

Upregulation of Tumor Necrosis Factor-Alpha by Stress and Substance P in a Murine Model of Allergic Airway Inflammation

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Key Words

Airway inflammation • Allergy • Eosinophils • Mouse •
Sound stress • Substance P • Tumor necrosis factor- α

Abstract

Objective: Clinical observation has suggested that stress and asthma morbidity are associated, though underlying mechanisms are not clearly understood. After having established a mouse model of stress-exacerbated allergic airway inflammation, we demonstrated a stress-mediating role for neurokinin-1 receptor, the main substance P (SP) receptor. Here, our aim was to investigate the influence of stress or exogenously applied SP on airway inflammation and on the local cytokine production of immune cells. **Methods:** BALB/c mice were systemically sensitized to ovalbumin (OVA) and repeatedly challenged with OVA aerosol. Sound stress was applied to the animals for 24 h, starting with the first airway challenge. Alternatively, one group of non-stressed mice received intranasal SP before airway challenges. Cell numbers were determined in bronchoalveolar lavage (BAL) fluid. Leukocytes from mediastinal lymph nodes were analyzed by flow cytometry to determine the percentages of T cells producing interleukin-4, interferon- γ and tumor necrosis factor- α . **Results:** In BAL fluids of stressed or SP-treated animals, significantly higher total cell counts were found compared to non-stressed mice. In lymph nodes, the per-

centage of TNF- α -positive T cells was higher in stressed mice and mice after application of SP. In contrast, the influence of stress did not increase the percentages of interferon- γ -positive CD3+ cells, meanwhile the application of SP increased the percentages of T cells positive for this cytokine. **Conclusion:** Our data provide further evidence for a stress-mediating neuroimmunological pathway that, putatively via SP, is able to influence the composition of immune cells in different compartments of allergic airway inflammation.

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Introduction

Stress has been related to acute and chronic alterations in the immune system, e.g. modification in cytokine expression profiles and changes in the distribution of lymphocytes in different tissues [1]. It has been shown that stress acts as a precipitating or provoking factor in inflammatory diseases like rheumatoid arthritis [2] or inflammatory bowel disease [3, 4]. Bronchial asthma is another example for a chronic disease that might be influenced by emotional stress. Various clinical studies have demonstrated an association between distressful experiences and the onset of asthma symptoms [5–7]. However, the pathophysiological role of stress in bronchial asthma remains largely unknown.

Table 1. Experimental groups and substances used for sensitization, pretreatment and challenge

Group	Mice n	Sensitization	Challenge	Stress	Pretreatment before challenge/stress
I	10	OVA/Al(OH) ₃	OVA	no	PBS
II	10	OVA/Al(OH) ₃	OVA	yes	PBS
III	7	OVA/Al(OH) ₃	OVA	no	SP intranasal

Stress has been defined as a stimulus that activates both, the hypothalamic-pituitary-adrenal axis as well as the sympathetic nervous system, thereby inducing neuronal and humoral changes that may finally lead to an adaptation of the individual to the changed environment [8]. Recent studies in animals indicate that stress may further lead to the release of neuropeptides, especially the tachykinin substance P (SP) [for review, see 9], and thereby induces or exacerbates inflammatory processes [10–13].

Regarding the pathophysiology of asthma, tachykinins are supposed to play an important role due to their capacity to induce neurogenic inflammation, bronchoconstriction and vascular leakage [14, 15]. In allergic airway inflammation, tachykinins are released into the airways after exposure to allergen. Higher levels of SP have been found in bronchoalveolar lavage (BAL) fluid of atopic subjects compared to non-atopic individuals [16]. These differences were further increased by allergen provocation [16]. In a guinea pig model of allergic airway inflammation, a significant increase in SP was detected in lung tissues after allergen challenge [17]. SP found in the airways presumably derives from afferent nerve fibers lining the mucosal epithelium that secrete tachykinins like SP and neurokinin A [18, 19]. Additionally, inflammatory and immune cells that are attracted to the airways during allergic inflammation, e.g. eosinophils [20–22], macrophages [23], lymphocytes [24] and dendritic cells [25], are able to secrete these mediators in response to various stimuli. SP modulates a variety of immune responses including mast cell degranulation [26], chemotaxis of eosinophils, neutrophils [27–29] and lymphocytes [30] and interleukin (IL)-2 production of human and murine T cells [31, 32].

