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**ENDOGENOUS AND EXOGENOUS
GLUCOCORTICOID EFFECTS IN A
MODEL OF ALLERGIC AIRWAY
INFLAMMATION.**

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“The more you learn, the more you realise how much you don’t know”

Own experience 2000-2008

*To my wonderful husband Peter,
and our lovely children Kasper, Linus and Sandra.*

ABSTRACT

Allergic asthma and rhinitis are inflammatory diseases of the airways. Allergic asthma is characterized by eosinophilic inflammation, reversible bronchoconstriction and airway hyperresponsiveness, whereas patients with rhinitis suffer from nasal inflammation, leading to rhinorrhea and nasal congestion. Glucocorticoids (GCs) are one of the major drugs for asthma and rhinitis treatment and can regulate leukocyte trafficking and mediator release. The hypothalamus-pituitary-adrenal (HPA) axis regulates the secretion of endogenous GCs, and is one of the major stress effector systems in the body. The main role for the HPA-axis is to facilitate the maintenance of neuroendocrine homeostasis, both at basal and pathological conditions. An understanding of the regulation and function of the HPA axis is important for the evaluation of pathological changes and possible side effects caused by GCs. In the studies presented in this thesis we have used a mouse model to investigate the effects of endogenous GC synthesis and GC receptor inhibition, as well as acute stress, and different doses of exogenous administered GCs on inflammatory cells in different cellular compartments in allergic airway inflammation. We also investigated the effects of acute stress on the neurotrophic mediator, nerve growth factor (NGF) in the airways, during allergic inflammation.

In the first study (I), we investigated the effects of endogenous GCs on eosinophilic airway inflammation. Inhibition of GC release with metyrapone (ME) induced an increase of bone marrow eosinophilia and when the ME treatment was combined with a GC receptor antagonist (RU 486) the allergen-induced bone marrow eosinophilia was further enhanced. ME treatment also induced an increase of CD4⁺ T lymphocytes in the nasal mucosa, both when employed as a single treatment and in combination with RU 486.

In the second study (II), timing and dose-dependent effects of dexamethasone (DEX) on eosinophilic airway inflammation were studied. This study provided evidence that a low dose of DEX (1 µg/kg) evoked a stronger inhibitory effect on the eosinophilic airway inflammation, as compared to the effect of the 500 times higher pharmacological dose (500µg/kg) if given before the allergen challenge. When administered simultaneously the allergen challenge, the two doses displayed similar effects.

The third study (III), was focused on the effects of timing of a short acute stress on allergic airway inflammation in upper and lower airways. Short stress applied before an allergen challenge decreased the allergen-induced eosinophilia in bronchoalveolar lavage fluid and lungs and also the inflammation in the nasal tissue. No effects on eosinophilia or inflammation were seen when stress was applied after allergen challenge or as a double stress both before and after challenge. The protective effects, seen in the mice stressed prior to the allergen challenge, were GC-dependent.

In the fourth study (IV), the aim was to assess how acute stress modulated NGF levels and eosinophils in the airways during non-allergic and mild allergic conditions. We found that short stress increased the levels of NGF locally in the airways in both allergic and non-allergic mice. We also demonstrated that airway eosinophils decreased when stress was applied after allergen-challenge in a model of mild airway inflammation. The stress-evoked increase in NGF and decrease in eosinophilia in the airways were dependent on endogenous GC-synthesis, as evident from pre-treatment with ME.

In summary, these results indicate that endogenous GCs may have a protective effect in both upper and lower eosinophilic airway inflammation. Our studies also demonstrate that the inhibitory effect of GCs on the allergic inflammation is timing-dependent.

By the use of a GC synthesis inhibitor, we show that the inhibitory effects on the eosinophilic airway inflammation during acute stress are GC-dependent. Acute stress increased local airway NGF and this effect is mediated by endogenous GC synthesis. Thus, we also conclude that NGF may function as a mediator in the psychoneuroimmunological stress-response.

