Alteration of endogenous corticosteroids and catecholamines in allergen-induced eosinophilic inflammation in Brown Norway rats

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INTRODUCTION

Asthma is characterized by an increase in airway hyperresponsiveness to various bronchoconstrictive stimuli and reversible airway obstruction. The pathogenesis of airway hyperresponsiveness has been linked to airway inflammation in human asthma.1 Human asthma is associated with infiltration of eosinophils and mononuclear cells into the lungs.2

Clinical investigations have demonstrated that corticosteroids are potent inhibitors of the late asthmatic responses (LAR) in the skin, lung, and nose.3–5 Prevention and treatment with exogenous corticosteroids are very effective for reducing airway inflammation, including eosinophil and T lymphocyte infiltration into the lungs.6

Original Article

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Abstract

Although various types of stress activate a pituitary adrenal response, the alteration of endogenous corticosteroids and catecholamines during asthma remains unclear. The aim of this study was to assess changes in endogenous corticosteroid and catecholamine levels in allergic eosinophilic inflammation in rats, using metabolic cages. Brown Norway rats (female, 6 weeks old) were sensitized with intraperitoneal injections of ovalbumin on days 0 and 2 and challenged with either an aerosol of ovalbumin or saline for 30 min on day 21. Levels of urinary 11-hydroxycorticosteroid (OHCS), a primary metabolite of corticosterone; epinephrine and norepinephrine were determined in pooled samples taken 0–24 h before and 8–32 h after the challenge. Serum adrenocorticotropic hormone, corticosterone levels and cell counts in bronchoalveolar lavage fluid were assessed 32–36 h after the challenge, as well as lung eosinophil peroxidase activity, an indirect index of eosinophil infiltration. The numbers of total cells and eosinophils in bronchoalveolar lavage fluid and lung eosinophil peroxidase activity were significantly increased in the ovalbumin-challenged rats compared with the saline-challenged rats. While urinary OHCS and serum corticosterone levels were significantly increased after challenge in the ovalbumin-challenged rats, compared with the saline-challenged rats (2.1 ± 0.1 × 10⁻¹ vs 1.7 ± 0.1 × 10⁻¹ mg/g creatinine, P < 0.05 and 482 ± 49 vs 348 ± 19 ng/mL, P < 0.02, respectively), serum adrenocorticotropic hormone levels did not differ between the two groups. Urinary epinephrine and norepinephrine excretion also did not differ between the two groups. It is concluded that endogenous corticosterone, but not catecholamine, increases as a pathophysiologic adrenal response, possibly to protect lung during allergic eosinophilic inflammation.

Key words: airway inflammation, asthma, Brown Norway rat, endogenous corticosteroid, metabolic cage, urinary catecholamine excretion, urinary 11-hydroxy corticosteroid excretion.
Endogenous corticosteroids may also be important. For example, metyrapone, an \(\text{11}^\beta\)-hydroxylase inhibitor that blocks the synthesis of corticosterone, has been found to enhance airway inflammation in response to an inhaled allergen in experimental animals.\(^7\) Thus, both exogenously administered and endogenously produced corticosteroids may down-regulate eosinophilic inflammation.

It is well documented that various types of stress, including infection, surgical procedures and inflammation, activate pituitary-adrenal responses by increasing cytokines and neuropeptides, thereby increasing production of endogenous corticosteroids and catecholamines in humans and experimental animals.\(^8,9\) However, Nakazawa et al. and Durham et al. have demonstrated that serum cortisol levels do not increase during LAR in human asthma.\(^10,11\) Turner et al. have reported that serum corticosterone levels increase in asthmatic Brown Norway (BN) rats beyond the normal range reported in the previous literature;\(^12\) however, it seems possible that stresses other than asthma, including inhalation, handling and blood sampling, may have increased serum corticosterone levels in this study. Because the alteration of endogenous corticosteroids during eosinophilic inflammation in asthma is still controversial, it is of interest to assess pathophysiologic adrenal responses with minimal stress in a simple experimental model of asthma.