In a murine model of allergen-induced airway inflammation, we have recently demonstrated that exogenously applied sound stress enhanced airway inflammation and airway hyperreactivity [33]. Furthermore, we established a role for the main SP receptor, neurokinin-1 receptor, in

mediating stress effects [34]. The aims of the present study were (1) to determine the influence of stress on the capacity of immune cells to produce cytokines and (2) to investigate whether an exogenously induced increase in the SP content in the airways with concurrent allergic inflammation might change the quality or quantity of inflammatory responses (in the lung and draining lymph nodes) similar to the changes seen after application of stress.

Methods

Animals

Balb/c mice were purchased from the Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV, Berlin, Germany) and maintained on a 12-hour light/dark cycle. Animal care and experimental procedures were followed according to the institutional guidelines and conformed to the requirements of the state authority for animal research conduct (LaGeSi, Berlin).

Protocol of Sensitization

Balb/c mice were sensitized by intraperitoneal injection of 10 µg chicken ovalbumin (OVA, grade VI; Sigma, Deisenhofen, Germany) emulsified in 1.5 mg Al(OH)₃ (Alum Imject; Pierce, Rockford, Ill., USA) on days 0, 14 and 21. Mice were challenged twice with OVA aerosol (1% OVA diluted in phosphate-buffered saline, PBS) via the airways on days 26 and 27, as described previously [35].

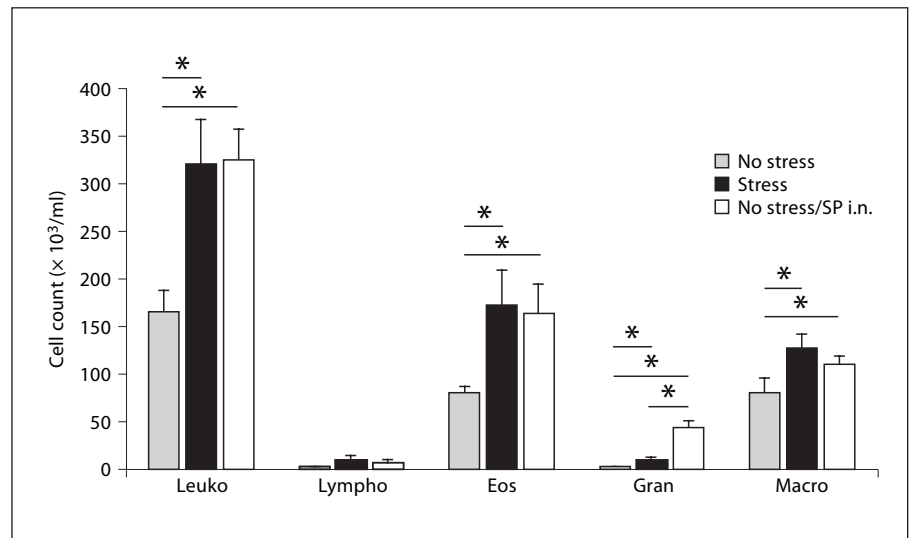
Stress Exposure/SP Application

After intraperitoneal sensitization, mice were randomized to one of three different experimental groups (table 1). Coinciding with the first OVA aerosol challenge, one group was exposed to sound stress for a single 24-hour period, while the other groups remained undisturbed. The stress device was placed in the mouse cage so that the mice could not escape perception of the sound. During exposure to the stressor in their home cage, stressed animals were kept in a different room separated from control animals. One group of non-stressed mice received intranasal SP. Application of SP (0.0625 nmol/mouse, Sigma) was performed 1 h before each airway challenge, preceded by intranasal application of phosphoramidon (5 µg/mouse) in order to prevent SP metabolism. The total volume of intranasal application was 50 µl. Mice were slightly anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (16 mg/kg) before application.

