

Original article

Impact of psychosocial stress on airway inflammation and its mechanism in a murine model of allergic asthma

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Keywords: psychosocial stress; asthma; glucocorticoid insensitivity; glucocorticoid receptor

Background It has already been recognized that psychosocial stress evokes asthma exacerbation; however, the mechanism of how stress gets inside the body is not clear. This study aimed to observe the impact of psychosocial stress on airway inflammation and its mechanism in the ovalbumin-induced asthmatic mice combined with social disruption stress.

Methods Thirty-six male BALB/c mice were randomly divided into: control group, asthma group (ovalbumin-induced), asthma plus social disruption stress group (SDR), and SDR group. The open field video tracking system was used to assess animal behaviors. The invasive pulmonary resistance (RL) and dynamic lung compliance (cdyn) test system from Buxco was applied to detect pulmonary function. The enzyme-linked immunosorbent assay (ELISA) was utilized to determine OVA-IgE, T-helper type 2 (Th2) cytokines (IL-4, IL-5, IL-13) and corticosterone in mouse serum, the Th2 cytokines (IL-4, IL-5, IL-13, IL-6, TNF- α) in bronchoalveolar lavage fluid (BALF), and IL-6 and TNF- α levels in the supernatant of splenocytes cultured *in vitro*. Hematoxylin-eosin (H&E) staining was used to assess airway inflammation in lung histology. The cell count kit-8 assay (CCK-8) was applied to evaluate the inhibitory effect of corticosterone on splenocyte proliferation induced by lipopolysaccharide (LPS). Real time-PCR and Western blotting were utilized to determine glucocorticoid receptor (GR) mRNA and GR protein expression in lungs.

Results The open field test showed that combined allergen exposure and repeated stress significantly shortened the time the mice spent in the center of the open field ($P < 0.01$), increased ambulatory activity ($P < 0.01$) and the count of fecal boli ($P < 0.01$), but decreased vertical activity ($P < 0.01$). Results from pulmonary function demonstrated that airway hyperresponsiveness (AHR) was enhanced by psychosocial stress compared with allergy exposure alone. The ELISA results showed that cytokines in serum and BALF were significantly increased ($P < 0.05$). Moreover, the lung histology showed that infiltrated inflammatory cells were significantly increased in the asthma-SDR group compared with the asthma group ($P < 0.05$). Interestingly, serum corticosterone was remarkably raised by psychosocial stress ($P < 0.05$). In addition, the inhibitory effect of corticosterone on IL-6 and TNF- α in LPS-stimulated splenocyte cultures *in vitro* was diminished in the asthma-SDR group compared to the asthma group. The CCK-8 test revealed that the inhibition effect of corticosterone on splenocyte proliferation induced by LPS was significantly impaired in the SDR and asthma-SDR groups, while no significant effect was observed in the control and asthma groups. Furthermore, expression of GR mRNA and GR protein were significantly reduced in the lung tissues of the asthma-SDR group ($P < 0.05$).

Conclusions Social disruption stress can promote anxiety behavior, activate the hypothalamic-pituitary-adrenal (HPA) axis, increase AHR and inflammation, and also impair glucocorticoid sensitivity and its function in a murine model of asthma. The down-regulation of GR expression induced by social disruption stress is in part associated with glucocorticoid insensitivity, which leads to asthma exacerbation.

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Despite the advances in management, asthma still is a serious public health problem throughout the world, affecting an estimated 300 million individuals.¹ In the last few decades, with the conversion from a biomedical model to a bio-psycho-social medical model, there is growing awareness that asthma is a disease characterized by airway inflammation and bronchial obstruction that can be triggered and exacerbated by psychosocial factors like low socioeconomic status² and urban living.^{3,4}

It is well known that exposure to many forms of acute stress activates the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic-adrenal-medullary axis, leading to increased secretion of the hormones cortisol, epinephrine, and norepinephrine.^{5,6} High levels of circulating cortisol

diminish inflammation in the airway and the periphery; similarly, beta-adrenergic agonists are potent

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bronchodilators. Taken together, these observations suggest the paradoxical conclusion that stressful experiences should ameliorate rather than exacerbate symptoms of asthma. However, the findings from studies of asthma patients are not consistent with this conclusion, as stress has been shown repeatedly to worsen the course of the illness.⁷ For example, in an 18-month prospective study of children with asthma, the experience of an acute negative life event (e.g., death of a close family member) increased the risk of a subsequent asthma attack by nearly 2-fold. Children exposed to high levels of acute and chronic stress showed a 3-fold increase in risk for an asthma attack in the two weeks that followed the acute event.⁸ Furthermore, school examinations induce asthma exacerbations in children.⁹ Lietzen et al,¹⁰ through a prospective, population-based cohort study of 16 881 males and females, found that stressful life events increased the onset of asthma. Besides acute stressful experiences triggered asthma exacerbations, chronic psychosocial stress, like inadequate parent support¹¹ and chronic caregiver stress in early childhood, were also associated with asthma exacerbation and development of allergy diseases.¹² Furthermore, increasing evidence supports a specific relationship between chronic psychological stress (depression, anxiety, panic, *et cetera*) and atopic diseases, as suggested by a recent review by Chida et al.¹³ For instance, the parental psychological state (anxiety) can affect the manifestation of asthma in the child, possibly by influencing the corticotrophin-mediated stress response.¹⁴

Undoubtedly, elucidating the mechanism of how psychosocial stress can “get under the skin” to effect asthma patients plays an important role in improving asthma management. However, studies on stress, immune response and asthma are hampered by the fact that peripheral blood samples are probably inadequate to reflect the changes occurring in the airways.¹⁵ Moreover, obtaining airway samples in human is often difficult and costly, and the sampling procedure itself (i.e. bronchoscopy) can be stressful and confound the effects of the psychological stress under study. A well-established murine model of allergic asthma is available,^{16,17} which is validated by demonstrating characteristic cellular and histological features of airway inflammation and airway hyperresponsiveness (AHR). Similarly, there is a reliable and valid murine model of social disruption stress which has been successfully built by our laboratory¹⁸ and others.¹⁹⁻²²

Based on these models, the purposes of this study were as follows: (1) observe the impact of psychosocial stress on behavior, AHR, inflammatory cytokines, pulmonary histopathology, HPA axis function and glucocorticoid sensitivity in a murine model of combined social disruption stress and allergy sensitization, and (2) examine the potential mechanism by which psychosocial stress “gets into the body” to affect the airway inflammatory responses.