Ovalbumin (OVA)-induced airway eosinophilia and hyperresponsiveness in the BN rat is a widely used system, which mimics human asthma in several respects. Pathophysiologic investigations have revealed a close relationship between antigen-induced infiltration of inflammatory cells, mostly eosinophils, and either LAR or airway hyperresponsiveness in this model, although the precise underlying mechanisms are not fully understood.\(^13,14\)

In the present study, we maintained animals in metabolic cages for assessment of the urinary excretion of \(\text{11}\)-hydroxycorticosteroid (OHCS), a primary metabolite of corticosterone, as well as epinephrine and nor-epinephrine during airway inflammation in asthmatic BN rats. In addition, serum adrenocorticotropic hormone (ACTH) and corticosterone levels were measured as well as indices of inflammation.

**METHODS**

**Animals**

Virus-free, inbred Brown Norway rats (5 weeks old; Charles River Japan Inc.) were kept in temperature-controlled rooms under a constant light cycle. Animals were allowed free access to water and a standard laboratory diet and the study protocol was approved by the animal care committee of Nagoya City University.

**Sensitization procedures and allergen challenge**

We randomly assigned animals into the following three groups: (i) naive (\(n = 6\)); (ii) saline-challenged (\(n = 9\)); and (iii) OVA-challenged (\(n = 9\)). Rats of the saline- and OVA-challenged groups were sensitized with intraperitoneal injections of \(1 \text{ mg OVA (Sigma, Nagoya, Japan), containing Al(OH)}_3 (100 \text{ mg/mL}) \text{ in } 0.9\% \text{ saline,}\) on days 0 and 2 and then challenged with either exposure to aerosol of 0.9% saline or 1% OVA, respectively, for 30 min on day 21. The naive group rats (non-sensitized and non-challenged) were kept in identical conditions to the sensitized rats for 3 weeks and studied thereafter at the same time points. The aerosol was generated with a pumping nebulizer (Yagami, Nagoya, Japan; particle size 1–10 \(\mu\)m, 5 L/min) connected to a plexiglass exposure chamber with a volume of 5L.

**Urine collection**

All groups of animals were kept in metabolic cages (Chubu Science, Nagoya, Japan), which separate urine from stools, from 1 day before the challenge with aerosol to the end of the experiments. Urine samples were pooled both for 0–24 h before and for 8–32 h after the challenge.

**Bronchoalveolar lavage and cell counting**

Bronchoalveolar lavages (BAL) were performed 32–36 h after challenge with saline or OVA aerosol (between 10.00 and 14.00 h) to minimize the influence of any circadian rhythms on the parameters. The abdominal cavity was incised under intraperitoneal anesthesia with sodium pentobarbital (60 mg/100 g weight). After blood was taken from the abdominal aorta, the trachea was cannulated with a catheter. Ten milliliters of \(\text{Ca}^{2+}\text{- and Mg}^{2+}\text{-free phosphate-buffered saline (PBS) with } 5 \text{ mmol/L ethylenediaminetetraacetic acid (EDTA) was instilled into the bronchoalveolar cavity and then withdrawn. This procedure was repeated four times. After BAL fluid (BALF) was collected, the lungs were removed, weighed and kept in a } –40\text{°C freezer until eosinophilic peroxidase (EPO) activity was determined. The blood was}
centrifuged and the serum stored in a – 40°C freezer for the subsequent analysis.

Cells in the BALF were sedimented by centrifugation (10 min at 200 g, 4°C) and resuspended in PBS. Total leukocyte counts were determined by autoanalyzer Model F300 (Sysmex Toa, Tokyo, Japan). Macrophages, eosinophils, neutrophils and lymphocytes in cyt centrifuge preparations were stained with Diff-Quick (Kokusai shiyaku, Kobe, Japan). Cells were identified on the basis of standard morphologic features. One thousand cells were counted under 400-fold magnification. The absolute and percentage numbers for each cell type were determined.

**Measurement of lung eosinophil peroxidase activity**

The lung (EPO) activity was measured as described by Schneider and Issekutz. Approximately 300–400 mg lung tissue (10–20% of the total lung) was homogenized in 20 mmol/L potassium buffer (pH 7.4) containing 1 mmol/L EDTA and centrifuged at 6000 g for 20 min at 4°C. The pellet was rehomogenized in 50 mmol/L acetic acid (pH 6.0) containing 0.5% cetyltrimethylammonium hydroxide and sonicated for 10 s. The EPO activity in the supernatant was then determined.

