

Chronic asthma results in cognitive dysfunction in immature mice



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ABSTRACT

Asthma is the most common chronic childhood illness today. However, little attention is paid for the impacts of chronic asthma-induced hypoxia on cognitive function in children. The present study used immature mice to establish ovalbumin-induced chronic asthma model, and found that chronic asthma impaired learning and memory ability in Morris Water Maze test. Further study revealed that chronic asthma destroyed synaptic structure, impaired long-term potentiation (LTP) maintaining in the CA1 region of mouse hippocampal slices. We found that intermittent hypoxia during chronic asthma resulted in down-regulation of c-fos, Arc and neurogenesis, which was responsible for the impairment of learning and memory in immature mice. Moreover, our results showed that budesonide treatment alone was inadequate for attenuating chronic asthma-induced cognitive impairment. Therefore, our findings indicate that chronic asthma might result in cognitive dysfunction in children, and more attention should be paid for chronic asthma-induced brain damage in the clinical therapy.

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Introduction

Asthma is the most common chronic childhood illness today (Yock et al., 2010). Both the number of children diagnosed with asthma and the severity of asthma have increased rapidly in recent years (Gozde et al., 2011). Asthma is an inflammatory disease that affects the airways. During an asthma attack, muscles that are around the airways tighten, which causes swelling of the airways' linings. The swelling allows less oxygen to be taken in by the body and used by vital organs. A long period of time without enough oxygen can affect brain function. Severe asthma can cause some degree of diffuse cerebral hypoxia (Brannan and Loughheed, 2012). If a child were to have a severe asthma attack and not receive adequate care in a certain window of time, the child could experience an anoxic insult including lack of oxygen to the brain (de Moraes et al., 2012).

Oxygen is vital to maintain the normal functions of almost all the organs, especially the brain which is one of the heaviest oxygen consumers in the body. The importance of oxygen to the brain is not only reflected in its development, but also depicted in various pathological processes of many cerebral diseases (Boroujerdi et al.,

2012; Hummler et al., 2012). Decreases in oxygen supply to certain brain regions will result in memory impairments along with other deficits. Hence, a child could experience cognitive delay due to the lack of oxygen to the brain. There have been many studies focusing on the effects of stroke, trauma, and as well as sleep apnea syndrome on learning and memory (Cengiz et al., 2011; Dore-Duffy et al., 2011; Stowe et al., 2011). However, the effects of chronic asthma-induced intermittent hypoxia on cognition of children remain unclear. Therefore, the present study used immature mice to establish chronic asthma model, by which the impacts of asthma-induced brain hypoxia on learning and memory were investigated. Moreover, the mechanisms underlying chronic intermittent hypoxia on cognition were elucidated too.

Materials and methods

Animals

Twenty-to-22-day-old female BALB/c mice, weighing 12 g to 15 g, were obtained from the Experimental Animal Center of Jiangsu. Mice were housed with free access to food and water in a room with an ambient temperature of 22 ± 2 °C and a 12:12 h light/dark cycle. All 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; asthmatic groups with saline treatment; asthmatic mice treated with budesonide (treatment details described as in the following).

Abbreviations: HIF-1 α , hypoxia induced factor 1 α ; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; fEPSP, field excitatory postsynaptic potential; HFS, high-frequency stimulation; LTP, long-term potentiation; BALF, bronchial alveolar lavage fluid; HE, hematoxylin and eosin; PFA, paraformaldehyde; VEGF, vascular endothelial growth factor; GPR124, G protein-coupled receptor 124; MWM, Morris Water Maze; IEG, immediate early gene; DG, dentate gyrus.

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Sensitization and inhalational exposure

Allergic mouse models of asthma are generated by first sensitizing animals to a foreign protein, most commonly ovalbumin. Then, the animal receives a further antigen exposure either directly to the lungs in the form of an aerosol. This elicits an inflammatory reaction in the lungs characterized by an influx of eosinophils, epithelial thickening, and airway hyperresponsiveness (AHR). Mice were sensitized via 2 intraperitoneal injections of 10 μ g of ovalbumin (grade V, $\geq 98\%$ pure, Sigma, St. Louis, Missouri, USA) with alum adjuvant on days 0 and 14 of the experiment. Starting on day 21, the mice, housed in whole-body exposure chambers, 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 aggravate condition. The temperature was kept at 20 °C to 25 °C and the relative humidity at 40% to 60%. An outline of the study procedures and time intervals is given in Fig. 1. The animals were subjected to Morris Water Maze test during the last week and were sacrificed for analysis on day 84.

Budesonide treatment

For mild chronic asthma, low-dose corticosteroids such as budesonide are recommended for preventing asthma symptoms due to its anti-inflammatory action. Micronized dry powder of budesonide was dissolved in 70% ethanol and diluted in sterile normal saline on the experimental day. Budesonide was given 1 h before OVA challenge or aggravation by intranasal administration (25 μ l each time, 350 μ g/kg) according to the treatment schedule in Fig. 1. Mice subjected to allergen challenge without budesonide treatment were treated with saline according to the treatment schedule in Fig. 1.

Airway inflammation analysis

The mice were anesthetized and placed in the 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 bronchial alveolar lavage fluid (BALF) was immediately centrifuged (5 min, 4 °C, 151 g/min). Cell pellets were resuspended in 1 ml PBS for total and differential cell counting. Differential cell counting was performed on hematoxylin and eosin (HE)-stained cytopspins. On each cytopspin 200 to 500 cells were counted. Subsequent to lavage, the lungs were isolated and instilled with 0.4 ml of 4% paraformaldehyde (PFA) and placed in PFA overnight for histology. PFA-fixed lung sections were stained with HE stain. Six to eight slices per mouse were used for evaluation.