METHODS

Main experimental materials and apparatus

Experimental animals

Thirty-six pathogen-free male BALB/c mice (test mice, aged 6–8 weeks old, weighed 18–20 gram, 3 per cage) and 15 male C57BL/6J mice (aggressors, 8–10 weeks old, weighed 20–22 gram) were purchased from Shanghai SLAC Co. (Shanghai, China) and used in this study.

Main reagents

Ovalbumin (OVA, Grade II), aluminum hydroxide, corticosterone, lipopolysaccharide (LPS) and methacholine (Mch) were all bought from Sigma Company, USA. ELISA kits for IL-4, IL-5, IL-6, IL-13 and TNF- α were purchased from Biosource, USA. The kit for corticosterone detection was from R&D Systems (USA). Lymphocyte Separation Medium (Mouse) was from Lichen Pharmaceutical Ltd., Shanghai, China. Pentobarbital sodium was obtained from Xitang Biotechnology Ltd., Shanghai, China. The cell count kit-8 (CCK-8) assay was from Dojindo Laboratories, Japan. Trizol and SuperScript II reverse transcriptase were from Invitrogen Co., Carlsbad, CA, USA. Anti-glucocorticoid receptor (GR) antibody and anti-GAPDH antibody were obtained from Abcam, USA. Fetal bovine serum (FBS) was from Gibco, USA.

Main apparatus

The invasive pulmonary resistance (RL) and dynamic lung compliance (CYDN) test system was from Buxco, USA. The open field video tracking system and the special software SOF-811 for analysis was from Med Associates, USA. The 5804R refrigerated centrifuge was from Eppendorf, Germany. The DFX 320 microscope was purchased from Leica, Germany. The YS9801 medical ultrasonic atomizer was from the Yisheng Technology Co. Ltd, Shanghai, China. The Rotor-Gene 3000 real-time PCR was purchased from Corbett Research, Australia. Software for primer design was from Primer Premier 5.0. The device for inducing asthma was a self-made plexiglass case of 30 cm×16 cm×14 cm.

Experimental methods

Experimental grouping

Fifteen male C57BL/6J mice used as aggressors arrived in the animal facility at 8–9 weeks old and were singly-housed in cages for four weeks in order to reduce inter-individual variability and increase the likelihood of social interaction-induced stress without creating overt phenotypic changes. Therefore, when the experiment began, the C57BL/6J mice were 12–13 weeks old. Thirty-six BALB/c mice that were studied for their stress and allergic responses were allowed to acclimate to their new environment for one week before the experiment and were maintained on a 12-hour light/dark schedule (lights on at 6:00 a.m.) with food and water available *ad libitum*. They were randomly assigned to four groups ($n=9$ per

group): A, no manipulation (Control); B, sensitized and antigen challenged (Asthma); C, sensitized, antigen challenged, and with social disruption (Asthma-SDR); D, social disruption model (SDR). All animal experiments were approved by the Fudan University Animal Care and Use Committee.

Allergic sensitization and challenge

Asthma and Asthma-SDR mice were sensitized and challenged by OVA according to the modified protocol reported previously.^{16,17} Briefly, the mice were immunized at the 1st and 8th days (seven days apart) by a peritoneal injection (i.p.) of 0.5 ml sterile saline containing 100 µg OVA and 4 mg aluminum hydroxide. Fourteen days after the first sensitization, the mice were challenged continuously for two weeks (30 minutes each day) by an inhalation of 1% OVA through an ultrasound aerification inhaler in a home-made plexiglass case.

Selection of aggressive intruders

Prior to the social disruption procedure, C57BL/6J mice were screened in a preliminary study for aggressive behaviors towards a separate cohort of BALB/c mice to ensure the defeat of the resident experimental mice, as previously described.²³ The heaviest C57BL/6J mice were selected for the aggression test. Latency of first attack and dominance status of mice were visually determined by observing key behaviors such as tail rattling, chasing or biting for dominance and escape and upright postures or immobility for submission.^{24,25} Mice being aggressive, dominant and presenting the lowest latency to attack were then selected to be aggressive intruders for the stress procedure.²⁶ Four C57BL/6J mice were excluded in the selection.

Social disruption model

The social disruption model had been extensively used and previously described by our laboratory¹⁸ and others, with slight modulation in this study. Briefly, an older and larger male C57BL/6J (the intruder) was placed into the cage containing an established social hierarchy of three young male BALB/c (the residents) for a 2-hour confrontation beginning at 4:30 p.m. Animals underwent six SDR cycles over a week; three nightly cycles, one night off, and three more cycles. Observations were made to ensure that the aggressors defeated the experimental subjects. To avoid habituation to the intruder, aggressors were rotated daily. An intruder displaying no aggressive behavior within the first 5 minutes towards resident mice was replaced by another one. However, when severe injuries occurred, the resident mouse was excluded. In the present study, no experimental animal was excluded. Different aggressors were used on consecutive nights. Mice in the control group were left undisturbed.