Key words: airway, allergy, asthma, eosinophil, glucocorticoids, hypothalamus-pituitary-adrenal (HPA) axis, inflammation, nerve growth factor (NGF), neuroimmunology, psychoneuroimmunology, restraint, stress



LIST OF PUBLICATIONS

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

- I. **Georén S**, Åhnblad P, Stjärne P, Wikström A-C, Stierna P.
Significance of endogenous glucocorticoid sensitivity for airway eosinophilia in a murine model of allergy.
Acta Oto-Laryngologica 2005; 125:378-385

- II. **Kumlien Georén S**, Tcacencu I, Wikström A-C, Stierna P.
Early timing of low-dose dexamethasone decreases inflammation in a murine model of eosinophilic airway disease.
Acta Oto-Laryngologica 2008; in press

- III. **Kumlien Georén S**, Olgart Höglund C, Tcacencu I, Wikström A-C, Stierna P.
Timing-dependent effects of restraint stress on eosinophilic airway inflammation in mice.
Neuroimmunomodulation 2008; in press

- IV. Kemi C*, **Kumlien Georén S***, Grunewald J, Stierna P, Olgart Höglund C.
Effects of acute stress on airway levels of NGF in a murine model of allergic asthma.
**contributed equally*
Submitted

LIST OF ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
AHR	Airway hyperreactivity
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
BDNF	Brain-derived neurotrophic factor
BM	Bone marrow
CA	Catecholamine
CRH	Corticotrophin releasing hormone
DEX	Dexamethasone
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
ELISA	Enzyme-linked immunosorbent assay
EPO	Eosinophilic Peroxidase
GC	Glucocorticoid
GM-CSF	Granulocyte macrophage-colony stimulating factor
GR	Glucocorticoid receptor
GRE	Glucocorticoid response elements
HPA	Hypothalamus-pituitary-adrenal
i.n.	Intra nasal
i.p.	Intra peritoneal
IL	Interleukin
LT	Leukotriene
MBP	Major basic protein
ME	Metyrapone
NGF	Nerve growth factor
NT	Neurotrophin
OVA	Ovalbumin
p75 ^{NTR}	p75 neurotrophin receptor
PAF	Platelet activating factor
RA	Rheumatoid arthritis
RU 486	Mifepristone
SAM	Sympathetic-adrenalmedullary
Th	T helper
TNF	Tumour necrosis factor
Trk	Tyrosine kinase receptors

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1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Asthma and airway allergy are considered to be major health problems in the Western countries and their prevalence has been increasing for the last decades, although a plateau of the increase has now been described [1]. The reason for the increase is not completely known, but environmental factors such as a Western life style, including the hygiene hypothesis, stress and pollution have been suggested as contributing factors. Asthma is characterized by airway inflammation and airway hyperresponsiveness, whereas the patients with rhinitis suffer from nasal inflammation, leading to rhinorrhea and nasal congestions. Today, treatment with glucocorticosteroids (GCs) is one of the most common therapeutics in both asthma and rhinitis patients [1].

Stress has long been suspected as a possible cause for enhancement of the allergic symptoms, such as increased number and extent of exacerbations [2], but improvement of the allergic symptoms, such as dyspnea has also been demonstrated [3, 4]. The mechanism for this dual response is not fully understood but it has been thought to involve alterations in the regulation and sensitivity to the stress hormones.

This work is focused on the allergic airway inflammation in both the upper and lower airways and the effects of glucocorticoids, both when given as exogenous drugs and when having effects on the modulation of the endogenous glucocorticoids, including studies with acute stress, and inhibition of the endogenous glucocorticoids in a murine model.

1.2 AIRWAY ALLERGY - A SYSTEMIC DISEASE

The allergic airway disease may be classified as a systemic disease. One of the major evidence for this could be the “allergic march” where atopic dermatitis, asthma and allergic diseases are co-morbidities. In some patients, skin symptoms and food allergy are the first allergic symptoms in life, then asthma starts after a few years and allergic rhinitis develops later [5]. Although, the allergic march is seen in many atopic patients, a large variety of disease and symptom development exists.

Stimulation of the nasal or bronchial mucosa by allergen provocation results in increased influx of inflammatory cells in airway mucosa, and also an increased maturation and production of eosinophils in the bone marrow [6-8]. (*Figure 1*)

The allergic response to an airborne allergen is divided into an immediate- and a late-phase response in both upper and lower airways. The immediate phase response in the lower airways causes a bronchial constriction, and in the upper airways, sneezing and rhinorrhea, minutes after the allergen inhalation. The effects in the airways are due to mediators, such as histamine released by mast cells, in the lower airways the mediators effects the smooth muscles and in the upper airways, the blood vessels. About 7-9 hours after the allergen exposure the late phase response occurs, as a result of the effect of an influx of inflammatory cells, such as leukocytes and eosinophils [9] nasal congestion and further narrowing of the lower airways may occur.