Morris Water Maze

The water maze consisted of a black pool (100 cm in diameter, 75 cm high, bottom 45 cm above floor level) filled with water (opaque

with ink) and a black platform (10 cm in diameter, 50 cm high) submerged 1 cm below the water surface. The water was maintained at 22 ± 2 °C, and the platform was placed in either of four virtual quadrants at 20 cm from the sidewall. The movements of mice were recorded with a video camera connected to a computer. Data were analyzed using a tracking program (version 2.5.2, XinTianDi Technology, Beijing, China). Tests were conducted between 0800 and 1300 h. One day before training, the mice were allowed to swim for 2 min. For learning, the offspring were given three trials on each day for 4 consecutive days. Each mouse was placed at one of the other three starting points that were used in a pseudorandom order so that each position was used once in each block of three trials. If the mice failed to find the escape platform within 60 s, the researcher would guide them to the platform where they were allowed to remain for 10 s. A 1-h interval was imposed before the beginning of the next trial. The platform location was not changed during the learning period. The amount of time the mice spent in looking for the submerged platform (escape latency) was recorded. One day after the learning period, the animals were subjected to a single 60-s probe test in which the platform was removed from the pool. The length of time that mice took to find and stayed at the designated quadrant was analyzed.

In vitro electrophysiology

The hippocampus was removed from the brains and sliced at 400 μ m using a vibratome, and then placed in a holding chamber for 1 h at room temperature. Three slices from each mouse were then transferred to the recording chamber. The slices were maintained at 33.5 ± 0.5 °C and continuously superfused with artificial cerebrospinal fluid (ACSF, 1.3–1.5 ml/min) that had been saturated with 95% O₂ and 5% CO₂ mixed gas. The composition of the ACSF (pH 7.4) is (in mM): 127 NaCl, 4.7 KCl, 12 NaH₂PO₄, 12 MgCl₂, 2.5 CaCl₂, 220 NaHCO₃, and 10 mM glucose. Field excitatory postsynaptic potential (fEPSP) of CA1 was recorded from the stratum pyramidale with a glass pipette filled with 2% pontamine sky blue and 0.5 M sodium acetate. The stimulating electrode was placed on a Schaffer collateral of CA3. A single test stimulus (200 s) was applied at intervals of 1 s and the stimulus intensity was set at a level when a population spike of 60–70% of the maximum was evoked. High-frequency stimulation (HFS) (100 Hz, 100 pulses) was applied to induce long-term potentiation (LTP). Before the induction of LTP, a baseline fEPSP was recorded for 20 min.

Electron microscopy

One mm³ CA3 from the right hippocampus of the three groups was dissected. The tissue was fixed in 2.5% glutaraldehyde for 1 h. After being washed, sections were post-fixed in a solution of 1% osmium tetroxide for 1 h, dehydrated through a graded series of ethanol, and embedded in an admixture of acetone and epon resin (1:1) for 2 h, and then in epon resin for 2 h. Blocks were polymerized at 60 °C for 48 h. The pyramidal layer of the CA3 was selected precisely

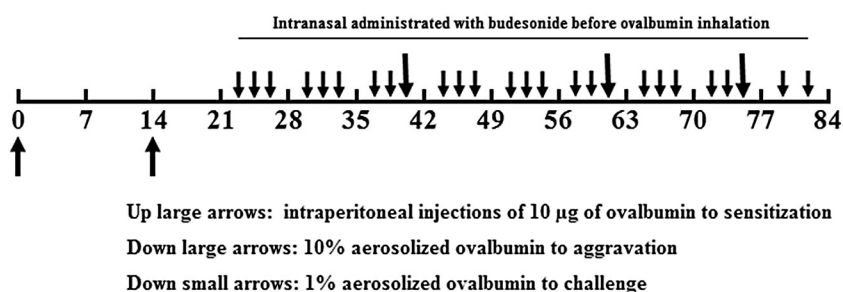


Fig. 1. Procedures for establishing chronic asthma models. The down small arrows indicated mice were exposed to 1% aerosolized ovalbumin to challenge; the down large arrows indicated that the mice were exposed to 10% aerosolized ovalbumin to aggravation; the up large arrows indicated the mice were sensitized via intraperitoneal injections of 10 μ g of ovalbumin; budesonide was given 1 h before OVA challenge or aggravation by intranasal administration.

after 1% Toluidine Blue staining. CA3 of ultrathin sections of silver interference color were cut and collected on 2×1 mm slot. Sections were stained with 2% uranyl acetate (20 min) followed by lead citrate (15 min), and examined with an electron microscope (Zeiss EM-9S) at a magnification of 20,000. Ten digital images of the sections were acquired along the axis of the cell layer in one mouse. On the digital images, synapses were identified by the presence of both pre- and postsynaptic densities, with an associated vesicle cloud in the presynaptic button.

Quantitative real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from mouse hippocampus using Trizol reagent (Invitrogen Life technologies, USA) followed by treatment with RNase-free DNaseI (Invitrogen Life technologies, USA). Reverse transcription was performed with the One-Step RNA-PCR Kit (Takara), according to the manufacturer's protocol. PCR primers were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene (forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCTGTTGCTGTA-3'); hypoxia induced factor 1 α (HIF-1 α , forward 5'-ATGGTAGGGTAGCCACAATTGCAC-3' and reverse 5'-CTTCATGATCCAGGCTTAAC-3'); c-fos (forward 5'-AAACCGCATGGA GTGTGTTGTTCC-3' and reverse 5'-TCAGACCACCTCGACAATGCATGA-3'); vascular endothelial growth factor (VEGF, forward 5'-GCACATAGG AGAGATGAGCTTCC-3' and reverse 5'-CTCCGCTCTGAACAAGGCT-3'). Quantitative real-time PCR was performed on a 7300 Real-Time PCR System using the SYBR Green PCR Master Mix. After the addition of primers, and template DNA to the master, PCR thermal cycle parameters were as follows: 95 °C for 10 min, 40 cycles of 60 °C for 60 s, and 95 °C for 15 s, and a melting curve from 60 °C to 95 °C to ensure amplification of a single product. The samples were run in triplicate and the experiments were repeated at least three times. The GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All values were expressed as fold increase or decrease relative to the expression of GAPDH. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_T : cycle number at which each PCR reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔC_T) between the C_T value of the sample for the target gene and the mean C_T value of that sample for the endogenous control (GAPDH). Relative expression was calculated as the difference ($\Delta\Delta C_T$) between the ΔC_T values of the test sample and of the control sample. Relative expression of genes of interest was calculated and expressed as $2^{-\Delta\Delta C_T}$, in which $2^{-\Delta\Delta C_T} = [(C_T \text{ target gene} - C_T \text{ endogenous control}) \text{ test sample} - (C_T \text{ target gene} - C_T \text{ endogenous control}) \text{ control sample}]$.