Sensitization, antigen challenge, and social disruption stress schedule

The challenge schedule is presented in Figure 1 and

briefly summarized below: day 1 and day 8, sensitization (i.p.); days 15 to 28, antigen challenge (inhalation); days 22 to 24, social disruption stress; and days 26 to 28, social disruption stress.

The open field test (OFT)

In order to study the effects of repeated psychosocial stress on behaviors in an OVA-induced murine asthmatic model, we used the open field video tracking system. On the morning immediately following the final cycle of social disruption stress, spontaneous activity under novelty stress was measured for 5 minutes. As previously described,²⁷ all mice were acclimatized to the test room for 1 hour and thereafter placed in a clear Plexiglas chamber measuring 270 mm×270 mm×200 mm with arrays of 16×16 photo detector, positioned 2.5 cm and 10 cm above the floor of the chamber. The software (Activity Monitor version 5, Med Associates) allowed a distinction to be made between repetitive interruptions of adjacent photo beams. As indices of ambulatory counts (number of individual horizontal movements registered when the mice walked on all four feet) and vertical counts (rearing counts registered when a mouse's body inclined vertically with hind paws on the floor and forepaws on the wall of the activity cage) were evaluated. The time spent at the centre of the chamber served as an index of anxiety.²⁸ The equipment allowed four mice to be tested at one time. Moreover, the number of fecal boli was counted at the end of each trial. In order to reduce anxiety caused by the environment, a low-level illumination was used throughout the experiment. After the test of one mouse, the chamber was wiped by 30% alcohol in order to avoid interference between different mice.

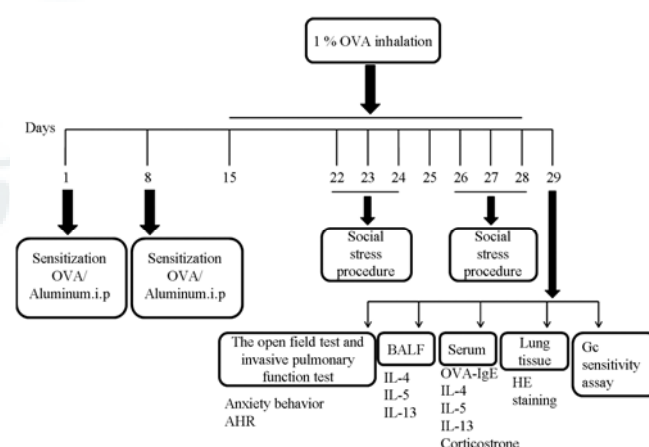


Figure 1. Sensitization: a peritoneal injection of sterile saline of 0.5 ml containing OVA of 100 µg and aluminum hydroxide of 4 mg at the 1st and 8th days (7 days apart). Antigen challenge: aerosolized antigen (1% OVA) for 30 minutes via an ultrasound aerification inhaler from day 15 to day 28 for 14 days. Psychosocial stress: experimental subjects underwent six SDR cycles over a week at Days 22–24 and Days 26–28. On the morning of day 29 immediately following the final cycle of social disruption, anxiety behaviors were measured in the OFT and AHR was assessed by invasive pulmonary function test and then animals were sacrificed for sample collection.

Measurement of AHR by Buxco's modular and invasive system

After the OFT, AHR was assessed more directly by measuring changes in RL and CYDN in response to increasing concentrations of inhaled Mch. Before Mch challenge, the mice were anesthetized with pentobarbital sodium (60–70 mg/kg i.p.). Then a tracheostomy was made, the trachea was cannulated, the pleural spaces were opened, and the mice were placed in a whole-body plethysmography (WBP) chamber for anesthetized animals.²⁹ After a stable baseline airway pressure (<5% variation over 2.5 minutes) was reached, saline and increasing concentrations of Mch (3.125 mg/ml, 6.25 mg/ml, 12.5 mg/ml) in succession were administered via a jet nebulizer into the head chamber. Minimum values for RL were determined and expressed as percent change from the baseline value.³⁰ The date was excluded if the mouse died during the detection, because the result of RL was extremely high and inaccurate.

Inflammatory cytokines and corticosterone assay in serum

Blood was collected by retro-orbital bleeding and separated in a refrigerated centrifuge at 4°C. Serum was stored at -80°C until assays. Levels of OVA-IgE, IL-4, IL-5, IL-13 and corticosterone in serum were measured using ELISA kits according to the manufacturer's instructions. Taking corticosterone as an example, 10 µl of serum and 0.5 µl of steroid displacement reagent was diluted in 990 µl of assay buffer, completing a 100-fold dilution. Plates were coated with 100 µl of anti-corticosterone antibodies overnight at 4°C, then blocked with assay buffer for 2 hours at room temperature. After two washes with PBS-Tween, 100 µl of standards and serum samples were added and incubated overnight at 4°C. Plates were washed four times before 100 µl/well of biotinylated anti-corticosterone was added. After 45 minutes at room temperature, plates were washed six times with PBS-Tween. After addition of 100 µl/well of a 1:1000 dilution of avidin-peroxidase, plates were incubated at room temperature for 30 minutes. After eight washes, 100 µl of substrate solution was added per well, and the enzymatic reaction was allowed to develop at room temperature. OD was measured at 405 nm on an ELISA plate reader. Corticosterone concentrations were quantified by comparison to the standard curves. Samples were analyzed in duplicate in a single assay; threshold of detection = 150 pg/ml, coefficient of variation limit = 9.6%, and concentration expressed in pg/ml.

Collection and analysis of bronchoalveolar lavage fluid (BALF)

In the invasive pulmonary function test, the mice's tracheas were cannulated, and their chests were opened. BALF was collected from the left lung by means of bronchial ligation and that was performed four times (0.8 ml PBS/lavage) through the tracheal cannula.³¹ The retrieved lavage aliquots were pooled and centrifuged. The supernatant was stored at -20°C for cytokine

detection. The levels of IL-4, IL-5, IL-6, IL-13 and TNF-α in BALF were detected by ELISA kits following the manufacturer's instructions.