Bronchoalveolar Lavage

BAL fluid was obtained 24 h after the second airway challenge (day 28). Animals were sacrificed and tracheas cannulated. Airways were lavaged twice with 0.8 ml ice-cold PBS and the total number of cells in BAL fluid was determined as previously described [35]. Cytospins were prepared for each sample by centrifugation of 50 µl BAL fluid. After fixation, cytopins were stained with Diff Quik (Dade Behring, Marburg, Germany) and differential cell counts were performed. Using standard morphological criteria, cells were classified as neutrophils, eosino-

Fig. 1. Total leukocyte number and cell type identification in BAL fluids of OVA-sensitized and airway-challenged BALB/c mice. BAL was performed 24 h after the last allergen aerosol challenge. Stressed mice and non-stressed mice with intranasal application of SP showed significantly higher total cell numbers than non-stressed mice, * $p < 0.017$. Means and SEM. Leuko = Leukocytes; Lympho = lymphocytes; Eos = eosinophil granulocytes; Gran = neutrophil granulocytes; Macro = macrophages.



phils, lymphocytes or macrophages counting at least 100 cells per cytospin.

Intracellular Cytokine Detection in Draining Lymph Nodes by Flow Cytometry

For examination of cytokine production, draining lymph nodes (mediastinal lymph nodes) were harvested and pooled per group. Single-cell suspensions were obtained by passage through a 100- μ m cell strainer (Falcon, Palo Alto, Calif., USA). For the single-cell detection of intracellular cytokines, cells were stimulated for 5 h at 10^6 cells/ml in 24-well plates with phorbol myristate acetate (PMA)/ionomycin (1 μ g/ml/10 ng/ml). After 2 h of stimulation, brefeldin A (Golgi Plug, 1 μ l/ml; BD Pharmingen, Heidelberg, Germany) was added. After washing, cells were fixed (formaldehyde 2%) followed by surface staining for CD3-FITC, permeabilization with saponin (0.5%, Sigma), and staining for intracellular cytokines with PE-labeled anti-tumor necrosis factor (TNF)- α monoclonal antibody or phycoerythrin-labeled anti-IL-4 or anti-interferon- γ , respectively. All antibodies were purchased from BD Pharmingen. Samples were analyzed on a FAC-Scalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif., USA). The lymphocyte population was gated and used for further analysis. Negative control samples incubated with fluorochrome-conjugated isotype-matched antibodies were performed in parallel.

Determination of Allergen-Specific Antibodies

Blood was sampled from the lateral caudal veins on day 28 of sensitization before animals were sacrificed. Blood was clotted at room temperature and centrifuged. Total IgE and allergen-specific IgE antibody titers were determined in sera by enzyme-linked immunosorbent assay as described previously [33]. Anti-mouse IgE antibodies were obtained from Pharmingen, Hamburg, Germany. The anti-OVA IgE antibody titers of the samples were related to pooled standard sera that were generated in the laboratory. Detection limits were 1.95 ng/ml for total IgE and 15 EU/ml for anti-OVA IgE.

Lung Histology

The right ventricles were cannulated and the lungs were rinsed with 10 ml of PBS to remove intravascular blood. Samples were snap-frozen and sectioned. Sections were stained with hematoxylin and eosin for evaluation of the lung morphology.

Statistics

Differences between groups were determined using the non-parametric Kruskal-Wallis test. The Mann-Whitney U test was used to compare two groups, respectively. Significance was set at $p \leq 0.05$, and the α level was adjusted using Bonferroni correction.