1.2.1 Airways

1.2.1.1 Upper airways

The most normal is to breathe through the nose, which acts as a filter, heat exchanger and humidifier for the inhaled air, thereby protecting the lower airways. The upper airways (nose to larynx) consist of ciliary epithelium and mucus glands with an extensive vasculature and innervation. The rich vasculature is a key feature of the nasal mucosa and changes in the vasculature may lead to severe nasal obstruction [10]. Symptoms in rhinitis patients are mainly nasal congestion, sneezing and rhinorrhea.

1.2.1.2 Lower airways

The lower airways (larynx to alveoli) are responsible for transportation of the air to the bronchiole/ alveoli and the gas exchange by diffusion in the respiratory bronchiole and the alveoli. The bronchi are characterized by the presence of smooth muscles from the trachea to the bronchioles, accounting for the bronchoconstriction of asthma [8]. In the lungs, symptoms such as hyperactivity and obstruction, increased mucus production and induced remodelling, are features known as hallmarks of asthma.

1.2.1.3 United airways

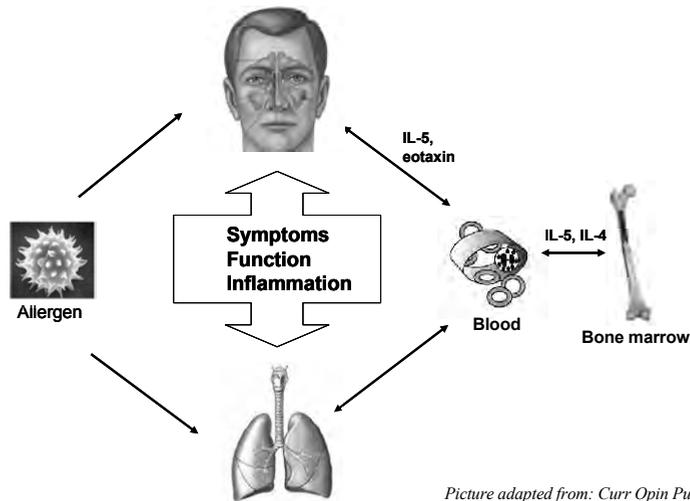
The overall view of the pathology of airway allergy has profoundly changed and evolved during the last 15 years in that particular attention has been paid to the relationships between asthma and rhinitis, i.e. between pathological processes in the upper and lower airways. This has led to the concept of “united airways”. The upper and lower airways are connected in several ways in addition to their apparent anatomical proximity, such as common immunological behavior. The paranasal sinuses are involved in both asthma and rhinitis, and there is also a possibility to modulate the clinical and immunological response in rhinitis patients in order to prevent asthma development [8, 11]. It has been shown that most of the asthmatic patients also display symptoms of rhinitis, whereas only few rhinitis patients have asthma [8].

In response to allergen provocation several inflammatory cells, such as eosinophils and lymphocytes are increased both in the nasal mucosa and the lung tissue. The nasal mucosa is rich in mast cells that are thought to contribute to the naso –bronchial connection through cytokine release [12]. It has been shown that in a “pure” upper airway inflammation in mice, with no allergen deposit in the lower airway mucosa, an inflammation and increased bronchial hyperresponsiveness occurs in the lungs [13].

In rhinitis patients, an association with non-specific bronchial hyperresponsiveness has been demonstrated after non-allergic stimuli, such as cold air and various irritants [14, 15]. A possible mechanism for this non-specific hyperreactivity could be the “neurogenic inflammation”, in which neuropeptides released from the sensory nerves (i.e. substance P and neurokinin) are involved [12, 14]. Activation of the sensory nerves can cause stimulation of the reflexes that then effects the end organs by, regulating the plasma extravasation and oedema. Thus a release of peptides from the sensory nerves in the airways may result in inflammation of the airways [16].

Despite that the united airway concept is well accepted in the clinic, only few animal models of allergic airway inflammation examine both upper and lower airways.

Figure 1: Suggestion of connections between upper and lower airways and bone marrow.



Picture adapted from: *Curr Opin Pul Med*
© 2003 Lippincott Williams & Wilkins,

1.2.2 Bone marrow

Several studies have shown activation of the bone marrow in allergic subjects and in animals that are exposed to allergen. Increased number of eosinophils and eosinophil precursors have been found in the bone marrow of atopic and asthmatic subjects [6, 17-19] and in animal models of allergen-induced airway inflammation [7, 20, 21]. A major theory for the increase of airway eosinophils in response to allergen provocation is increased egression of the eosinophils from the bone marrow.