Western blotting

Mouse hippocampus tissue was homogenized and solubilized in lyses buffer (Bio-Rad, Hercules, CA, USA). Protein concentrations were determined using the Micro BCA Kit (Pierce Biotechnology, Rockford, IL). Proteins were separated on Tris-HCl polyacrylamide gels (Bio-Rad) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking, the blots were incubated with anti-HIF-1 α (1:800, Cell Signaling Technology Inc), anti-c-fos (1:400, Santa Cruz, CA, USA) and anti-G protein coupled receptor 124 (GRP124, 1:1000, Cell Signaling Technology Inc) antibodies in TBST overnight at 4 °C, and then with horseradish peroxidase (HRP) conjugated secondary antibodies (Santa Cruz, CA, USA). Immunoreactive bands were detected by enhanced chemiluminescence (ECL) plus detection reagent (Pierce, Rockford, IL, USA), and analyzed using an Omega 16ic Chemiluminescence Imaging System (Ultra-Lum, CA, USA).

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- μ m-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-Arc antibody (1:400, Santa Cruz, CA, USA) and rabbit polyclonal anti-Ki67 (1:500, Cell Signaling Technology Inc). Then, incubated with secondary antibody, goat anti-mouse TRITC (red, 1:200, Chemicon) or HRP-conjugated secondary antibody (1:800, Chemicon) for 1 h. Ki67 immunoreactivity was visualized by incubation in DAB. Control staining was performed without the primary antibodies. For cell counting, every sixth hippocampal section was kept for immunohistochemistry. For each mouse, ten non-overlapping sections were analyzed, the number of immunopositive cells was determined from the values obtained from the ten brain sections. The mean number of immunopositive cells in the ten sections of each mouse was taken as $n = 1$. The number of immunopositive cells was expressed as the mean $\pm$ SEM for each group ($n = 5-6$).

Measurements of serum VEGF levels

Serum VEGF levels were measured using Mouse VEGF Immunoassay kits (R&D, USA) according to the manufacturer's recommendations.

Statistical analysis

All values are expressed as the means $\pm$ S.E.M. Comparisons between groups were made using a two-tailed, unequal-variance Student's *t*-tests. Differences were considered significant at $P < 0.05$.

Results

Chronic asthma results in inflammatory response in mouse airway

HE staining showed that there was evident allergen challenge induced inflammation in the airway of asthmatic mice, including eosinophilic and mononuclear cell inflammation, mucus occlusion of airways and widespread deposition of subepithelial collagen. There was typical airway remodeling with goblet cell hyperplasia, epithelial hypertrophy, and either subepithelial or peribronchiolar fibrosis (Fig. 2A). The bronchus basilar membrane of asthmatic mice was significantly thicker than that of control groups. Intranasal administration of budesonide could relieve the basilar membrane hypertrophy (Fig. 2B).

The numbers of eosinophils and neutrophils in BALF were remarkably increased in asthmatic mice compared with saline groups. Budesonide treatment significantly inhibited the infiltration of inflammatory cells (Fig. 2C). Also, the total numbers in BALF of asthmatic groups were increased robustly as compared to the control groups (Fig. 2D). Budesonide treatment could prevent the cell increase in BALF, confirming that budesonide could inhibit airway inflammation.

Chronic asthma impairs learning and memory ability of immature mice in Morris Water Maze test

Fig. 3A showed the learning curves for latency period in Morris Water Maze over the course of 4-day training. Mean latency periods were similar for three groups at the first training day. From the second day to the fourth day, the latency periods of the control groups were significantly shorter than the ova groups. Budesonide treatment

