Participation of Antidiuretic Hormone (ADH) in Asthma Exacerbations Induced by Psychological Stress via PKA/PKC Signal Pathway in Airway-Related Vagal Preganglionic Neurons (AVPNs)

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\textbf{Key Words}

Psychological stress \cdot Asthma \cdot Antidiuretic hormone (ADH) \cdot PKA/PKC

\textbf{Abstract}

\textbf{Aims:} Present study was performed to examine whether ADH was implicated in psychological stress asthma and to explore the underlying molecular mechanism. \textbf{Methods:} We not only examined ADH levels in the cerebrospinal fluid (CSF) via radioimmunoassay, but also measured ADH receptor (ADHR) expression in airway-related vagal preganglionic neurons (AVPNs) through real-time PCR in all experimental mice. Western blotting was performed to evaluate the relationship between ADH and PKA/PKC in psychological stress asthma. Finally, the role of PKA/PKC in psychological stress asthma was analyzed. \textbf{Results:} Marked asthma exacerbations were noted owing to significantly elevated levels of ADH and ADHR after psychological stress induction as compared to OVA alone (asthma group). ADHR antagonists (SR-49095 or SR-121463A) dramatically lowered higher protein levels of PKA\textalpha{} and PKC\textalpha{} induced by psychological stress as compared to OVA alone, suggesting the correlation between ADH and PKA/PKC in psychological stress asthma. KT-5720 (PKA inhibitor) and Go-7874 (PKC inhibitor) further directly revealed the involvement of PKA/PKC in psychological stress asthma. Some notable changes were also noted after employing PKA and PKC inhibitors in psychological stress asthma, including reduced asthmatic inflammation (lower eosinophil peroxidase (EPO) activity, myeloperoxidase (MPO) activity, immunoglobulin E (IgE) level, and histamine release), substantial decrements in inflammatory cell counts (eosinophils and lymphocytes), and decreased cytokine secretion (IL-6, IL-10, and IFN-\textgamma{}), indicating the involvement of PKA/PKC in asthma exacerbations induced by psychological stress. \textbf{Conclusion:} Our results strongly suggested that ADH participated in psychological stress-induced asthma exacerbations via PKA/PKC signal pathway in AVPNs.
Introduction

Asthma is a common chronic airway inflammatory disease that features airway hyperresponsiveness, involving eosinophils, mast cells, and lymphocytes [1]. Immune response in asthma is primarily elicited by T-helper 2 (Th2) cytokines, especially interleukin (IL)-4, IL-5 and IL-13 that cause immunoglobulin E (IgE) production, eosinophil accumulation, and mucus hypersecretion [2-4]. Several studies also showed that activated neutrophils could free metalloproteinase (MMP-9), MPO and elastase, which all contributed to asthma exacerbation [5, 6]. Interaction of genetic and environmental factors is implicated in the onset, as well as the course of asthma. Smoke, pollutants and viral infections are common environmental inducers of asthma [7, 8]. Intriguingly, psychological stress has also been established the role of enemy within in the progression of asthma. Actually, early in the 19th century, Sir William Osler [9] has defined asthma as "neurotic affection". Subsequently, mounting evidence suggests that chronic stress greatly affects life quality of patients with asthma and is linked to depressed medication compliance [10-12]. From an immune point of view, chronic stress can alter immune balance and may be responsible for increasing prevalence and worse severity of asthma, at least partially [13, 14]. Psychological stress is also linked to neuro-endocrino-immune network in asthma [15]. Accordingly, asthma is regarded primarily as a psychological disease and psychological stress is considered to exert impact on neuroendocrine-immune modulation, thus influenced the progression of asthma.

Antidiuretic hormone (ADH), a neuropeptide hormone secreted from pituitary gland, exerts significant effects in cardiovascular and renal disease via binding to ADH receptor (ADHR), primarily V1A and V2 receptors [16, 17]. Earlier studies have noticed elevated levels of ADH in patients with status asthmaticus during acute asthma, suggesting a potential link between ADH and the propagation of asthma [18, 19]. Iikura et al [20] further found that increased ADH levels in acute asthma in children had a fall in the case that asthma got improvement, indicating that ADH levels may be associated with clinical course of asthma. Moreover, ectopic hormone secretion was detected with spaceflight, including ADH, adrenocorticotropic (ACTH), and aldosterone, etc., companied with aberrant numbers of T-lymphocytes and natural killers, partly reflecting the network between neuroendocrine and immune system [21]. Accordingly, we inferred that neuro-endocrino-immune axis had an impact on ADH secretion in asthma progression. Thus, in view of the link between neuroendocrine-immune axis and psychological stress in the development of asthma, the hypothesis was that ADH participated in the course of psychological stress asthma. Furthermore, considering that the well-known ADHR subtypes (V1A, V1B, and V2) are members of the seven transmembrane domain G-protein-coupled receptor family [22], and cAMP-protein kinase (PKA) and Ca²⁺-dependent/independent protein kinase (PKC) are important signal transduction factors implicated in cell signaling pathways linked to G-protein-coupled receptors [23], hence, in this study, we not only examined ADH and ADHR levels, but also clarified the role of PKA/PKC signal pathway in a mouse model of psychological stress asthma.