1.3 INFLAMMATORY CELLS IN AIRWAY INFLAMMATION

All patients with asthma or rhinitis have a specific pattern of inflammation in the airways that is characterized by degranulated mast cells, infiltration of eosinophils and an increased number of activated Th2-cells [22, 23]. This specific pattern of inflammation probably underlies the clinical features of asthma and rhinitis.

1.3.1 Eosinophils

The eosinophil was discovered in 1879 by Paul Ehrlich as a blood cell that had the remarkable ability to bind the acidic dye eosin. Eosinophils are non-dividing bone marrow-derived cells and are present in the blood as they travel to their final site of deposition in the tissue. In the tissue the eosinophil life span is in the range of two to four days, whereas in the blood the half-life is less than a day. In healthy individuals, they constitute less than 5% of the circulating white blood cells. Eosinophil accumulation is associated with the pathology and symptomatology of several diseases, such as different allergic disorders and helminth-induced inflammation. Hence, the eosinophils have been demonstrated as major effector cells in the inflammatory process underlying much of the pathogenesis of asthma and other allergic diseases [24].

Asthma severity has been correlated with the number of eosinophils in peripheral blood, sputum and bronchoalveolar lavage fluid (BALF) or eosinophil cationic protein levels [25] and large number of activated tissue eosinophils are typically observed in airway biopsies, sputum and BALF from asthmatic patients [24].

1.3.1.1 Eosinophilic granule proteins and inflammatory mediators

The eosinophil secretes an array of cytotoxic granule cationic proteins, such as eosinophil peroxidase (EPO), eosinophil cationic protein (ECP), eosinophil derived neurotoxin (EDN) and major basic protein (MBP) all of which are capable of inducing tissue damage and impaired function [26]. MBP, EPO and ECP are toxic to a variety of tissues, such as heart, brain and bronchial epithelium, MBP and ECP can alter smooth muscle contraction e.g. by inducing mast cell degranulation and ECP can also stimulate airway mucus secretion [27].

Activated eosinophils produce inflammatory mediators such as leukotrienes (LTs) (mainly LTC₄) and platelet activating factor (PAF) that may enhance the allergic response due to increased vascular permeability and inducing eosinophil chemotaxis. Activated eosinophils also secrete cytokines and chemokines, such as transforming growth factor (TGF)-alpha, IL-2, IL-4, IL-5, IL-10, IL-12, TNF- α and CC and CXC chemokines, such as Eotaxin and RANTES [27].

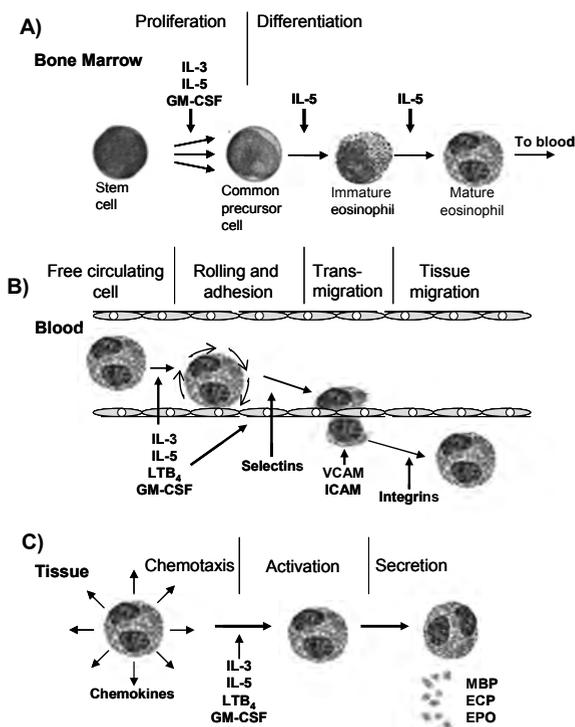


Figure 2: A simplified model of the A) maturation, B) migration, and C) activation of the eosinophil.

Adapted from: Mygind N, Dahl, R, Pedersen S, Thestrup-Pedersen K. *Essential Allergy*. 1996 Blackwell Science Ltd

1.3.1.2 Eosinophilopoiesis

Eosinophils develop from CD34⁺ hematopoietic stem cells, under the influence of IL-3, IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) [27]. IL-5 is the primary regulator for eosinophil migration from the bone marrow into the circulation. Circulating eosinophils subsequently interact with the endothelium by processes involving rolling, adhesion and diapedesis. Eosinophils cross the endothelium into tissues by a regulated process involving interactions between chemokines and adhesion receptors on the endothelium [27]. (Figure 2)

1.3.1.3 Time course of eosinophils in airway inflammation

Allergen-induced airway inflammation causes stimulation of the bone marrow and is associated with enhanced eosinophilopoiesis in atopic subjects [18, 19] and in animal models of allergic airway inflammation [20].