Lung histological analysis

After the left lung had been lavaged, the middle lobe of the right lung was excised from each mouse, fixed in 4% formalin, and embedded in paraffin. Sections 3–4 µm thin from blocks were stained with H&E. Histopathological assessment (light microscopy) was performed blind on randomized sections. Inflammatory changes were graded using a semi-quantitative scale of 0–5 for perivascular eosinophilia, peribronchiolar eosinophilia, epithelial damage, and edema.³²

Splenocyte isolation and corticosterone sensitivity assay

Glucocorticoid-induced cell death was quantified, as described previously.^{22,33–35} A single-cell splenocyte suspension was prepared, and added in triplicate (2.5×10^5 cells/well) to flat-bottom 96-well plates at a volume of 100 µl/well for cell proliferation assays and 300 µl/well to flat-bottom 48-well plates for cytokine analysis. Cells were stimulated with 1 µg/ml LPS and corticosterone (dose range 0.01–5 µmol/L) at 37°C in a 5% CO₂ incubator. After 18 hours of incubation, cell supernatants were harvested and frozen at -20°C for cytokine analysis. Other cultures were incubated for 44 hours, at which time cell viability was determined by CCK-8 assay, and read at 450 nm by an ELISA plate reader.³⁶ Cell viability was expressed as percentage of OD of the no-corticosterone control.

PCR detection using real-time fluorescent quantitation

The total RNA was extracted with Trizol. For cDNA synthesis, total RNA (2 µg) was used to generate cDNA in each sample by using SuperScript II reverse transcriptase with 0.5 µg oligo (dT) primers. The OD₂₆₀ of prepared total RNA was measured with spectrophotometer and then used to determine the RNA concentration. One hundred nanograms of total RNA from each sample was applied to 1% agarose gels to determine the purity and quantity of RNA by ethidium bromide stain. The intensity of 28S rRNA bands, determined by densitometry, was used to control RNA amounts used in real-time PCR analysis. One microliter of cDNA was amplified in each PCR reaction mixture containing 200 nmol/L of sense and antisense primers of selected genes. The internal control gene used was GAPDH. For PCR amplification, the following mouse-specific sense and antisense primers were used: GR, 5'-GGA ATA GGT GCC AAG GGT CT-3' (forward) and 5'-GAG CAC ACC AGG CAG AGT TT-3' (reverse); GAPDH, 5'-GGG GAG CCA AAA GGG TCA TC-3' (forward) and 5'-GAC GCC TG C TTC ACC ACC TTC TTG-3' (reverse). PCR amplification was performed on a Light Cycler FastStart DNA Master SYBR Green I. The amplification was performed in a Roche Light Cycler 2.0 instrument under the following condition: initial denaturation at 95°C for 10 minutes, 40 cycles of amplification at 95°C for 20 seconds, annealing at 56°C

for 15 seconds, and then extension at 72°C for 20 seconds.

Western blotting evaluated the GR expression in lung tissues

Lungs were homogenized in lysis buffer and incubated for 30 minutes at 4°C. Cell debris was removed by micro-centrifugation, and supernatants were quickly frozen. The protein concentration was determined by the BSA method. Thirty micrograms of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, then electroblotted onto a PVDF membrane. The immunoblot was incubated for 1 hour with 5% milk at room temperature, and then incubated overnight at 4°C with a 1:1000 dilution of anti-GR antibody and 1:10000 dilution of anti-GAPDH antibody. Blots were washed three times with Tween-20/Tris-buffered saline (TTBS) and then incubated with a 1:1000 dilution of HRP-conjugated secondary antibody for 2 hours at room temperature. Blots were again washed three times with TTBS and then developed by enhanced chemiluminescence. Band intensities were quantified using UN-SCAN-IT gel analysis software (version 6). The optical density for glucocorticoid receptors was shown as a proportion of GAPDH optical density.

Statistical analysis

Experimental data was analyzed with the SPSS17.0 package. The results were presented as mean $\pm$ standard deviation (SD). We did multiple statistical analyses to compare two groups. If there were more than two groups, we first used a normal testing method and homoscedasticity analyses via the Shapiro-Wilk method and the Levene method. If the data consisted of a normal distribution and homoscedasticity, we did one-factor analysis of variance (ONE-ANOVA). If the data were not consistent with a normal distribution, we did a rank test whose statistical significance was set at $P < 0.05$.

RESULTS

Repeated psychosocial stress increased anxiety behaviors in the OVA-induced murine asthmatic model in the OFT

The SDR and Asthma-SDR mice had markedly reduced time spent in the center of the open field (Figure 2A), increased ambulatory activity (Figure 2B), decreased vertical activity (Figure 2C), and an increased count of fecal boli (Figure 2D). Vertical activity was significantly decreased (Figure 2C) and the count of fecal boli was increased (Figure 2D) in the Asthma group compared to the Control group.