Materials and Methods

Animal and reagents

Six to eight-week-old female mice (BALB/c, 20-24 g) obtained from Shanghai Experimental Animal Center (Shanghai, China) were given standard diet and water. All the experimental protocols were approved by the Animal Ethics Committee of Shanghai Jiao Tong University. Rhodamine (X-rhodamine-5-(and-6)-isothiocyanate), ovalbumin (OVA, Grade V), SR-49059 (V1A antagonist), SR-121463A (V2 antagonist), KT-5720 (PKA inhibitor), Go-7874 (PKC inhibitor), hexadecyltrimethylammonium bromide (HTAB), and 3,3',5,5'-tetramethylbenzidine (TMB) were collected from Sigma-Aldrich (St Louis, MO). Southern Biotech (Birmingham,USA) provided HRP labelled anti-mouse IgE secondary. ELISA kits of IL-6, IL-10, and IFN-γ were supplied from Biolegend (San Diego, CA)
Retrograde fluorescent labeling of airway-related vagal preganglionic neurons (AVPNs)

The vagus nerve which mainly derives from AVPNs are responsible for airway functions [24]. Thus, we retrogradely fluorescent labelled AVPNs in experimental mice to clearly observe ADHR expression, as described previously [25]. Briefly, BALB/c mice were firstly placed on a glass box (5 × 5 × 5 cm) filled with halothane. 30 s later, the mice lost consciousness, but their breathing were still normal. Then ice water-filled bags were adopted to slow the heart rate of mice. After breathing stopped, 1% rhodamine (0.5µl) was injected into extrathoracic trachea wall between the fourth and eighth tracheal cartilage, a thermo pad benefited the recovery of animals.

Animal experiments

To explore the effect of psychological stress on asthma, we randomly divided the mice into three groups (n= 8): Control, Asthma, Asthma+psychological stress (PS). On the first day after grouping, Control group were intraperitoneally injected with 0.2 mL of normal saline once a day, and other two groups were sensitized via intraperitoneal (i.p.) injection with 0.2 mL of sensitization fluid containing 10 µg OVA and 1 mg aluminum hydroxide in phosphate buffered saline (PBS) once a day. Two weeks later, mice in Control group inhaled the atomization of normal saline once a day for 20 min from day 14 to day 28, while other two groups were challenged with 1% (W/V) OVA solution in PBS, and Asthma+PS group were simultaneously stimulated with restriction and tail clamp for stress once a day, as described previously [26, 27]. To further investigate the relationship between ADH and PKA/PKC in Asthma+PS group, SR-49059 or SR-121463A were used 2 h before every OVA challenge by i.p. injection at the same concentration of 10^{-8} M. Finally, to assess the effects of PKA/PKC on Asthma+PS, KT-5720 or Go-7874 (5 mg/kg; 0.5 µl) was administered each day before every OVA challenge by intracerebroventricular (i.c.v.) injection. KT-5720 or Go-7874 was dissolved in sterile saline.

Measurement of biochemical stress parameters

24 h after the final challenge (day 29), blood in mice was sampled by facial vein (cheek) and caudal vein (tail) phlebotomy and automatically (ABS) from mice. Plasma corticosterone levels were quantified by ELISA, as described previously [28].

Body weight (BW), daily food intake (DFI) and daily water intake (DWI) were recorded each morning once a day for 5 days.

RNA isolation and Real-Time PCR

Total-RNA was extracted from AVPNs using TRizol (Invitrogen, Carlsbad, CA, USA), Superscript™ Reverse transcriptase and oligo(dt) 18 primer were employed for cDNA synthesis following the manufacturer’s guidances (Life Technologies, Gaithersburg, MD, USA). Real-time PCR reactions were performed using a SYBR Green PCR kit (GenePharma, Shanghai, China). GAPDH was used as the internal control.