Results

Airway Inflammation

To determine the cellular constituents of leukocytes infiltrating the airways of the animals, BAL fluids were analyzed, and a significantly higher number of total leukocytes was found in stressed animals (group II, $321 \pm 47 \times 10^3/\text{ml}$) compared to non-stressed mice (group I, $166 \pm 23 \times 10^3/\text{ml}$, Mann-Whitney $Z = -3.027$, $p = 0.002$; fig. 1). Cell differentiation revealed that the difference between the two groups was predominantly based on an increased number of eosinophils (172 ± 37 vs. $81 \pm 7 \times 10^3/\text{ml}$, Mann-Whitney $Z = -2.873$, $p = 0.004$; fig. 1). The application of SP in non-stressed animals resulted in increased total cell numbers in the same range as seen in stressed mice (group III, $325 \pm 32 \times 10^3/\text{ml}$, Mann-Whitney $Z = -2.982$, $p = 0.003$ vs. group I; fig. 1), and like in stressed mice mainly enhanced eosinophil numbers (164 ± 31 , Mann-Whitney $Z = -2.440$, $p = 0.015$ vs.

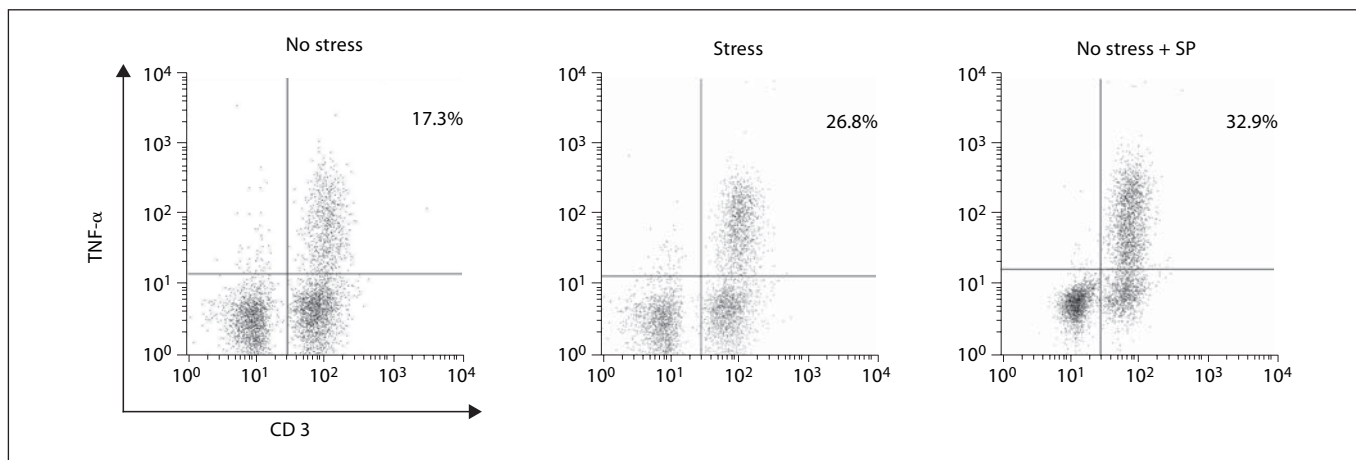


Fig. 2. Percentages of TNF- α -producing T cells in lymph nodes of OVA-sensitized and airway-challenged BALB/c mice. Cells were obtained from non-stressed mice, stressed mice or non-stressed mice with intranasal SP application and cultured for 6 h in the presence of PMA and ionomycin.