Repeated psychosocial stress enhanced AHR in the OVA-induced murine asthmatic model

AHR was significantly enhanced in the Asthma and Asthma-SDR group compared with the Control and SDR group at Mch concentrations of 3.125 mg/ml–12.50 mg/ml (Figure 3). Although there was no statistically

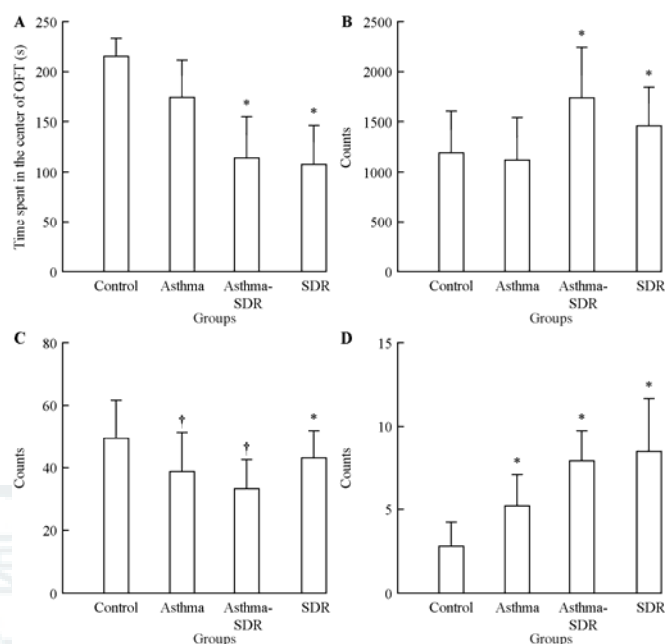


Figure 2. In the OFT, social disruption stress induced anxious behaviors ($n=9$). **A:** The time the mice spent in the center of OFT was significantly shortened in the Asthma-SDR and SDR groups, which served as an index of anxiety. **B:** Social disruption stress markedly increased horizontal activity, which indicated that the mice were active in the periphery region of OFT. This result demonstrates that social disruption stress induces anxiety but not depression when the mice face with a new environment. **C:** The reduction of vertical activity in the Asthma-SDR and SDR groups was also found in the Asthma mice. **D:** Social disruption stress increased the counts of fecal boli during the OFT. * $P < 0.05$, compared with the control group and asthma group in A and B. * $P < 0.05$, † $P < 0.01$, compared with the control group in C and D.

significant difference, RL was higher in the Asthma-SDR group than in the Asthma group at all Mch concentrations.

Repeated psychosocial stress increased serum corticosterone in the OVA-induced murine asthmatic model

To observe the impact of social disruption stress on HPA axis, we detected the level of corticosterone in serum. The corticosterone levels in SDR and Asthma-SDR groups were significantly higher than those in Control and

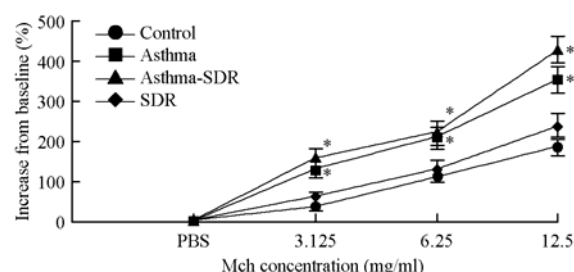


Figure 3. AHR responses to Mch were significantly enhanced in antigen exposed mice compared to the control mice. Although there was no significant difference between the Asthma-SDR group and the Asthma group, the social disruption increased the airway resistance in the allergic mice. Control group, $n=8$; Asthma group, $n=8$; Asthma-SDR group, $n=9$; SDR group, $n=6$. * $P < 0.05$, vs the control group and the SDR group.

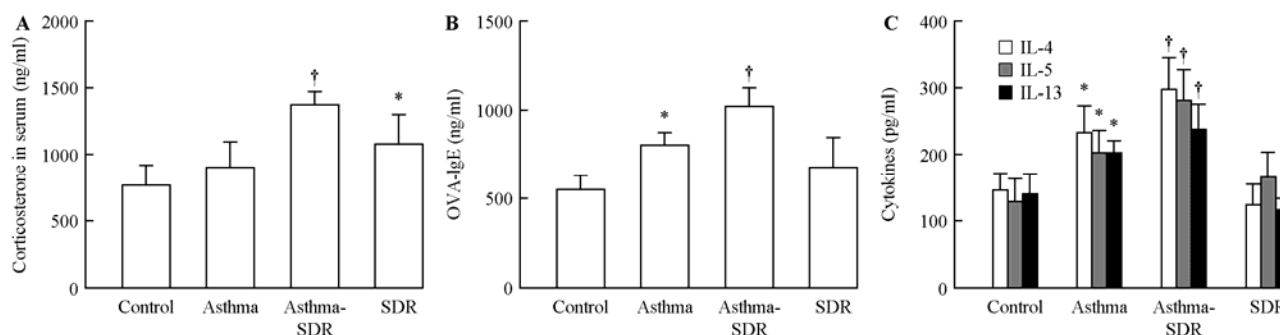


Figure 4. The inhibitory effects of corticosterone on inflammatory cytokines are seriously impaired owing to social disruption stress. **A:** Social disruption stress significantly increases circulating corticosterone in serum, which indicates that stress could activate the HPA axis. **B:** The level of OVA-IgE is significantly increased in the asthma group and asthma-SDR group. **C:** The levels of Th2 inflammatory cytokines (IL-4, IL-5, IL-13) involved in asthma are increased in the serum of the mice with combined antigen exposure and stress compared with antigen exposure alone ($n=9$). $^*P < 0.05$, $^{\dagger}P < 0.01$, compared with the control group and asthma group in **A**. $^*P < 0.05$, $^{\dagger}P < 0.01$, compared with the control group and SDR group in **B** and **C**.

Asthma groups, which indicated the activation of the HPA axis by social disruption stress (Figure 4A).

Repeated psychosocial stress increased inflammation cytokines in serum and BALF in the OVA-induced murine asthmatic model

Serum OVA-IgE levels, which were barely detectable in control mice, were increased by OVA-sensitization and challenge (Figure 4B). Levels of IL-4, IL-5 and IL-13 (Figure 4C) in serum were significantly increased in the Asthma and Asthma-SDR groups compared with those in the Control and SDR groups. Moreover, the Asthma-SDR group produced more OVA-IgE, IL-4 and IL-5 in serum than the Asthma group. The levels of IL-4, IL-5, IL-13 and TNF- α in BALF were significantly increased in Asthma-SDR group compared to the Asthma group (Figure 5). However, there was no significant difference between the Asthma-SDR group and Asthma group in the level of IL-6.