To determine EPO activity, aliquots were diluted 1/50 in 50 mmol/L HEPES pH 8.0, added to the EPO sub-strate solution, consisting of 50 mmol/L HEPES (pH 8.0), 6 mmol/L KBr, 3 mmol/L o-phenylene diamine, 4.5 mmol/L H2O2, and then incubated at 37°C for 3 min. The reaction was stopped by adding 150 μL of 4 mol/L H2SO4 and the absorbance at 490 nm was determined.

**Measurement of urinary 11-OHCS**

An aliquot of 4 mL urine was adjusted to pH 5.0 with 10% Na2CO3 and 2.4 mL pipetted into a glass-stoppered test tube, containing 0.4 mL acetate buffer and 2000 Fishman units of β-glucuronidase. After incubation without a cap at 48°C for 15 h, 0.5 mL 0.25 mol/L NaOH was added. The urine was shaken for about 40 s with 5 mL methylene chloride and then centrifuged, the urine layer removed by suction and the extract washed with 0.1 mol/L NaOH in 1.0 mL 10% Na2SO4. After the last wash, the extract was centrifuged and the aqueous layer carefully removed. Aliquots of the extract (3 mL) were pipetted into each of two test tubes, with 0.3 mL ethanol. Phenylhydrazine-sulfuric acid solution (0.5 mL) was added to one tube and 0.5 mL diluted sulfuric acid to the other. After shaking for 30 s, the test tubes were left for 15 min. The methylene chloride layer was then removed by suction and the remaining phase incubated in 60°C in a water bath for 30 min. During the incubation, the remaining methylene chloride vaporized. After incubation, the optical densities of the samples against blanks were measured at 410 nm in 10 mm cuvettes.

**Hormone measurements**

Serum corticosterone levels were determined using a commercially available radioimmunoassay kit (coated-A-count, Los Angeles, CA, USA). Plasma ACTH was assayed immunoradiometrically employing a commercial kit (Mediphysix, Tokyo, Japan).

**Data analysis**

Unless stated otherwise, group results are expressed as mean ± SEM. Concentration–response curves were tested with an analysis of variance (ANOVA, two-way). Comparisons among the groups were performed with one-way ANOVA using the StatView data analysis system, version 4.5 (Abacus Concepts, Inc., Berkeley, CA, USA). Statistical significance was concluded at the 5% level of confidence.

**RESULTS**

**Bronchoalveolar lavage**

Ovalbumin-challenged rats had significantly increased total cell counts, including eosinophils and lymphocytes, in BALF at 32–36 h after the challenge compared with the naive and the saline-challenged rats (P < 0.0001, P < 0.0001 and P < 0.05, respectively, Fig. 1). Eosinophil counts in BALF in the naive, saline-challenged and OVA-challenged rats were 1.1 ± 0.8 × 10, 3.3 ± 1.2 × 10 and 4.4 ± 0.2 × 10/μL, respectively. There were no significant differences in the numbers of macrophages and neutrophils in BALF among the three groups.
Lung eosinophil peroxidase activity

Lung EPO activity at 32–36 h after the challenge significantly increased in the OVA-challenged rats compared with the naive and saline-challenged rats (2.93 ± 0.39 × 10^-1 vs 0.94 ± 0.53 × 10^-1 and 1.25 ± 0.24 × 10^-1; P < 0.01, Fig. 2).

Urinary 11-hydroxycorticosteroid excretion

Urinary 11-OHCS excretion for 8–32 h after the OVA challenge significantly increased compared with the values for 0–24 h before the challenge (P < 0.04, Fig. 3a). The values before the challenge were 1.69 ± 0.1 × 10^-1 and 1.79 ± 0.1 × 10^-1 mg/g for the OVA- and saline-challenged rats, respectively, and after the challenge were 2.03 ± 0.1 × 10^-1 and 1.74 ± 0.1 × 10^-1 mg/g, respectively.

Serum corticosterone and adrenocorticotropic hormone levels

Serum corticosterone levels at 32–36 h after the challenge were significantly increased in the OVA-challenged rats, compared with the naive and saline-challenged rats (482 ± 49 vs 348 ± 19 and 341 ± 33 ng/mL, respectively; P < 0.02 for both, Fig. 3b).