Western blotting

Western blotting was performed as reported earlier [23]. Lung tissues detached from the mice were homogenized and centrifuged, then 20 µl of supernatant and gel loading solution were mixed. The specimens were loaded onto 10% SDS-PAGE mini-gel and proteins were transferred to a polyvinylidene difluorid (PVDF) membrane (Beyotime Biotech, China). After blocking for 1 h with 5% non-fat dry milk in PBS, membranes were incubated overnight at room temperature with anti-PKAα, anti-PKCα, and anti-β-actin (Santa Cruz Biotechnologies, Santa Cruz, CA), then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit IgG) for 1.5 h at room temperature, followed by the detection with chemiluminescence (ECL) reagent (Amersham, Buckinghamshire, UK). β-actin served as the loading control.

Histomorphological Analysis

The lung samples were dissected and fixed in 4 % formaldehyde for over 24 hours in room temperature. After being decalcified in 10 % ethylene diamine tetraacetic acid (EDTA) solution for over 2 weeks, the samples were embedded in paraffin. The specimens were cut into 4 µm thick sections and stained with haematoxylin-eosin (H&E).
Measurement of Cerebrospinal fluid (CSF) ADH levels

Measurement of CSF ADH levels was performed as described previously [29]. CSF was withdrawn from the mice, and ADH levels in CSF were examined using a kit supplied from Nichols Institute (San Mateo, CA, USA). Samples were kept on ice immediately after collection, then centrifuged at 4°C. We added anti-vasopressin and the samples were incubated at 4°C for 24 h. 125I vasopressin was also added and the samples were incubated at 4°C for additional 24 h. Then the antibody-bound radiolabeled vasopressin was numbered in a gamma counter.

Collection of bronchoalveolar lavage fluid (BALF)

Collection of BALF in models of asthma and psychological stress asthma was done as previously described [30]. Briefly, mice were sacrificed 24 h after the last challenge by an overdose of pentobarbital (50 mg/kg), then tracheotomy was carried out and PBS (0.5 mL) was dropped into the lung. BALF was obtained after three successive operations (1.5 mL) through tracheal cannulation. Haemocytometer was employed to evaluate total cell counts in BALF and Giemsa was prepared for differential count. Five fields at × 40 were randomly selected and cells were determined based on morphological characteristics.

Eosinophil peroxidase (EPO) assay

EPO activity was detected in lungs as described previously, with some alterations [31]. Firstly, lung was homogenized and centrifuged at 6000 rpm for 15 min. Then the cell pellet was resuspended and homogenized again followed by three freeze thaw cycles. After the mixture with reaction solutions containing 1.5 mM O-phenyladenine (OPD) and 6.6 mM hydrogen peroxide (H2O2), the samples was incubated at 20°C for 30 min in dark. Finally, the reaction was finished using 4 M H2SO4 and results were presented in absorbance units.

Myeloperoxidase (MPO) activity

MPO activity was examined to evaluate pulmonary neutrophil infiltration [32]. Lung homogenate was firstly prepared in 50 mM potassium phosphate buffer (pH 6.0) and experienced three freeze cycles at -80°C. Then the homogenate was mixed with the reaction solution containing 0.002% H2O2 and 0.167 mg/ml O-dianisidine dihydrochloride in 300 µl volume. MPO activity was detected through monitoring the change in absorbance at 460 nm over 20 min.

IgE level

We also examined OVA specific-IgE as described by Chauhan et al. [33]. Firstly, ELISA plates were covered with OVA at the concentration of 100 µg/ml overnight at 4°C. Then the plates were cleaned and blocked with 2% bovine serum albumin for 2 h at room temperature. Subsequently sera were dropped onto the plates and incubated for 24 h at 4°C before washing. After an additional incubation with biotinylated anti-IgE (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) for 2 h at 4°C, the plates were incubated with streptavidin horseradish peroxidase (Southern Biotechnology) for 1 h at room temperature. Then plates were washed and incubated with TMB substrate solution (100 µl/well, Becton Dickinson Biosciences) for 10 min at room temperature. The reaction was terminated with 0.18 M H2SO4 and the plates were detected at 450 nm.

Histamine estimation

Histamine levels were also examined using flat bottom black well plate as reported by Chauhan et al. [33]. Briefly, 180 µl of diluted serum in 0.1 M HCl was mingled with 36 µl 1 M NaOH and 9 µl 1% O-phthalaldehyde (OPT). After the incubation with 18 µl 3 M HCl for 20 min, the reaction was finished. 360 nm excitation and 450 nm emission filters were adopted to determine fluorescence intensities.