The eosinophils have been shown to be increased in lungs, BALF, bone marrow and blood after an allergen challenge. A few hours after the challenge an increase in peripheral blood eosinophils concurrent with decreased bone marrow eosinophilia has been demonstrated [20, 28]. The increase in the blood is likely to be a result of mature eosinophils exiting from the bone marrow into the bloodstream. After approximately a half day a decrease of blood eosinophils occurs and at the same time tissue eosinophils increase [20, 28]. Mature eosinophils leave the bone marrow and are attracted to sites of allergic inflammation by the actions of pro-inflammatory mediators e.g. IL-4, IL-5 and IL-13 [29]. It has been shown that after allergen provocation performed on three consecutive days in mice, the increase of tissue eosinophils can persist for up to a month [28].

The bone marrow response after an allergen challenge may consist of several distinct periods, where the decrease of eosinophils seen immediately after allergen exposure is a consequence of a lower eosinophil production rather than egression. After a period of increased production rate occurs and the eosinophil levels restore to normal or higher. The next period persists for the duration of the local airway inflammatory response and is characterized by elevated bone marrow production of eosinophils, whereas their progenitor levels have returned to normal [30].

1.3.2 Lymphocytes

The most important function of the lymphocyte is to respond directly to exogenous antigens. T lymphocytes form the majority of lymphocytes in the peripheral blood and are widely distributed throughout the body in lymphoid organs, including the spleen and thymus, and at mucosal surfaces. T lymphocytes have the capacity to influence the production, lifespan and activation of granulocytes and other cell types. All mature post-thymic T lymphocytes express the markers CD2 and CD3. T cells can then be divided into two different subgroups depending on their receptors and functions; CD4⁺ T-helper cells and CD8⁺ cytotoxic T-cells [9].

CD4⁺ T cells and their secreted cytokines are considered to have a central role in initiating and orchestrating the inflammatory process, including tissue eosinophilia. The naïve CD 4⁺ T cells differentiate under influence of different cytokines and dendritic cells into either T-helper cells type 1 (Th1) or T-helper cells type 2 (Th2) [9]. Th1 cells produce IL-2, which is required for the proliferation of CD8⁺ T cells, and IFN- γ , that is crucial for activation of macrophages [9]. Th2 cells produce IL-4, IL-5, IL-10, and IL-13 which are considered important for the development of allergic diseases.

The levels of Th2-associated cytokines are increased in the airways of asthmatics [31] and in mouse models of allergic inflammation [e.g., Ref. 20, 32, 33], therefore asthma is mainly regarded as a Th2-driven disease.

The CD8⁺ T-cells kill a target cell that displays peptide fragments of cytosolic pathogens, most notably viruses [9]. CD8⁺ T-cells release the cytotoxins perforin and granzyme, which cause an infected/dysfunctional cell to undergo lysis. CD8⁺ T-cells can also induce apoptosis in a target cell by release of granzyme. The cytotoxic T cell has been suggested to be involved in the late asthmatic reaction, and functions as a proinflammatory cell in the airways [34].

Regulatory T-cells (previously known as suppressor T cells) are a specialized subpopulation of T-cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and prevent pathological self-reactivity, i.e. autoimmune disease [9]. In atopic disease, regulatory T-cells have been shown to decrease the eosinophilia in the airways and dysregulated in atopic individuals regulatory T-cells have been suggested to be less efficient in inhibiting Th2-type cytokine production than in healthy individuals [35, 36].

B lymphocytes play an important role in the humoral immune response. When the B lymphocytes become activated, they transform into plasma cells that produce and secrete antigen-specific antibodies. IgE antibodies are produced by plasma cells under the influence of Th2 cells producing IL-4 and IL-13 [9].

1.3.3 Neutrophils

Neutrophils play an important role in the inflammatory response, e.g. in resolution of inflammation and in host defence through their capacity to phagocytose and destroy bacteria. They appear during the immediate early response in the nose and in BALF after allergic airway challenge [37-39], and they are also increased in BALF of patients with acute exacerbations of asthma [38]. However, their increase is short in duration as compared to the increase in eosinophils. The precise role of neutrophils in allergy and asthma is not fully understood, but they have been connected to airway remodelling [38] and they are thought to be central in the pulmonary inflammation process occurring in asthma, by release of mediators that promote asthma symptoms [40].