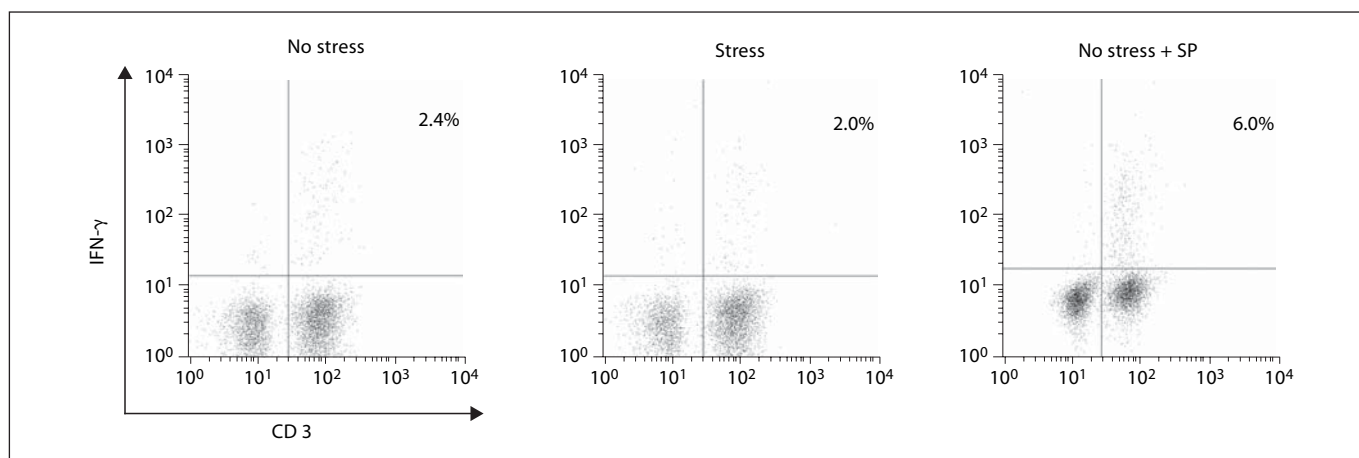


Fig. 3. Percentage of interferon (IFN)- γ -producing T cells in lymph nodes of OVA-sensitized and airway-challenged BALB/c mice. Cells were obtained from non-stressed mice, stressed mice or non-stressed mice with intranasal SP application and cultured for 6 h in the presence of PMA and ionomycin.

group I) caused an increase in total leukocyte count. Additionally, the number of granulocytes increased significantly, too.

Airway inflammation was confirmed by analysis of lung tissues upon hematoxylin-eosin staining. All animals showed peribronchial infiltration with inflammatory cells.

Cytokine Production in Draining Lymph Nodes

We measured cytokine-producing lymphocytes from peribronchial (mediastinal) lymph nodes by flow cytometry.

The frequency of TNF- α -producing T cells in non-stressed mice (group I) was 17.3%. Compared to non-stressed mice, animals with application of stress (group II) or SP (group III) showed an increased frequency of CD3+/TNF- α + cells (26.8 and 32.9%; fig. 2).

The percentage of interferon- γ -secreting T cells in lymph nodes was not affected by stress application (2.0% of cells from stressed mice vs. 2.4% from non-stressed ones) while in animals with intranasal application of SP the frequency was 6.0% (fig. 3). The frequency of IL-4+ CD3+ cells was marginal in all groups (fig. 4).