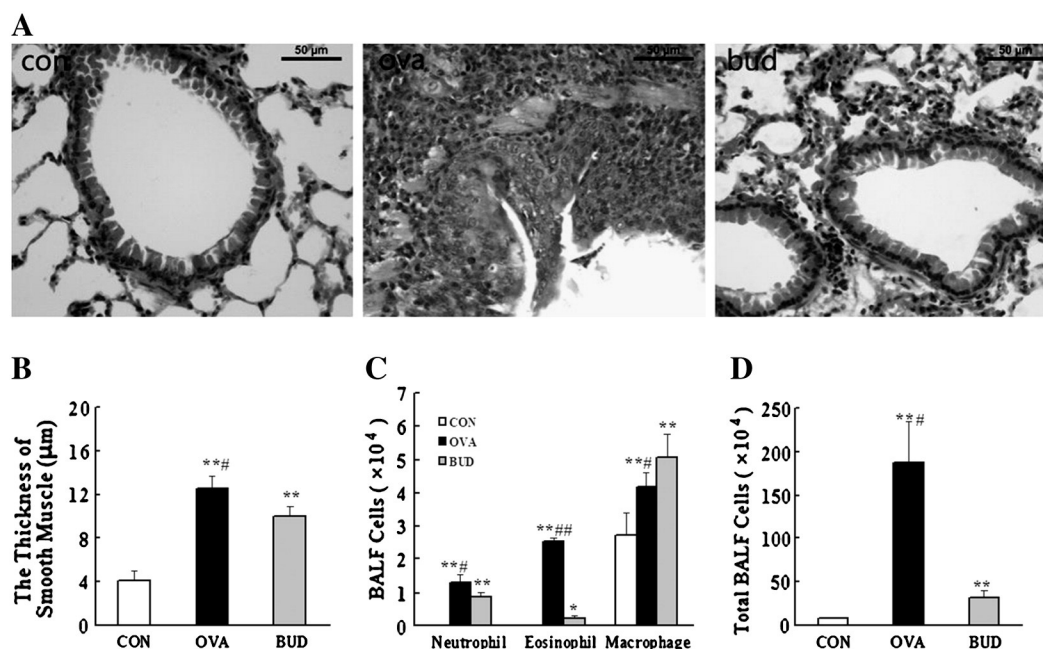


Fig. 2. Effects of chronic asthma on the pathology of lung and the number of inflammatory cell in BALF. A. Representative HE staining photos in airway of three groups. B. The thickness of airway smooth muscle in three groups ($n = 5$). C. Numbers of neutrophil, eosinophil and microphage in BALF of three groups ($n = 8$). D. The total cell numbers in BALF ($n = 8$). Data represent the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. CON group; # $p < 0.05$, ## $p < 0.01$ vs. BUD group. CON or con: control group; OVA or ova: asthma model group; BUD or bud: budesonide-treated group.

failed to ameliorate asthma-induced damage in learning ability. In the probe test, which was a trial without the platform, the control groups rapidly found the position where the platform had been, and their escape latency was significantly shorter than their previously acquired level. Moreover, during the probe trial, the time that the control mice stayed in the targeted quadrant was significantly longer than the asthmatic groups and budesonide-treated groups (Fig. 3B).

Chronic asthma impairs LTP maintaining without affecting LTP induction

To further investigate the mechanism of learning of memory, we tested the induction of LTP in the CA1 region of mouse hippocampal slices. LTP is a cellular model of spatial learning and memory and was measured by the changes in fEPSPs before and after HFS in the CA1 area of the hippocampus. After a stable baseline was established, LTP was induced by the delivery of HFS (100 Hz for 1 s) and recorded for 60 min. Under the control group, the fEPSP slope was induced to $148 \pm 6\%$, $142 \pm 6\%$, $139 \pm 7\%$, of the baseline at peak, from 30 min

to 60 min after HFS (Figs. 4A, E). In the asthmatic groups, the fEPSP slope was induced to $152 \pm 6\%$, but a dramatic reduction in the fEPSP slope to $105 \pm 11\%$, $100 \pm 8\%$ of baseline at peak, 30 min and 60 min after HFS was observed, indicating that chronic asthma-induced hypoxia impaired LTP maintaining without affecting induction of LTP (Figs. 4B, E). In budesonide-treated groups, the fEPSP slope was induced to $150 \pm 7\%$, but fEPSP slope was maintained to $122 \pm 6\%$, $127 \pm 8\%$ (Figs. 4C, E). We also examined the basal transmission of synapse in the CA1 region by plotting the fractional changes in EPSP slope induced by the stimulus at 0.2–1.2 mA. As shown in Figs. 4D and F, the slopes of EPSP in the asthmatic groups were significantly decreased compared with the control groups. Intranasal administration of budesonide could rescue chronic asthma-induced descent of EPSP slope in mice.

Chronic asthma destroys hippocampal ultra-structure of mice

To understand the mechanisms underlying impaired synaptic function in asthmatic mice, synapse morphology was analyzed by

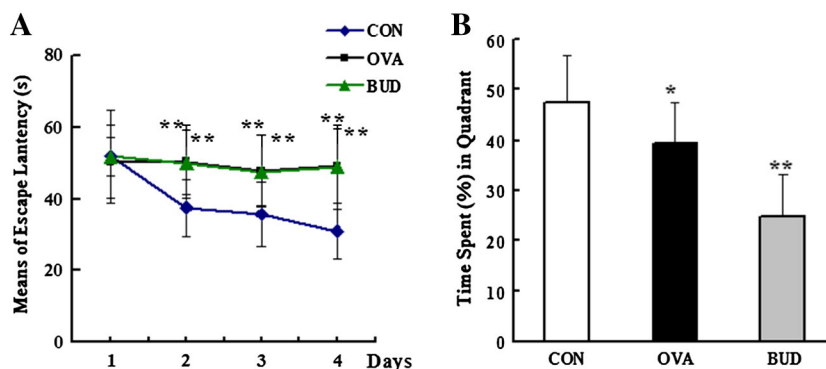


Fig. 3. Effects of chronic asthma on the learning and memory ability of mice in the water maze test. A. Latency periods to find the hidden platform over 4 consecutive training days were shown. B. The percentage of time spent in target quadrant in three groups. Data represent the mean $\pm$ SEM. $n = 20$, * $p < 0.05$, ** $p < 0.01$ vs. CON group. CON: control group; OVA: asthma model group; BUD: budesonide-treated group.

