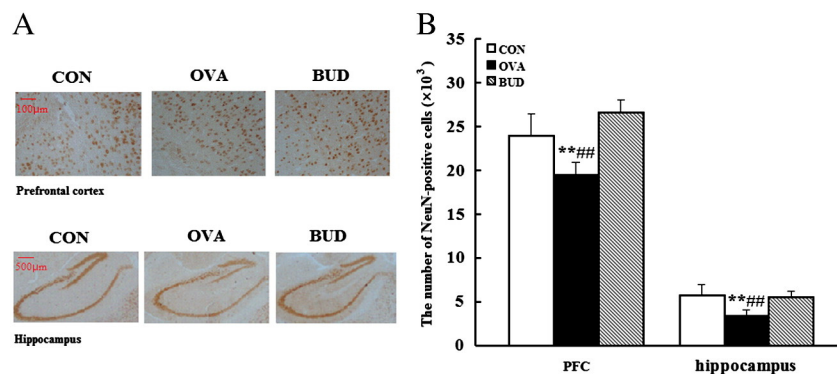


Fig. 5. Effects of budesonide on chronic asthma-induced losses of NeuN-positive cell in the mouse PFC and hippocampus. A. Representative micrographs of NeuN-positive cells in the different groups; B. The numbers of NeuN-positive cells in the mouse PFC and hippocampus ($n = 5$). Scale bar: 100 μ m (PFC), 500 μ m (hippocampus). Data represents the mean $\pm$ SEM, ** $p < 0.01$ vs. con group; ### $p < 0.01$ vs. bud group. CON: control group; OVA: asthma model group; BUD: budesonide-treated group; PFC: prefrontal cortex.

larger in the PFC and hippocampus of asthmatic group, and the cd11b expressions were robustly increased. Simultaneously, there were increases of TNF α and IL-1 β in the PFC and hippocampus of asthmatic mice. Budesonide treatment could strongly inhibit microglial activation, and decrease TNF α and IL-1 β levels, but increase levels of the anti-inflammatory factors, TGF β and IL-10. In the present study, we first revealed that chronic asthma-induced peripheral inflammation could exacerbate inflammatory injury in the brain, and found that anti-inflammatory treatment with budesonide could effectively ameliorate chronic asthma-induced neuroinflammation.

It has been suggested that abnormal activation or deregulation of hypoxia-induced transcriptional pathways may be responsible for inflammation pathogenesis (Grenz et al., 2012). Increasing evidence indicates that hypoxia-inducible factor 1 α (HIF-1 α) and NF κ B are linked in a regulatory loop that confirms the implication of hypoxia in inflammation (Mamlouk and Wielockx, 2013). Asthma-induced airway inflammation and hypoxia could result in the stabilization of hypoxia-inducible factor 1 α (HIF-1 α), and thereby increase the levels of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF α) and interleukin-1 β (IL-1 β). Toll-like receptor 4 (TLR4) is the receptor for the endotoxin of gram-negative bacteria. In addition to bacterial endotoxin, TLR4 can also be activated by endogenous molecules or “danger signals” released during tissue injury, such as inflammatory factors (Zhou et al., 2014). The increased pro-inflammatory factors by chronic asthma could activate TLR4 as endogenous molecules. Subsequently, the activated TLR4 led to activations of transcription factors such as AP-1 and NF- κ B, and resulted in the further production of pro-inflammatory cytokines (Tewari et al., 2012). Therefore, our results suggest that up-regulations and activations of TLR4 and NF κ B signaling have been implicated in the development of adaptive immune responses in chronic asthma. Glucocorticoids, acting through glucocorticoid receptors, negatively modulate the transcriptional activity of genes encoding pro-inflammatory proteins such as cytokines, chemokines and adhesion molecules, and are an anti-inflammation drug for asthma. In the present study, we found that chronic asthma resulted in up-regulations of TLR4 and p65/NF κ B in mouse brain. Budesonide treatment could reverse chronic asthma-induced increases of TLR4 and p65/NF κ B, which might be contributed to budesonide-mediated anti-neuroinflammatory effects.

Accordingly, we found the neuronal loss in the asthmatic mouse PFC and hippocampus, indicating that chronic asthma could induce brain damage, due to hypoxia or inflammation. Inhaled budesonide could reverse the neuronal loss. So, our findings suggest that budesonide might play neuroprotective roles via its anti-inflammatory action.

In conclusion, the present study revealed for the first time that chronic asthma resulted in neuronal damage, partly due to neuroinflammation. Inhaled budesonide could protect against neuroinflammation, which might be contributed to attenuate brain injury in asthmatic mice.

Acknowledgment

This study was supported by grants from the National Natural Science Foundation of China (no.81273495) and the Major Project of Jiangsu Provincial Department of Education (no.12KJA310002).

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