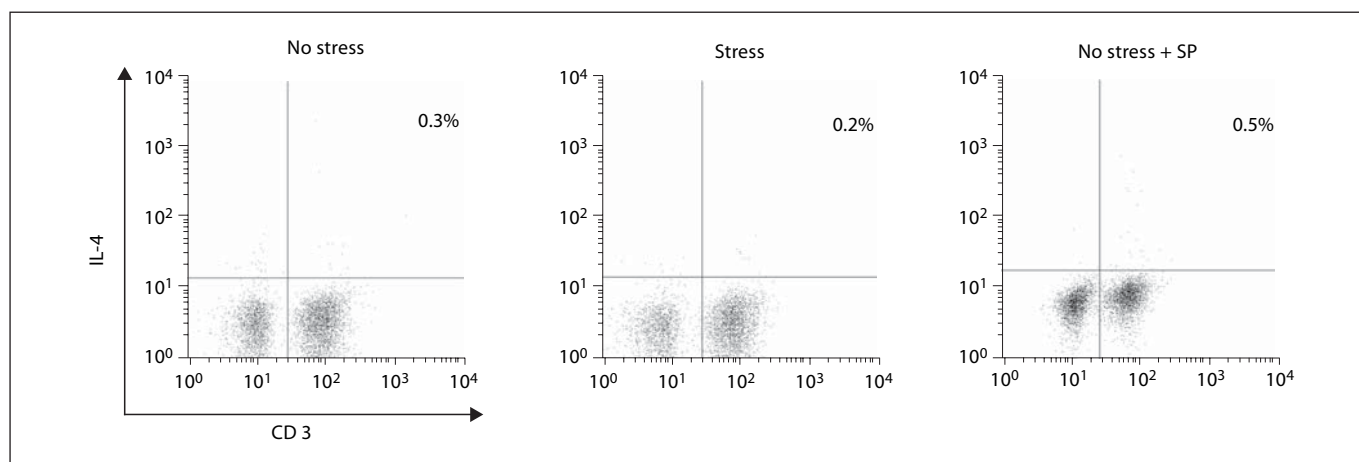


Fig. 4. Percentage of IL-4-producing T cells in lymph nodes of OVA-sensitized and airway-challenged BALB/c mice. Cells were obtained from non-stressed mice, stressed mice or non-stressed mice with intranasal SP application and cultured for 6 h in the presence of PMA and ionomycin.

OVA Sensitization and Immunoglobulin Synthesis

BALB/c mice, sensitized to OVA by intraperitoneal injections, developed high anti-OVA IgE antibody titers and increased total IgE as evaluated by enzyme-linked immunosorbent assay. No difference was detected between the three groups (data not shown).

Discussion

Utilizing a model of allergen-induced airway inflammation we found that sound stress or exogenously applied SP resulted in an enhanced influx of leukocytes into the airways. Further, we demonstrated that sound stress results in increased expression of TNF- α in T cells of the draining lymph nodes, while IL-4 and interferon- γ were slightly decreased with stress. SP application increased percentages of all measured cytokines in lymph node T cells.

During the last decades, evidence has accumulated that there is a bidirectional interaction between the nervous and the immune system. Thus, neuronal and hormonal changes characterizing the stress response may induce or exacerbate an inflammatory process. So far, the focus of experimental research regarding the modulating effects of stress on the inflammatory response has been the activation of the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system and the resulting release of cortisol and catecholamines with their influence on cell trafficking, proliferation and function. How-

ever, there is increasing evidence that neuropeptides like the tachykinin SP play an important role as mediators of stress reactions regulating endocrine, behavioral, cardiovascular and immunological responses to stress [for review see ref. 36].

In support of our previously published data we confirmed the enhancing effect of stress exposure on allergen-induced airway inflammation. Stress-induced enhancement of inflammatory cell influx is in line with recently published data from Forsythe et al. [37], who found increased cell numbers in BAL fluids of mice after chronic stress. Similar results were found after acute or chronic stress in rats [38, 39].

Furthermore, we found that in animals subjected to allergen sensitization and challenge, the qualitative and quantitative changes in the cellular contents of BAL fluids after exogenously applied SP are similar to changes seen in sensitized and challenged animals after stress. These findings further support the hypothesis that SP might act as a stress-mediating peptide.

Animal experiments have revealed that SP increases in response to stress and promotes inflammation. Stressed mice showed elevated immunoreactive SP in peritoneal wash fluid and increased IL-6 secretion from peritoneal macrophages. The stress-induced augmentation of IL-6 secretion was prevented by depletion of sensory neurons with capsaicin or by application of a neurokinin-1 receptor antagonist [11]. In rats, neonatal denervation of afferent SP-containing nerve fibers with capsaicin reduced stress-provoked activation of mast cells, which appear to

play a significant role in the pathophysiology of stress-sensitive skin disorders and interstitial cystitis [12, 13]. SP mediation in stress-promoted inflammation has also been demonstrated in animal models of abortion and hair growth inhibition [10, 40, 41].