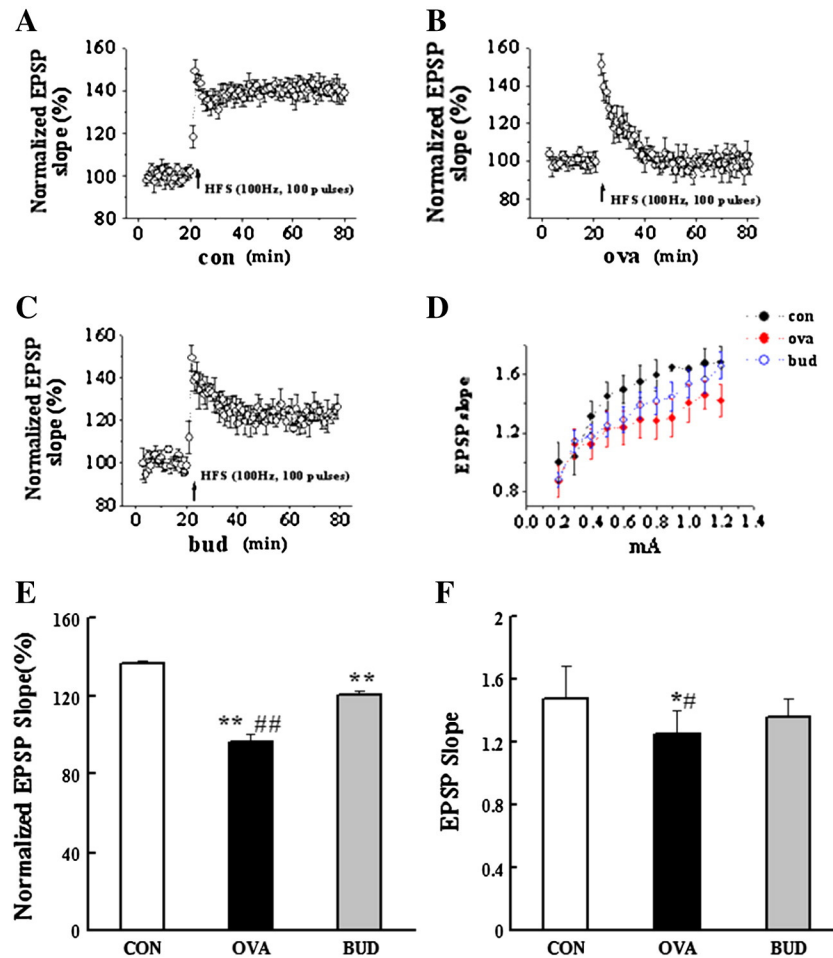


Fig. 4. Effects of chronic asthma on LTP induction and maintaining. A. EPSP in control groups before and after HFS. B. EPSP in control groups before and after HFS in asthmatic groups. C. EPSP in control groups before and after HFS in budesonide-treated groups. D. Effects of chronic asthma on basal synaptic transmission. E. The normalized slope at 60 min in three groups. F. EPSP slope in three groups at 0.8 mA. Data represent the mean $\pm$ SEM. $n = 5$, * $p < 0.05$, ** $p < 0.01$ vs CON group; # $p < 0.05$, ## $p < 0.01$ vs BUD group. CON or con: control group; OVA or ova: asthma model group; BUD or bud: budesonide-treated group.

electron microscopy. The general appearance of presynaptic terminals, spines, and postsynaptic densities at excitatory asymmetric synapses in the CA3 of control groups were remarkable more than that in the asthmatic groups, intranasal administration of budesonide failed to ameliorate the reductions of synapse densities. Synaptic vesicle numbers also showed a significant reduction in asthmatic mice (Figs. 5A, B).

Dramatically, swollen and vacuolated mitochondria were remarkably increased in the hippocampus of asthmatic mice. The damaged mitochondria were markedly swollen, with broken or disrupted cristae or incomplete membranes. Some mitochondria exhibited condensed or polymorphic matrix. The area and perimeter of mitochondria in asthmatic group were larger than control groups. Budesonide treatment could attenuate mitochondrial damage (Figs. 5A, C, and D).

Chronic asthma up-regulates HIF-1 α expression but down-regulates c-fos expressions and Arc-positive neuronal numbers in mouse hippocampus

The cellular response to hypoxia is regulated by a family of transcription factors called the hypoxia-inducible factors (HIFs). HIF-1 α is the most ubiquitously expressed and widely studied HIF isoform. HIF-1 α heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT, also known as HIF-1 β) forming the functional transcription factor HIF-1 α . HIF-1 α regulates the expression of more than 100 genes, such as cytokines. As shown in Fig. 6A, asthmatic mice showed higher protein levels of HIF-1 α in the hippocampus compared

with the control groups. Budesonide treatment could reverse the elevation of HIF-1 α .

To investigate the possible mechanisms of the effects of intermittent hypoxia intervention on learning and memory, we measured the level of the most important immediate early gene, c-fos and Arc, for memory formation and synaptic plasticity. The levels of c-fos protein in the hippocampus of the control group were significantly higher than that in the asthmatic group. However, budesonide treatment could not improve asthma-induced inhibition of c-fos expression (Fig. 6B).

Moreover, chronic asthma robustly decreased number of Arc-positive neuronal cells in the mouse hippocampus. But budesonide treatment failed to attenuate the reduction of Arc-positive cells (Fig. 6C). These results suggested that chronic asthma-induced disruption of c-fos and Arc expression might contribute to the impairments of LTP maintenance and the consolidation of spatial memory training.

Chronic asthma inhibits cell proliferation in subgranular zone of hippocampus

Ki67 staining was used to evaluate neuronal proliferation in the subgranular zone of the hippocampus. As shown in Fig. 7, there were only faintly immunoreactive Ki67-positive cells in asthmatic mice. Asthma resulted in reduction of cell proliferation to 54% compared with the control mice. Intranasal administration of budesonide significantly improved cell proliferation in the hippocampus (Fig. 7B).