Serum ACTH levels at 32–36 h after challenge did not significantly differ among the three groups, although the values were slightly higher in the OVA-challenged rats (data not shown).

Correlation between urinary 11-OHCS excretion and serum corticosterone levels

There was a significant correlation between urinary 11-OHCS and serum corticosterone levels among the values for the saline- and OVA-challenged rats (r = 0.574, P < 0.01, n = 17; Fig. 4).
Urinary epinephrine and norepinephrine excretion before and after challenge did not significantly differ among the three groups (Table 1).

**DISCUSSION**

The present study clearly demonstrates that urinary 11-

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**Fig. 3** (a) Urinary 11-hydroxycorticosteroi (OHCS) excretion in the naive \((n = 6)\) rats and 0–24 h before \((n = 9; \square)\) and 8–32 h after \((n = 9; \square)\) challenge in the saline- and ovalublin (OVA)-challenged rats. *\(P < 0.04\) compared with the saline-challenged rats after challenge. i\(P < 0.05\) compared with the OVA-challenged rats before challenge. (b) Serum corticosterone levels in the naive rats \((n = 6; \square)\) and at 48 h after challenge in saline- \((n = 8; \square)\) and OVA-challenged rats \((n = 8; \square)\). *\(P < 0.02\) compared with the naive or saline-challenged rats.

**Fig. 4** Correlation between urinary 11-hydroxycorticosteroid (OHCS) excretion and serum corticosterone levels for the saline- and ovalublin-challenged rats \((r = 0.574, n = 17)\).

**Table 1** Effects of saline and ovalublin challenge on the urinary excretion of epinephrine and norepinephrine in ovalublin-sensitized rats

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Before</th>
<th>After</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine</td>
<td>Naive</td>
<td>4.70 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>3.75 ± 0.56</td>
<td>4.31 ± 0.58</td>
<td>—</td>
</tr>
<tr>
<td>OVA</td>
<td>2.44 ± 0.78</td>
<td>3.48 ± 0.27</td>
<td>—</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>Naive</td>
<td>52.1 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>44.1 ± 9.4</td>
<td>51.9 ± 6.7</td>
<td>—</td>
</tr>
<tr>
<td>OVA</td>
<td>70.6 ± 10.1</td>
<td>50.7 ± 3.8</td>
<td>—</td>
</tr>
</tbody>
</table>

OVA, ovalublin.

OHCS and serum corticosterone levels are significantly increased, whereas serum ACTH levels and urinary epi-

epinephrine and norepinephrine excretion are not altered, during allergen-induced eosinophilic inflammation in BN rats.

The OVA aerosol challenge caused an increase in the numbers of total cells and eosinophils in BALF and in lung EPO activity, an indirect index of eosinophil infiltration into the lungs, at 32–36 h after challenge. Elwood et al. have also reported a significant increase in eosinophil recovery in BALF (i.e. airway inflammation) both 5–8 h and 18–24 h after allergen challenge. Thus, the period from 8 to 36 h after the challenge can be considered to coincide with eosinophilic inflammation in this model.
Because the adrenal glands of the rat lack the 17α-hydroxylase that is needed to convert progesterone into cortisol, corticosterone is the major endogenous corticosteroid and was therefore chosen for determination in the present study. Diurnal variation is known to be substantial, with the normal non-stressed serum corticosterone level ranging from 50 ng/mL at approximately 6 h to a peak of 400 ng/mL 12 h later. It would be ideal to continuously monitor the serum corticosterone levels during the experimental period to assess the pituitary-adrenal response in the rat, but this is impossible without inflicting stresses. Previous studies have demonstrated that the stress of tail shock caused increases in endogenous ACTH and corticosterone levels in rats. We hypothesized that animal handling, immobilization and injection into the tail may have a profound influence on the animal’s pituitary-adrenal system. Therefore, we assessed urinary 11-OHCS excretion, a main metabolite of corticosterone, using metabolic cages during eosinophilic inflammation and also measured serum corticosterone levels. Although we found a weak but significant correlation between urinary 11-OHCS excretion and serum corticosterone levels, measurement of urinary 11-OHCS excretion that reflects the amount of endogenously secreted corticosterone may be particularly useful and reliable.