Impact of repeated psychosocial stress on lung histology in the OVA-induced murine asthmatic model

Histological analysis revealed typical pathological features of asthma in the Asthma and Asthma-SDR mice. Numerous inflammatory cells, including eosinophils, had

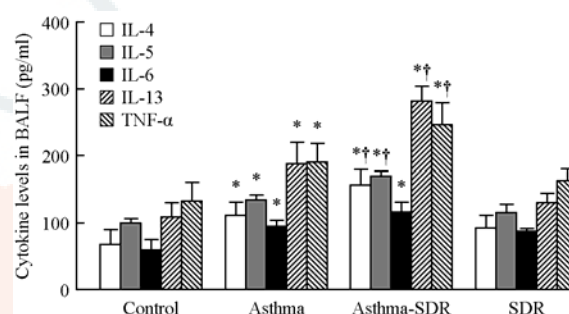


Figure 5. Combined allergy exposure and stress significantly increase the levels of IL-4, IL-5, IL-13, and TNF- α compared to antigen exposure alone in BALF ($n=9$). $^*P < 0.05$, compared with the control group and SDR group. $^{\dagger}P < 0.05$, compared with the Asthma group.

infiltrated around the bronchioles, and mucus and debris had accumulated in the lumens of bronchioles (Figure 6B and 6C) compared with the Control and SDR mice (Figure 6A and 6D). To obtain evidence for the severity of inflammation in lung tissues, we graded perivascular eosinophilia, peribronchiolar eosinophilia, epithelial damage, and edema by using a semi-quantitative scale. Airway inflammation was significantly more severe in the lungs of the Asthma-SDR group compared with those in the Asthma group (Table 1).

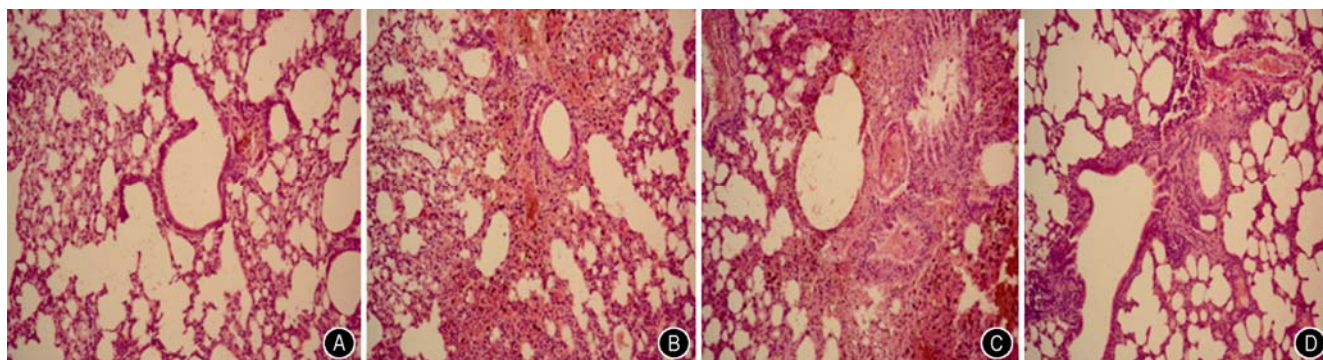


Figure 6. Social disruption stress exacerbated the airway inflammation in the allergic mice from lung histopathology (HE staining, original magnification $\times 100$). According to the lung biopsy with HE staining, the inflammatory cell, (especially eosinophil) infiltration into the perivascular and peribronchiolar was more severe in the Asthma group (**B**) and Asthma-SDR group (**C**) than in the Control (**A**) and SDR (**D**) groups.

Table 1. Inflammatory changes were graded using a semi-quantitative method for perivascular eosinophilia, peribronchiolar eosinophilia, epithelial damage and edema

Groups	Perivascular eosinophilia	Peribronchiolar eosinophilia	Edema	Epithelial damage
Control	1 (1–2)	0 (0–1)	1 (1–1)	1 (1–1)
Asthma	3 (3–5)*	2 (2–3)*	4 (3–5)*	3 (3–4)*
Asthma-SDR	4 (3–4)*	3 (2–4)*†	5 (4–5)*†	4 (3–5)*†
SDR	1 (1–2)	0 (0–1)	2 (1–3)	1 (1–2)

Allergy exposure with or without stress had higher scores than the control and stress alone groups. Moreover, the asthma-SDR mice had severe peribronchiolar eosinophilia, epithelial damage and edema compared to allergy exposure alone.

* $P < 0.05$, compared with the Control group and SDR group. † $P < 0.05$, compared with the Asthma group.

Repeated psychosocial stress *in vitro* mediated splenocyte glucocorticoid insensitivity and impaired glucocorticoid function of inhibition cytokines production in the OVA-induced murine asthmatic model

Splenocyte function, including the release of cytokines, should normally be significantly inhibited by corticosteroids. Such dose-dependent inhibition is clearly shown in Figure 7A in the cells derived from control and asthma mice. However, in splenocytes derived from the SDR group, the inhibitory effects of corticosterone were completely abolished, but strangely, the asthma-SDR mice had not shown obviously impaired corticosterone inhibitory function on splenocytes proliferation. The levels of IL-6 and TNF- α in supernatant demonstrated that the corticosteroid responsiveness of immune cells in the spleen of asthma-SDR mice was significantly impaired (Figure 7B and 7C). The combination of stress and allergy appeared synergistic in reversing the inhibitory effects of corticosterone.

Repeated psychosocial stress induced reduction of GR protein and GR mRNA expression in the lung tissues on the OVA-sensitized murine asthmatic model

To confirm the effects of SDR combined with OVA-exposure on GR expression locally in the lung, we studied GR gene activation in the lung tissues of mice.