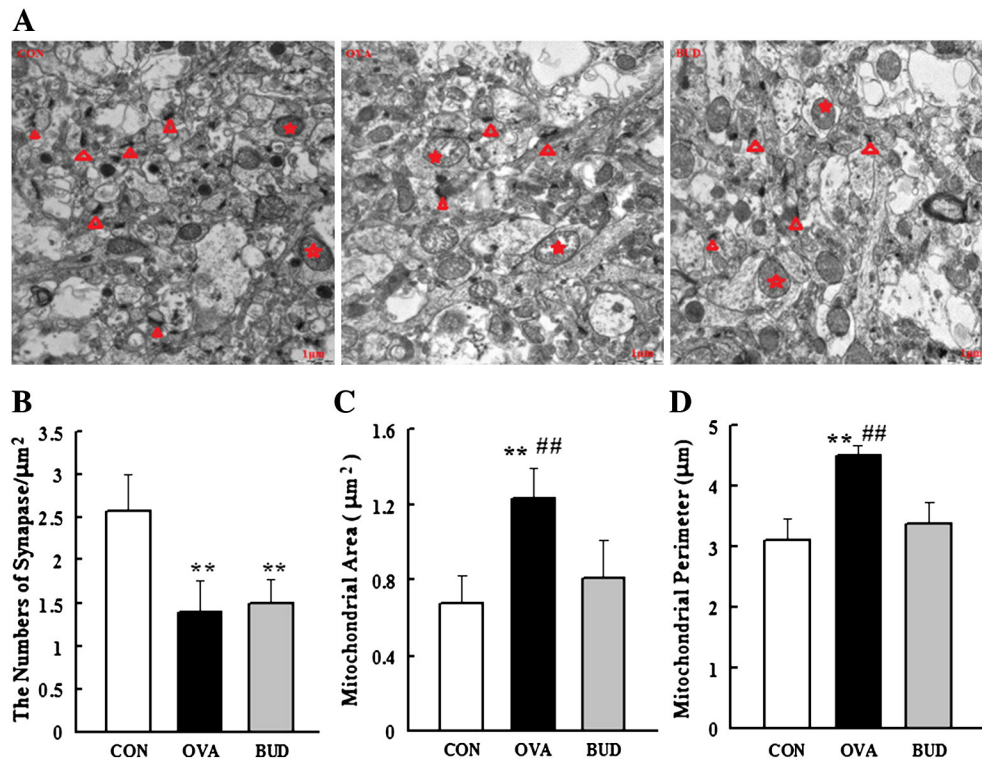


Fig. 5. Effects of chronic asthma on the CA3 ultra-structures of mouse hippocampus. A. Representative electron micrographs of CA3 (20,000×). B. Synapse numbers of CA3 in control groups, asthmatic groups and budesonide groups. C and D. Areas and perimeters of mitochondria in the three groups. Data represents the mean $\pm$ SEM. $n = 4$, ** $p < 0.01$ vs. con group; ## $p < 0.01$ vs. bud group. CON: control group; OVA: asthma model group; BUD: budesonide-treated group. “ Δ ” indicated synapse; “ $\star$ ” indicated mitochondria.

Chronic asthma improves angiogenesis in hippocampus of mice

VEGF has been proved to be a key regulator of angiogenesis in response to tissue hypoxia and plays an important role in vascular vasodilation. Hypoxia-induced expression of VEGF is mainly regulated by HIF-1. Therefore, we further determined the levels of VEGF in the hippocampus of mice. The results showed that the serum concentrations and the mRNA expressions of VEGF in asthmatic mice were remarkably increased compared to the control groups. Budesonide treatment decreased the levels of VEGF (Figs. 8A, B).

GPR124, an orphan member of the adhesion family of G protein-coupled receptors, was previously identified on the basis of its over-expression in tumor vasculature. Previous studies reveal a role for GPR124 as a critical regulator of angiogenesis and barrierogenesis of the developing brain. Therefore, we determined the protein expressions of GPR124 in our study. Consistently, the protein expressions of GPR124 in asthmatic mice were incremented by 127%. Intranasal administration of budesonide could further elevate the GPR124 level (Fig. 8C). These results suggested that chronic asthma-induced hypoxia might improve angiogenesis in brain.

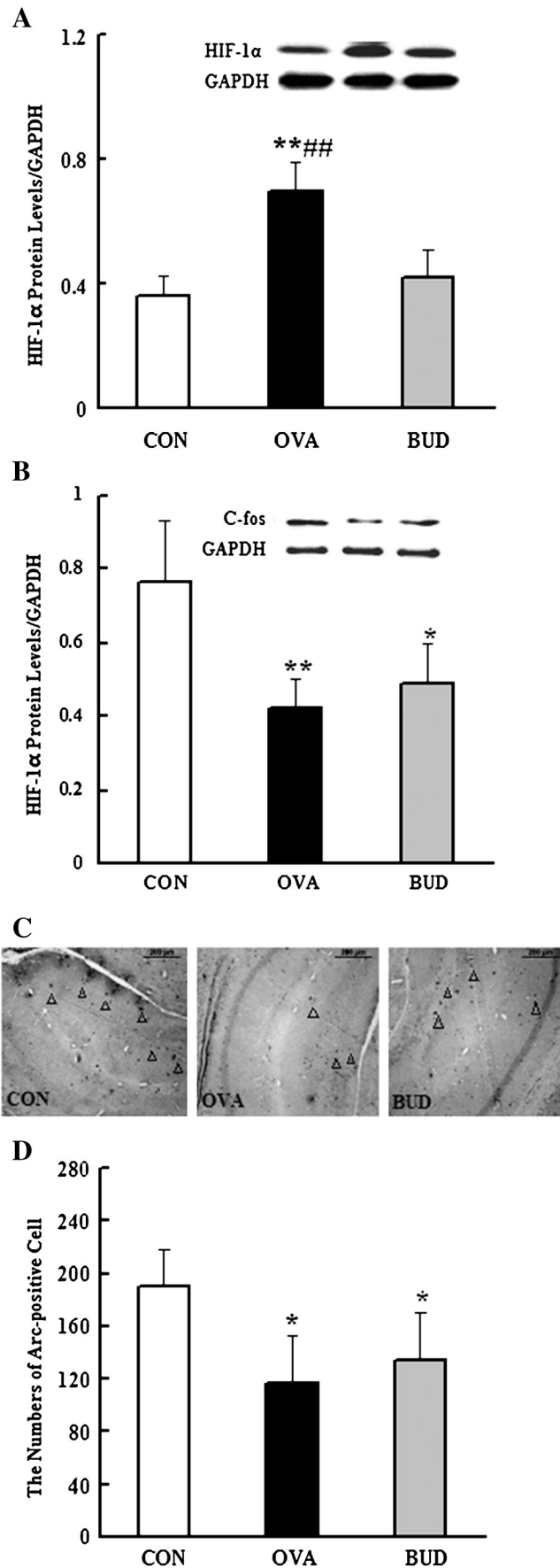
Discussion

Brain tissue is extensively sensitive to oxygen level. The oxygen supply to the brain might be influenced by various brain insults including stroke, trauma and asthma (Benarroch, 2009). Hypoxia could lead to irreversible damages and produce changes in the structure and functions in the brain (Feng et al., 2012; Schneider et al., 2012). In our work, we found that in Morris Water Maze (MWM) test, the latency periods of asthmatic mice were significant longer during training time, and the time spent in the targeted quadrant during the probe trial was shorter compared with the control groups. Budesonide treatment failed to ameliorate the impairment of learning