In contrast to our findings, Durham et al. have reported that serum cortisol levels at 3 h and 9 and 24 h after allergen inhalation are virtually identical to those observed with PBS in man, with no relationship to either the size of the LAR or circulating eosinophil counts. Similarly, Nakazawa et al. have also investigated the serum cortisol levels in two types of asthmatic response in the rat, but this is impossible without inflicting stresses. Previous studies have demonstrated that the stress of tail shock caused increases in endogenous ACTH and corticosterone levels in rats. We hypothesized that animal handling, immobilization and injection into the tail may have a profound influence on the animal’s pituitary-adrenal system. Therefore, we assessed urinary 11-OHCS excretion, a main metabolite of corticosterone, using metabolic cages during eosinophilic inflammation and also measured serum corticosterone levels. Although we found a weak but significant correlation between urinary 11-OHCS excretion and serum corticosterone levels, measurement of urinary 11-OHCS excretion that reflects the amount of endogenously secreted corticosterone may be particularly useful and reliable.

In contrast to our findings, Durham et al. have reported that serum cortisol levels at 3 h and 9 and 24 h after allergen inhalation are virtually identical to those observed with PBS in man, with no relationship to either the size of the LAR or circulating eosinophil counts. Similarly, Nakazawa et al. have also investigated the serum cortisol levels in two types of asthmatic response in atopic patients with asthma and have found a slight increase during the immediate response but a decrease during LAR. They have speculated that LAR may be caused by decreased synthesis of endogenous corticosteroids. The reason for this discrepancy is unclear. However, we speculate that patients with asthma have a more complex psychoneuroendocrine system and serum cortisol responses than those encountered in a rat asthma simple model.

Corticosteroids were originally thought to protect organisms during periods of stress via an enhancement of normal defense mechanisms. More recent studies have demonstrated that corticosteroids act in a suppressive manner, probably to protect against host defense mechanisms activated by stress. Furthermore, it is generally accepted that serum cortisol levels increase in response to stress following stimulation of corticosteroid-releasing factor and ACTH. However, serum ACTH levels at 32–36 h after allergen inhalation were not significantly increased in the present study. We think that the lack of increase in serum ACTH may be explained by the previous finding that serum ACTH levels transiently increase prior to the increase in corticosteroids and that ACTH is metabolized very quickly in vivo. We speculate that eosinophilic inflammation may cause certain types of stress to the host and a consequent increase in serum corticosterone. Thus, endogenous corticosterone may increase as a pathophysiologic adrenal response to protect the lung, although this is insufficient to suppress eosinophilic inflammation. Our data do not support the hypothesis that exhaustion of endogenous corticosterone causes LAR in asthma.

Catecholamines are released into the circulation in response to stress states, such as hypotension, hypoxia, exercise and fear. High levels of both plasma epinephrine and norepinephrine have been shown in life-threatening illnesses, such as acute myocardial infarction, major trauma and cardiac arrest. Norepinephrine is released from sympathetic nerve terminals, while epinephrine and dopamine are released from the adrenal medulla. It has been demonstrated that norepinephrine levels are increased, while epinephrine levels remain at normal levels during acute moderate asthma in man. Similarly, Parke et al. have reported that, despite clinically severe respiratory obstructions, few patients had raised catecholamine levels. This is generally consistent with our findings, indicating that urinary epinephrine and norepinephrine excretion are not increased in the present model. Thus, we can speculate that catecholamines may not increase beyond the physiologic range during acute, mild or moderate asthma, whereas they may increase during more severe asthma with circulatory failure and the response governing endogenous secretion of epinephrine and norepinephrine may be less sensitive than that for corticosterone in this mild asthma model.

In conclusion, endogenous corticosterone, but not epinephrine and norepinephrine, is increased during antigen-induced eosinophilic airway inflammation in the rat. Measurement of urinary levels of endogenous factors appears to be a useful approach to assess pathophysiologic changes with minimal stress in asthma rats. Further application of this method in the rat asthma model should enable us to elucidate the roles of various types of endogenous factors, including other hormones and cytokines, during eosinophilic inflammation.
REFERENCES