Cytokine analysis

Enzyme-linked immunosorbent assay (ELISA) was performed to analyze the levels of IFN-γ, IL-6, and IL-10 in BALF in accordance with the manufacturer’s recommendations. Microplate reader (EL × 800, BioteK) was employed to measure the optical density at 450 nm. The concentration of cytokines were shown in pg/ml.
Statistical analysis

Data were presented as mean ± SEM, and analyzed using SPSS 16 (SPSS Inc., Chicago, IL, USA). After Student t-test and ANOVA, Tukey’s test was employed to identify differences between multiple groups. P<0.05 was considered statistically significant.

Results

**ADH and ADHR are significantly increased in psychological stress asthma**

Considering that airway functions are under the control of vagus nerve which centrally results from AVPNs [24], we firstly examined the expression of ADHR in the AVPNs which were retrogradely fluorescent labeled by rhodamine through real-time PCR and western blotting among three groups (Control, Asthma, and Asthma + PS). Restriction and tail clamp for psychological stress resulted in the significant change in BW, DFI, DWI, and plasma corticosterone (Fig. 1), suggesting the feasibility of restriction and tail clamp for the induction of psychological stress. Compared with Control and Asthma group, the expression of ADHR was prominently elevated in Asthma + PS group, both in mRNA and protein level (Fig. 2A and B). Similarly, significantly increased ADH levels in the CSF were also observed in Asthma + PS group (Fig. 2C).

**ADH is associated with PKA/PKC in psychological stress asthma**

Fig. 1 has revealed massively increased ADH and ADHR in Asthma + PS group, considering that the well-known ADHR subtypes (V1A, V1B, and V2) are members of the seven transmembrane domain G-protein-coupled receptor superfamily [34], and PKA/PKC are involved in cell signaling pathways linked to G-protein-coupled receptors [23], thus, ADHR antagonists were employed to explore the potential link between ADH and PKA/PKC in psychological stress asthma. In view of the potential regulatory network between ADHR subtypes (V1A, V2) and PKA/PKC in vascular smooth muscle cell (VSMC) [35] and other cells [36, 37], SR-49059 (V1A antagonist) and SR-121463A (V2 antagonist) were selected. As shown in Fig. 3A and B, significantly higher protein levels of PKAα and PKCα

**Fig. 1.** Body weight (BW) in relation to baseline values (A), Daily food intake (B), Daily water intake (C), and plasma corticosterone levels (D) were measured 24 h after the final challenge (day 29), each morning once a day for 5 days. Blood sampling for mice were sampled by facial vein (cheek) and caudal vein (tail). The data are shown as means ± SEM. # Control vs. PS (P<0.05).
were noted in Asthma + PS group as compared to Asthma group, but that was reduced after ADHR antagonists treatment, suggesting the correlation between ADH and PKA/PKC in psychological stress asthma. Besides, H&E staining of lung histology revealed asthma exacerbation after psychological stress induction, and that ADHR antagonists treatment resulted in obvious amelioration of asthma exacerbation in Asthma + PS group (Fig. 4), further indicating the correlation between ADH and PKA/PKC in asthma exacerbation induced by psychological stress.

**PKA/PKC is implicated in psychological stress asthma**

The results in Fig. 3 indicated the participation of PKA/PKC in psychological stress-induced asthma, we further verified this result by using KT-5720 (PKA inhibitor) and Go-7874 (PKC inhibitor) (Fig. 5A and B). Apparently, significantly higher protein levels of PKAα and PKCα induced by psychological stress were significantly reduced after KT-5720 or Go-7874 treatment, directly indicating the involvement of PKA/PKC in psychological stress asthma.
Effect of PKA/PKC on asthmatic inflammation in psychological stress asthma

KT-5720 and Go-7874 were used to assess effects of PKA/PKC on psychological stress asthma. Significantly higher EPO activity, MPO activity, IgE level, and histamine release were noted after psychological stress induction, whereas, either KT-5720 or Go-7874 treatment resulted in reduced asthmatic inflammation with lower levels of these mediators (Fig. 6). Inflammatory cell counts in the BALF which were significantly increased after psychological stress induction were also conspicuously decreased with reduced infiltration of eosinophils and lymphocytes to the lung after KT-5720 or Go-7874 treatment (Fig. 7). However, neither KT-5720 nor Go-7874 had effect on lung inflammation in Control group. All the results suggested that PKA/PKC took part in asthma exacerbations induced by psychological stress characterized by large numbers of eosinophils and lymphocytes as well as higher EPO activity, IgE level, and etc.
Effect of PKA/PKC on cytokine release in psychological stress asthma