Real-time PCR showed that in the Asthma-SDR mice GR mRNA expression was significantly inhibited in the lung (Figure 8A). Furthermore, Western blotting analysis of lung extracts from the mice showed that both sensitization and challenge with OVA and experiencing social stress significantly reduced GR expression compared with the non-SDR mice (Figure 8B and 8C).

DISCUSSION

In the present study, we used a unique murine model that combined psychological stress and allergen exposure to investigate how the psycho-endocrine-immunological axis regulated airway inflammation. Social disruption, a well-characterized psychological stressor, had been used by our laboratory¹⁸ and others in order to assess the impact of a semi-natural stressor on immune function. In this model, a previously established social hierarchy of a stable cohort of younger male mice was disrupted by a large, aggressive male that confronted and repeatedly defeated the cage residents. Previous studies demonstrated that repeated defeat leads to physiological and behavioral responses (collectively referred to as the stress response), such as development of anxiety-like behaviors²⁸ and glucocorticoid release,^{37,38} which were also shown in the present study. The evidence of this was that the time the mice spent in the center of the OFT was significantly shortened, ambulatory activity and counts of fecal boli were marked increased, reflecting panic mood, and vertical activity remarkably diminished because of social disruption stress. The control mice had none of these reactions when faced with the new environment in the OFT. Thus, we regard the model of social disruption as successful in the present study. Besides, the OFT results revealed that anxious behaviors also emerged in the allergy exposure alone mice, in agreement with clinic research. Recently, Vazquez et al³⁹ found that patients with near-fatal asthma showed higher psychological morbidity, notably anxiety, even years after the near-fatal asthma episode. Notably, the interaction between asthma

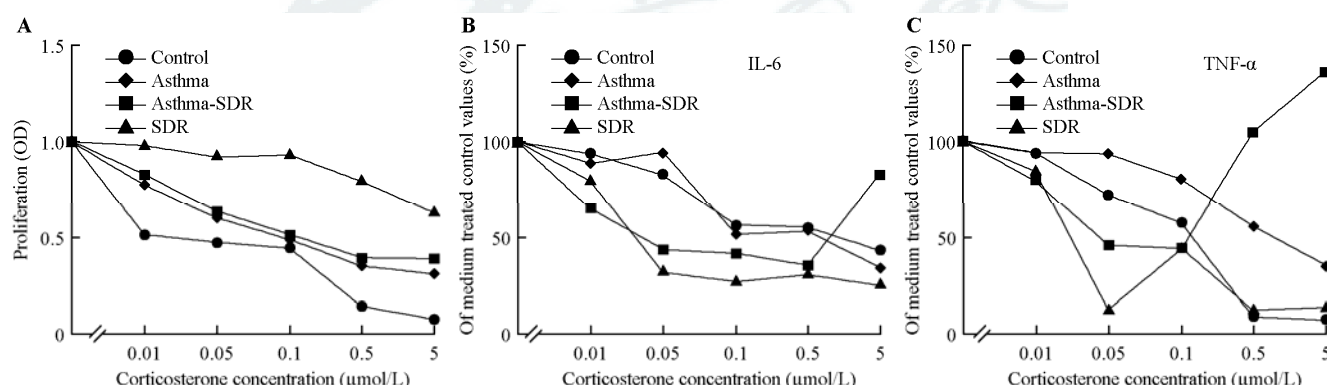


Figure 7. Dose-dependent inhibitory effects of corticosterone on LPS-stimulated splenocytes were abolished by the combination of stress and allergen. Splenocytes were cultured in the presence of LPS (1 $\mu\text{g/ml}$) and 0, 0.01, 0.05, 0.1, 0.5, or 5 $\mu\text{mol/L}$ corticosterone for 44 hours. Cell proliferation was determined using a CCK-8 assay. Cytokine levels in the supernatant were measured by ELISA in each group. Data are presented as the percentage of the number of cells or the cytokine concentration of control values measured in the absence of corticosterone. **A:** Stress by itself markedly diminished inhibitory effects of GC on splenocyte proliferation stimulated by LPS, but additional OVA exposure of the stressed mice did not enhance this effect. **B, C:** The release of IL-6 and TNF- α could not be inhibited by corticosteroids, reflecting impaired glucocorticoid function.

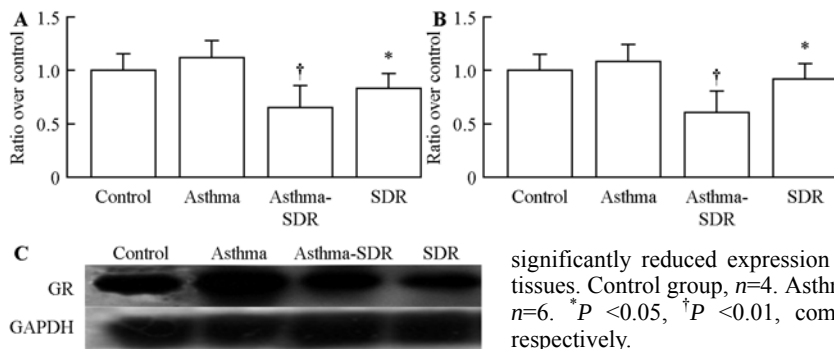


Figure 8. Social disruption stress significantly decreased the expressions of GR protein and GR mRNA in the lung tissues. Total protein and mRNA were isolated from the lungs of mice. Western blotting and real time-PCR were performed. Expression was assessed using densitometric analysis and GAPDH as control. Data were analyzed using the $\Delta\Delta C_t$ method. The combination of antigen exposure and SDR

significantly reduced expression of GR mRNA (**A**) and GR protein (**B**) in the lung tissues. Control group, $n=4$. Asthma group, $n=6$. Asthma-SDR group, $n=6$. SDR group, $n=6$. $^*P < 0.05$, $^{\dagger}P < 0.01$, compared with the control group and asthma group, respectively.

outcomes and psychological disorders is extremely complicated, and our data only showed that asthma might contribute to anxiety and mood disorders.