In asthma patients that are difficult to treat with GCs, neutrophils are often more predominant than eosinophils [41].

1.3.4 Macrophages

Macrophages are bone marrow-derived cells. They differentiate in the blood into monocytes, and finally reside in the tissue as mature macrophages. They contain a wide variety of enzymes and mediators, such as eicosanoids, interleukins and complement factors that are involved in inflammatory reactions. The main function of macrophages is to remove foreign material, through phagocytosis, antigen presentation to T lymphocytes and they can also secrete a variety of enzymes and mediators involved in inflammation reactions, including eicosanoids and interleukins [26].

It has been suggested that macrophages contribute to the airway inflammation of asthma through their capacity to secrete mediators. It has been reported that the BALF contains approximately 80% macrophages or more and they have been shown to be increased in atopic individuals [26].

1.3.5 Mast cells

The mast cells are important effector cells in allergic diseases. They are key players during the early phase response with a massive release of mediators. Mast cells are usually distributed throughout normal mucosa and connective tissues, especially in the skin and respiratory system [26]. Mast cells are able to produce the neurotrophins NGF, BDNF and NT-3 [42]. NGF can function as a chemoattractant for mast cells [43] and regulate mast cell degranulation [44]. Activated mast cells can rapidly release large amounts of e.g., histamine, neutral proteases, heparin, LTB₄ and LTC₄ [26].

1.4 STRESS AND AIRWAY ALLERGY

Stress has been shown to have dual effects in asthma and airway inflammation. Acute stress often causes a stimulation of the immune system, whereas chronic stress seems to down-regulate the immune responses. When acute stress persists in time, and ceases to be interrupted by basal unstressed periods, the stress becomes chronic. Where the exact difference between acute and chronic stress occurs is not fully understood, but most likely this is subject to individual variation. Acute stress has been shown to improve allergen-induced airway obstruction and dyspnea in severely asthmatic women [3, 4]. Chronic stress has been shown to increase the number of asthmatic exacerbations [2], induce a drive toward T helper(Th)2 predominance in the balance between Th1/Th2 cytokines [45, 46]. Furthermore, the levels of airway eosinophils have been shown to be elevated in asthmatics following stress [46].

1.5 THE HYPOTHALAMUS PITUITARY ADRENAL AXIS

The hypothalamus pituitary adrenal (HPA) axis is a complex set of direct influences and feedback interactions between: the hypothalamus, the pituitary gland, and the

adrenal glands. The homeostatic interactions between these organs constitute the HPA axis that regulates almost all neuroendocrine responses in both physiological and pathological conditions (*Figure 3*). It has previously been shown that there is a large inter-individual regulation of the HPA axis and that the pattern of cortisol release is stable and reproducible over time [47].

Activation of the HPA axis leads to release of corticotrophin releasing hormone (CRH) from the hypothalamus [48]. CRH is the main regulator of adrenocorticotrophic hormone (ACTH) release from the pituitary. ACTH activates the adrenal glands to produce glucocorticoid hormones (GC), mainly cortisol (corticosterone in mice). The GCs are the final effectors of the HPA axis and they exert their effects through intracellular receptors [48]. A negative feedback mechanism maintains the optimum activation of the HPA axis by inhibiting the production of CRH and ACTH.

One way to inhibit the HPA axis is to use a cortisol synthesis inhibitor e.g. metyrapone (ME). ME is a drug that can be used to diagnose adrenal insufficiency and to treat Cushing's syndrome. ME inhibits the release of cortisol (corticosterone in mice) by inhibiting the synthesis of the enzyme 11-beta-hydroxylase and also increases the release of ACTH in a dose-dependent manner [49]. It has been shown that an i.p. injection of ME decreases the plasma corticosterone in animals [50, 51]. The inhibitory effect on corticosterone lasts for at least 24 hours, but the upregulating effect on the HPA axis (among others, the increased ACTH) remains for several days [52].

Cortisol is the most abundant circulating steroid secreted by the adrenal cortex in humans and the levels never attain true steady state conditions, being characterized by a series of peaks, resulting from an episodic endogenous secretion [53]. Cortisol increases gluconeogenesis, is catabolic for proteins, increases blood pressure and has anti-inflammatory effects.