To determine whether PKA/PKC affected cytokine secretion into the BALF, the levels of IL-6, IL-10, and IFN-γ in the BALF were examined through ELISA 24 h after the last challenge, the results were illustrated in Fig. 8. Significantly enhanced cytokine secretion (IL-6, IL-10, and IFN-γ) induced by psychological stress as compared to OVA alone were markedly suppressed after KT-5720 or Go-7874 treatment, while, neither KT-5720 nor Go-7874 had effect on Control group.
Discussion

In some earlier studies, psychological stress has been suggested to exacerbate airway inflammation in a murine model of asthma [27, 38]. ADH, a neuropeptide hormone secreted from pituitary gland, is also aberrantly increased in patients with asthma [39, 40]. Since human response to psychological stress is associated with neuroendocrine-immune network [41], therefore, in this present study, the potential relationship between ADH and psychological stress asthma was explored in a mouse model. Marked asthma exacerbations were noted where ADH, and ADHR were found significantly elevated in psychological stress asthma as compared to OVA alone, in view of the correlation between ADH level and clinical course of asthma [20]. Moreover, ADH has been proven to exert effects through binding to ADHR, primarily V1A and V2 receptors [16, 42]. Given that common ADHR subtypes (V1A, V1B, and V2) are members of the seven transmembrane domain G-protein-coupled receptor superfamily [34], and the significant participation of PKA and PKC in cell signaling pathways linked to G-protein-coupled receptors [23], the hypothesis was tested whether PKA and PKC were associated with ADH in psychological stress asthma, as studied using two different ADHR antagonists. We observed that ADHR antagonists signally suppressed higher protein levels of PKAα and PKCα after psychological stress stimulation as compared to OVA alone, indicating the correlation between ADH and PKA/PKC in psychological stress asthma.

Currently, mounting evidence suggests that PKA and PKC are related to airway inflammation in asthma. Zhu et al. [43] reported that glucagon like peptide-1 (GLP-1) significantly ameliorated airway inflammation and mucus secretion via a PKA-dependent inactivation of nuclear factor-κB (NF-κB) signal pathway in a mouse model of OVA-induced asthma. Many studies also addressed the relationship between the cAMP/PKA pathway and the pathogenesis of asthma in some clinical studies [44-46]. PKC signal pathway is also one of the key players in asthma pathogenesis [47]. PKC is a serine/threonine phosphotransferase implicated in inflammation, immune response and some other physiological functions. Previous studies showed that inhibition of PKC suppressed the activation of lymphocytes, and lowered the expression of Th2 cytokines (IL-4 and IL-5) in asthmatic patients [48, 49]. In a OVA-sensitized pig model of asthma, the activation of PKC, especially PKCα and PKCε, plays a key role in lymphocyte infiltration, initiation of airway hypersensitivity (AHR), and asthma pathophysiology [50]. Moreover, PKC isoforms (δ, ζ) are also crucially implicated in the migration of asthmatic eosinophils [51]. In this study, we found that PKC and PKA inhibitors significantly lowered psychological stress-induced large numbers of eosinophils and lymphocytes, and enhanced Th2 cytokines secretion (IL-6, and IL-10) after psychological stress stimulation was also significantly suppressed after PKC and PKA.
inhibitors treatment, strongly supporting the role of PKC in asthma [48, 49]. Surprisingly, we found that Th1 cytokine, IFN-γ, was also significantly induced after psychological stress stimulation, which was line with previous reports that asthma did not completely fit Th1/Th2 shift paradigm [52, 53]. Actually, regulatory T cells (Treg) are also critical regulators of asthma [54]. PKC activation plays a key role in the modulation of IgE-mediated histamine release from basophils [55]. Here, PKC and PKA inhibitors strikingly reduced higher IgE level, histamine release, MPO activity, and also EPO activity induced by psychological stress. Our findings demonstrated that PKA/PKC was implicated in psychological stress-induced asthma exacerbations, in view of the relation between ADH and PKA/PKC in psychological stress asthma, we concluded that ADH participated in asthma exacerbations induced by psychological stress through PKA/PKC signaling pathway.

Overall, our results reveal a novel ADH-PKA/PKC regulatory network in asthma exacerbations induced by psychological stress, and provide a promising approach of targeting specific pathways towards the treatment of psychological stress asthma.

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Disclosure Statement

None.

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