ability of mice in the MWM test. Memory formation, maintenance, and retrieval are a dynamic process and involve cellular molecular, gene transcription and protein synthesis in the hippocampus. The LTP of synaptic transmission is the most widely studied model for neuronal change that occurs during learning, memory and storing information in the brain (Goh and Manahan-Vaughan, 2013; Hennigan et al., 2009). So, we further investigate the impacts of chronic asthma on the LTP. Consistently, we found that chronic asthma-induced hypoxia impaired LTP maintaining without affecting induction of LTP. Moreover, the slopes of EPSP in the asthmatic groups were significantly decreased indicating that chronic asthma induced synaptic dysfunction. Budesonide treatment just could rescue chronic asthma induced-descent of EPSP slope in mice. Therefore, our results revealed that chronic asthma impaired cognitive function in immature mice. Budesonide treatment could significantly attenuate airway inflammation but was not adequate to improve learning and memory ability of asthmatic immature mice.

Moreover, our study found that the presynaptic terminals, spines, and postsynaptic densities at excitatory asymmetric synapses in the CA3 of asthmatic mice were remarkably decreased. Also, the damaged mitochondria such as swollen mitochondria with broken or disrupted cristae or incomplete membranes, and the mitochondria that exhibited a condensed or polymorphic matrix, were significantly increased in the asthmatic groups. Intranasal administration of budesonide improved mitochondrial damage perhaps due to its anti-inflammatory actions, but could not ameliorate synaptic injury. These ultra-structural changes suggested that chronic asthma resulted in structural damages in the brain, in addition to cognitive functional impairment.

Many studies indicated that intermittent hypoxia could modify the structure and function of the brain such as learning and memory, synapse connection, brain plasticity and cellular proliferation (Luo et al., 2012; Satriotomo et al., 2012; Xie and Yung, 2012). Memory formation is associative to a series of gene transcription, translation,



protein production and synapse connection (Albasser et al., 2007). The relationships between immediate early gene (IEG) and memory have been established (Albasser et al., 2007). Inducible IEG activated by intermittent hypoxia such as c-fos is suggested to act as messengers that widely implicate the processes underlying learning and memory formation. C-fos, is considered as a key factor of memory formation (Suge et al., 2010). Specific suppression of c-fos translation impaired the memory in different behavioral paradigms (Amelchenko et al., 2012). C-fos expression in the hippocampus of rats was required for acquisition and recall of a socially transmitted food performance. In other words, activation of c-fos helps in memory formation (Igelstrom et al., 2010). Our data revealed that the amount of c-fos in the hippocampus of asthmatic groups was lower than that of the control groups. Budesonide treatment failed to up-regulate the levels of c-fos. These results suggest that chronic asthma-induced hypoxia would impair memory and learning by reducing the activation of c-fos.

The stabilization of change in synaptic strength requires rapid de novo RNA and protein synthesis (Ota et al., 2010). Candidate genes, which could underlie activity-dependent plasticity, have been identified on the basis of their rapid induction in brain neurons (Bramham et al., 2008). Arc, another IEG, is considered a marker for neuronal excitability that is enriched in dendrites of hippocampal neurons where it associates with cytoskeletal proteins (Liu et al., 2012), and has been found to play a fundamental role in the stabilization of activity-dependent hippocampal plasticity and which is coupled to cognitive function (Czerniawski et al., 2011). The findings in the current study regarding decrement of Arc-positive neuronal cell in the hippocampus of asthmatic mice, support the theory of impaired cognitive function in which Arc is suggested to be involved in synaptic plasticity. Budesonide treatment failed to improve the down-regulations of Arc. Our studies show that down-regulation of Arc protein expression may impair the maintenance phase of LTP without affecting its induction and impairs consolidation of long-term memory for spatial water task training.

The dentate gyrus (DG) of the hippocampus is one of the few regions of the mammalian brain where new neurons are generated throughout adulthood. This neurogenesis has been proposed as a novel mechanism that mediates spatial memory. Spatial learning is positively correlated to the rate of neurogenesis (Hays et al., 2012). Therefore, we further investigated cell proliferation in the asthmatic hippocampus. Our results showed that chronic asthma resulted in significant reduction of cell proliferation. Some studies reported that hypoxia intervention in normal neonatal rats inhibited the development of the brain, reducing neurogenesis, disrupting synaptic pathway and finally leading to memory impairment (Wei et al., 2008; Zheng et al., 2012). The implication of DG neurogenesis in spatial relational memory is also supported by spatial relational memory deficits in water maze after specifically disrupting the DG (Dominguez-Escriba et al., 2006; Tzeng et al., 2013). Therefore, depletion of neurogenesis could lead to deficits in temporal ordering of spatial information. Our present work revealed that reductions of cell proliferation in the hippocampus induced by chronic asthma might contribute to cognitive dysfunction in asthmatic mice.

The discovery of protein HIF-1α is the first key of the mechanism involved in intermittent hypoxia-mediated modifying the structure and function of the brain. When the cells undergo the onset of hypoxia, HIF-1α protein is continuously synthesized from mRNA (Mowat et al., 2010). Consequently, HIF-1α causes the transcription of its regulated downstream genes, including erythropoietin and VEGF (Cunningham et al., 2012). Accordingly, we also found that chronic asthma

Fig. 6. Effects of chronic asthma on the expressions of HIF-1α and c-fos, and on the numbers of Arc positive cells in hippocampus. A. The expressions of HIF-1α protein in three groups (n = 4). B. The expressions of c-fos protein in three groups (n = 4). C. Microphotographs of Arc-positive cells in the different groups. D. The numbers of Arc-positive cells in hippocampus (n = 5). Scale bar = 200 μm. Data represent the mean ± 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.