The magnitude of an inflammatory response may be restrained through the anti-inflammatory action of GCs that are released as a result of the immune activation or to stressors. Endogenous GCs have been suggested to have anti-inflammatory effects in the allergic reactions [50, 51, 54]. The effects of endogenous GC on peripheral target organs depends on three factors: the basal level and its normal fluctuations, possibilities for dynamic release and the peripheral sensitivity.

In patients with inflammatory atopic diseases, a blunted HPA axis responsiveness has been observed [55, 56] and patients with atopic eczema displayed a hypo-responsive HPA axis [57].

1.6 GLUCOCORTICOIDS (GC)

Glucocorticoids are the most potent anti-inflammatory agents available for asthma [58] and are used for the treatment of both asthma and allergic rhinitis [1, 59]. The purpose with the GC treatment is to gain control of the underlying disease process and to maintain this control for as long as possible, with the minimum of side effects [58]. GC administration affects virtually all organ systems, since the glucocorticoid receptor (GR) is present in all cell types and long term treatments may cause side effects like, Cushing-like syndromes and osteoporosis [60].

There are various synthetic GCs available for therapeutic use, e.g. dexamethasone, prednisolone, budesonide and mometasone furoate. The synthetic GCs have been developed to optimize therapeutic potential i.e. potency and minimize the adverse reactions, including systemic side effects.

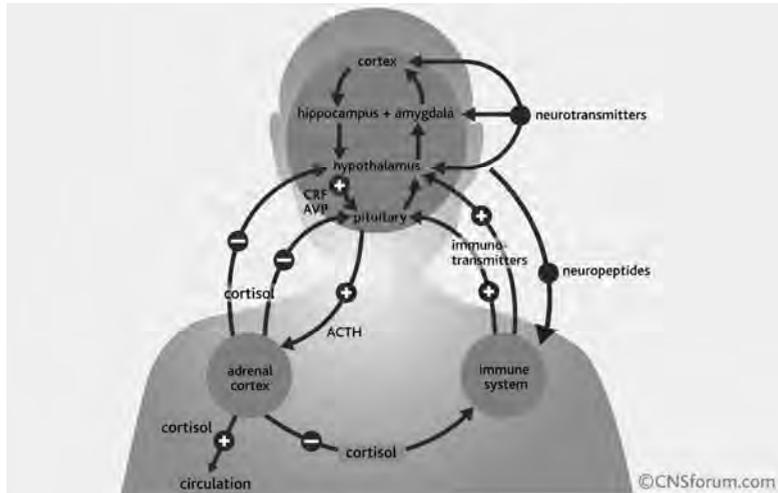


Figure 3: Hypothalamic-pituitary-adrenal (HPA) axis. The secretion of cortisol from the adrenal glands regulated by negative feedback mechanisms and neuroimmune interactions.

1.6.1 Glucocorticoid receptor (GR)

The anti-inflammatory effects of steroids are mediated through activation of an intracellular GC receptor (GR). GR belongs to the nuclear hormone receptor superfamily that also includes mineralocorticoid, progesterin, estrogen and other receptors. There are two main variants of the GR, namely GR- α and GR- β . GR- α functions as a hormone-dependent transcription factor, and up-regulates transcription by binding to glucocorticoid response elements (GREs) located in the promotor region of target genes. GR- α can also inhibit gene transcription by binding to negative GREs [61]. GR- β cannot bind GCs, although it can bind to GREs. GR- β acts as an inhibitor of the GR- α induced transcription.

In steroid resistant asthma, several mechanisms causing the glucocorticoid non-responsiveness have been suggested, such as mutations in the GR gene that may result in abnormal GR structure and resistance caused by Th2 type cytokines that may induce a reduction in affinity of GR for GC in inflammatory cells, resulting in a local resistance to the antiinflammatory actions of the GCs [reviewed in Ref. 62] Also increased levels of GR- β has been suggested as a cause for GCs insensitive asthma [61].

RU 486 (Mifepristone) is a GR antagonist that also has effects on the progesterone receptor, which has led to its use in medical abortions. RU 486 binds with higher

affinity to the GR than both cortisol and the synthetic steroid dexamethasone [63]. Administration of RU 486 leads to a clinical picture that mimics generalized GC resistance, with disinhibition of the HPA axis compensating for the peripheral GC blockade. Administration of RU 486 has also shown partial agonistic effects on the ACTH levels, although to a much lesser extent than cortisol [63, 64].