In an earlier study [33] we demonstrated that the stress-induced increase in airway inflammation is not associated with increased IL-4 or IL-5 levels in BAL fluids, suggesting that the effect is mediated via a mechanism not primarily involving the local Th2-type immune response. Here, we investigated whether the T-cell cytokine profile in draining lymph nodes is affected by stress or external SP application. Draining lymph nodes are the site where immune responses are initiated. After they acquire antigens in the periphery, antigen-presenting cells migrate to the draining lymph nodes, where they present processed antigen to T cells and induce T-cell activation. In this study, the percentage of T cells producing the pro-inflammatory cytokine TNF- α in mediastinal lymph nodes dramatically increased with stress or SP application suggesting activation of T cells by these stimuli. Stress showed no increasing effect on IL-4 production of T cells in lymph nodes, supporting our observation that pro-inflammatory mechanisms rather than a further shift towards the local Th2 type cytokine profile are responsible for increased cell influx into the airways after stress.

There is clinical, experimental and pharmacological evidence for TNF- α as a mediator of allergic airway inflammation. TNF- α is present in the lavage fluid of asthmatic patients [42], and increased TNF- α mRNA expression in BAL cells from asthmatic patients compared to controls has been reported [43]. Cembrzynska-Nowak et al. were able to show that TNF- α release from BAL leukocytes is increased in asthmatic patients [44]. Eosinophilic airway inflammation seems to be related to TNF- α since acute blockade of TNF- α can attenuate eosinophilic airway inflammation in sensitized and challenged animals [45, 46]. Our data are consistent with these findings as increased percentages of TNF- α in lymph node T cells are associated with increased eosinophilic airway inflammation.

TNF- α released at sites of allergic inflammation may contribute to the recruitment of leukocytes from the circulation by induction of endothelial cell adhesion molecule expression. TNF- α is an important stimulus for the expression of endothelial cell adhesion molecules such as ICAM-1, VCAM-1, and E-selectin [47–49]. Further studies will be necessary to prove this possible pathway in stress-induced aggravation of allergic airway inflammation.

SP-immunoreactive nerve fibers in tracheobronchial lymph nodes have been reported [50] and SP might be released by afferent sensory neurons after stress, as has been hypothesized to occur in stress-related dermatoses [51] and hair loss [52]. SP-mediated TNF- α production has been noted in peritoneal macrophages in response to cold water stress [11], and in a murine model of stress-induced abortion [10, 53]. Thus, the increased number of inflammatory cells observed in the bronchoalveolar lavage from our stressed animals could be due to the higher percentage of TNF- α -positive T cells in local lymph nodes. The fact, that SP application induces a comparable increase together with a high percentage of TNF- α -positive lymph node T cells suggests a stress-mediating role for the tachykinin SP in this model of stress-exacerbated airway inflammation.

Based on our recent data, the question of how stress might promote the upregulation of SP in the airways arises. Stress and stress-induced activation of the hypothalamic-pituitary-adrenal axis induce various mediators involved in the execution of stress effects. The neurotrophin nerve growth factor (NGF) has been found to be increasingly expressed after external stress [54, 55]. Aggressive behavior, a form of social stress, is associated with increased serum and hypothalamic NGF levels [56–58]. NGF induces SP in airway sensory nerves [59], thus exposure to stress may lead to an upregulation of SP in the airways via NGF. Future investigation will identify the exact role of NGF and other mediators involved in the transmission of stress-induced effects.

Acknowledgment

This study was supported by a grant from the German Research Association to R.A.J. (DFG Jo 365/2-1).

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