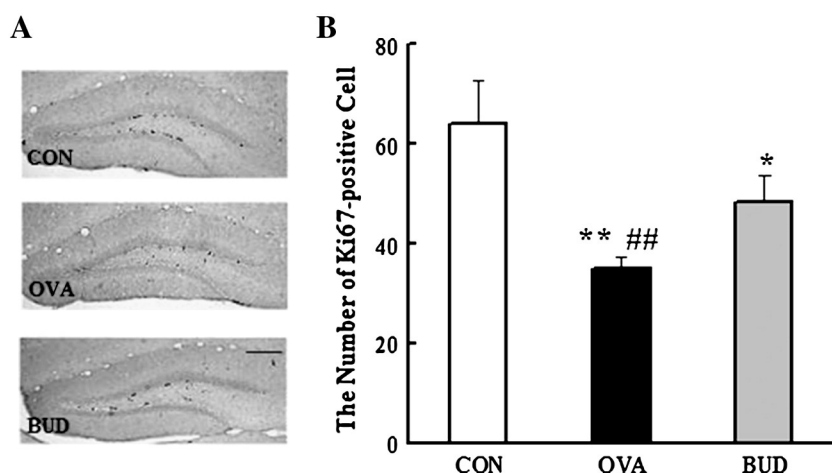


Fig. 7. Effects of asthma and budesonide on the cell proliferation in the subgranular zone (SGZ). A. Microphotographs of Ki67-positive cells in the different groups. B. Quantitative analysis of the immunocytochemistry for Ki67 in the SGZ. Scale bar = 200 μ m. Data represents the mean $\pm$ SEM. $n = 4-5$, * $p < 0.05$, ** $p < 0.01$ vs. CON group; ## $p < 0.01$ vs. BUD group. CON: control group; OVA: asthma model group; BUD: budesonide-treated group.

significantly increased HIF-1 α expressions. It has been reported that HIF-1 α could induce VEGF expression, and formation of new blood vessels of the targeted area in the brain, thereby providing increased blood flow, oxygen supply and reduced harmful responses to ischemia (Lemus-Varela et al., 2010; Sato et al., 2012). Angiogenesis is a complex process that involves multiple gene products expressed by different cell types. A large number of genes involved in different steps of angiogenesis have been shown to be increased by hypoxic challenge (Murukesh et al., 2010). Among them, VEGF is the most potent endothelial-specific mitogen, and it directly participates in angiogenesis by recruiting endothelial cells into the hypoxic and avascular area and stimulates their proliferation (Morita et al., 2013; Wang et al., 2011). Therefore, the induction of VEGF and various other proangiogenic factors leads to an increase in the vascular density and hence a decrease in oxygen diffusion distance (Benderro and Lamanna, 2011). In addition to improving angiogenesis, VEGF can improve neurogenesis, and enhance learning and memory ability (Sun et al., 2010). Ischemic brain VEGF exerts an acute neuroprotective effect, as well as longer latency effects on survival of new neurons and on angiogenesis (Morimoto et al., 2011). Interestingly, our present study found that chronic asthma increased VEGF and GRP124, indicating compensatory angiogenesis might occur in the brain. Budesonide administration inhibited VEGF levels but further increased GRP124 protein level. It has been well demonstrated that glucocorticoids inhibit VEGF and angiogenesis (Maiolino et al., 2012; Shikatani et al., 2012), which are consistent with our results. But there is no information about the effects of glucocorticoids on GRP124. Therefore, the potential actions of vascular supply and plasticity in the

asthmatic brain, and the signal pathways involved in budesonide regulating GRP124 expression should be further explored.

Conclusions

Chronic asthma impairs cognitive function and synaptic plasticity in immature mice. Down-regulation of c-fos, Arc and neurogenesis induced by intermittent hypoxia during chronic asthma might contribute to learning and memory dysfunctions. Budesonide treatment alone is inadequate for attenuating chronic asthma-induced structural and functional impairment. Based on our findings, more attention should be devoted for chronic asthma-induced brain damage in the clinical therapy.

Conflict of interest statement

The authors declare no conflict of interest.

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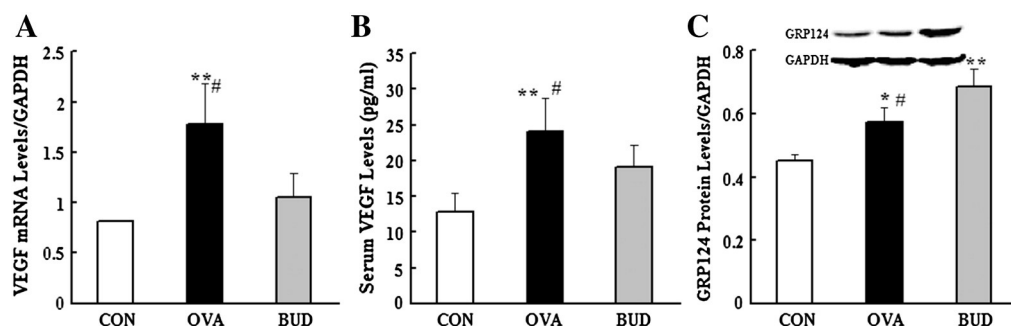


Fig. 8. Effects of asthma on the levels of VEGF and GPR124 in hippocampus. A. The mRNA levels of VEGF in three groups ($n = 4$). B. The serum levels of VEGF in three groups ($n = 16$). C. The protein expressions of GPR124 in three groups ($n = 4$). Data represents the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. CON group; # $p < 0.05$ vs. BUD group. CON: control group; OVA: asthma model group; BUD: budesonide-treated group.

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