This study aimed at observing the impact of psychosocial stress on airway inflammation in the murine model of allergic asthma. The results demonstrated that psychosocial stress exacerbated the airway inflammation response to allergen exposure. The enhanced inflammatory changes included increased AHR to Mch, increased inflammatory cytokines in BALF and serum, and elevated numbers of infiltrating cells into the lung. We used the invasive method for directly assessing AHR in anaesthetized mice in order to reduce the unpredictable stress to the mice under consciousness conditions. The result showed that there was no significant difference between the Asthma-SDR group and Asthma group, but RL was higher in the Asthma-SDR group. The reason for this phenomenon might be related to the lower concentrations of Mch that were not enough to induce significant differences. Besides the pulmonary function, levels of inflammatory cytokines, especially IL-4, IL-5, IL-13 and TNF- α , in BALF were significantly increased in the Asthma-SDR group compared with those in Asthma group, which demonstrated that psychosocial stress aggravated local airway inflammation. Lung histological analysis also showed that social disruption stress seriously induced inflammatory cell (especially eosinophil) infiltration into the lungs of mice with stress and allergy exposure.

Our data had shown that social disruption stress induced the release of corticosterone and activated the HPA axis, similar to the physical response when human experience stressful events.^{40,41} However, the above heightened inflammation occurred in the presence of elevated serum corticosterone in mice exposed to social stress and allergen inhalation. Thus, we speculate that psychosocial stress might induce a diminished inhibitory action of endogenous corticosteroids. In order to explain the mechanisms of synergistic effects between repeated psychosocial stress and the allergic immune response, we employed the technique which has been widely reported by others to evaluate corticosteroid sensitivity *in vitro*.^{21,35,42} Briefly, we used the CCK-8 assay to measure the splenocyte proliferation induced by LPS while in the presence of corticosterone (a steroid that binds both the

glucocorticoid and mineralocorticoid receptor with high affinity). In control mice, the proliferation of splenocytes induced by LPS could be inhibited by corticosterone. However, psychosocial stress by itself markedly decreased the inhibitory effect of corticosterone on immune cell proliferation and cytokine production, and strangely, combining OVA exposure with stress did not enhance this effect. This observation is consistent with results from previous studies.^{22,42} Therefore, the synergistic effects on the reversal of glucocorticoid sensitivity between OVA inhalation and stress could not be explained and will need further study.

In our preliminary study,¹⁸ we found that glucocorticoid receptors played a critical role in glucocorticoid function. Thus, we detected the expressions of GR mRNA and GR protein in the lung tissues. The data demonstrated that social stress significantly diminished GR expression in the lung tissues of mice when we combined stress and allergy exposure, compared with those with allergy exposure alone. Based on above results, we propose that psychosocial stress could induce GR down-regulation, leading to the impaired glucocorticoid function, which might be the potential mechanism that psychosocial stress triggers and exacerbates asthmatic airway inflammation. This hypothesis has previously been examined among subjects with asthma.¹¹

Although we are the first to report the murine model of combined SDR and OVA exposure, the impact of stress on asthma has been studied in other animal models. In a murine model of ultrasound stress and OVA-induced allergic airway sensitization, stressed mice displayed not only increased leukocyte (i.e., eosinophil) numbers in BALF, but also increased release of IL-4, IL-5, eotaxin and TNF- α in comparison with non-stressed mice.⁴³⁻⁴⁵ Using a sound stress model with pregnant BALB/c mice, Pincus-Knackstedt et al⁴⁶ showed that maternal stress increased susceptibility of the offspring to allergic sensitization to OVA. Forced swimming combined with allergy exposure that shifted mice towards a Th2 response and pulmonary inflammation was not reduced among allergic stressed mice in spite of elevated glucocorticoids.⁴⁷ However, studies demonstrated that airway inflammation response to acute and chronic stress was different. Kang et al⁴⁸ used an "open-field" as the psychological stress to observe Th1 and Th2 airway

cytokine responses and Th1:Th2 cytokine ratios in BALF in response to acute and repeated stress. They found a significant shift toward Th2 predominance in asthmatic mice under acute stress but not chronic stress, and suggested that it might be a mechanism underlying exacerbation of asthma.

Our study not only suggested that GR modulation might be in part involved in the underlying mechanism by which psychosocial stress worsened asthma outcomes, but also raised some intriguing questions. For example, preliminary studies had suggested that a failure to respond to GC might result from reduced GC binding to GR, reduced GR expression, enhanced activation of inflammatory pathways, or lack of corepressor activity.⁴⁹ Thus, reduced GR expression was just one of the reasons for glucocorticoid insensitivity. Whether other factors are involved in the process that psychosocial stress exacerbates asthma is not known. In addition, not all types of stress activate the human HPA axis, somewhat different from that of the social disruption stress.⁵⁰ The function of HPA axis is relevant to the time response, duration to the stress and so on. Thus, the complex interaction between behavioral, neural, endocrine and immune processes, both in human and animals, needs further study.

In summary, we utilized a novel murine model that combined social disruption stress and OVA exposure to study the impact of psychosocial stress on airway inflammation and its underlying mechanism. Our data show that psychosocial stress might induce the down-regulation of GR and then impair the inhibitory effects of GC on the inflammatory response and cytokine production, which enhances the airway allergic response. Undoubtedly, these findings are meaningful because they could guide us to find new interventional targets overcoming the negative effects of stress on asthma and improving the management of asthma.

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