1.6.2 GC effects in inflammation

The most general effect of GCs is to inhibit synthesis, release and/ or effect of cytokines and other mediators that promote immune and inflammatory reactions. The cytokines affected are for example; IL-1, IL-2, IL-3, IL-4, IL-5, IL-6 and IL-12, IFN- γ and TNF- α [65].

GCs have been shown to decrease both airway and circulating eosinophils [19]. Sites where steroids may interfere with the eosinophilic inflammation include the production of eosinophilic mediators, expansion of the eosinophil progenitor cell population in the bone marrow, terminal differentiation of eosinophils in the bone marrow, release of mature eosinophils into circulation, and recruitment of eosinophils into the airway [32]. One of the main theories today is that GCs probably inhibit eosinophil maturation by inhibiting the production and/ or release of IL-5 and other eosinopoietic factors from cells within the bone marrow [66]. GCs are very active as inhibitors of T-cell activation and cytokine production and mainly act on the Th2 type of the CD4⁺ T lymphocytes [34, 67].

Neutrophils have been suggested to be less sensitive for GCs than eosinophils and T lymphocytes [66] and the precise effect of GCs on neutrophils is unknown.

1.7 PSYCHONEUROIMMUNOLOGY

The interactions between the nervous system, the endocrine system and the immune system are more and more considered in both health and disease. The stress response is known to affect the interplay between these systems. The concept that stress in certain conditions may induce physiological changes leading to disease was first explored by Hans Selye, who called stress “distress” [68].

Acute stress is known as the “fight or flight” reaction, upon which the biological system is regulated back to normal, whereas in chronic stress the systems are activated over long periods of time, which in turn may lead to adaptive changes, such as receptor desensitization and tissue damage. This is called “allostatic load” and refers to the price the tissue or organ pays for an overactive response [68].

In response to stress, the peripheral stress systems; HPA-axis and/ or the sympathetic-adrenalmedullary (SAM) system are activated [48]. Upon activation these systems release their hormones, such as cortisol, CRH, acetylcholine and the catecholamines (CA) (adrenaline and noradrenaline) {Glaser & Kiecolt-Glaser, 2005} and it has been demonstrated that cognitive stressors, such as student exams and public speaking are correlated with high levels of CA and GCs [69, 70]. Almost all immune cells express the receptors for the stress hormones released by the HPA- and SAM-axis. Depending on the type of stress, different factors such as various cytokines and lipid mediators of inflammation are secreted and act on the components in the HPA axis, mostly to

potentiate its activity [48]. The three major “inflammatory cytokines” TNF- α , IL-1 and IL-6 are produced at inflammatory sites and are associated with an activation of the HPA axis [48]. Stress has also been shown to induce a redistribution of leukocytes from the bone marrow to more peripheral tissues [71-73].

1.8 NEUROTROPHINS

The neurotrophins are a family of polypeptides that promotes survival, growth and differentiation of neurons. The neurotrophins consist of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3 and NT-4/5. The neurotrophins exert their functions through the tyrosine kinase receptors (Trk) and the p75 neurotrophin receptor (p75^{NTR}) [74]. There are three different Trk receptors: TrkA, TrkB and TrkC. NGF binds to the TrkA, BDNF and NT-4/5 binds to TrkB and NT-3 to TrkC [74]. The Trk receptors transmit positive signals for enhanced survival and growth of injured neurons, whereas p75^{NTR} transmits both survival and apoptosis of neurons [74]. The two receptors can function to either enhance or suppress each other [74].

1.8.1 NGF

NGF was the first neurotrophin and was discovered more than 50 years ago by Levi-Montalcini [75]. NGF is not only important for neuronal growth, but is also involved in inflammation, such as the one in asthma and allergy. Elevated levels of NGF have been detected in blood and in the airways from asthmatic patients [76-78]. NGF may contribute to enhance airway hyperreactivity (AHR) in several ways [79-85]. The p75^{NTR} receptor has been suggested to play a role in the hyperreactivity and activation of the sensory nerves [86]. NGF has also been shown to enhance survival, activation and mediator release in inflammatory cells, such as eosinophils and lymphocytes [87, 88]. NGF is produced by several cells, neurons, inflammatory cells, such as lymphocytes or mast cells, and structural cells, such as fibroblast, epithelial or smooth muscle cells [86, 89-91].

It has been demonstrated that NGF increases in serum in response to several stressful events, such as parachuting, in caregivers for a cognitively impaired spouse and also in asthmatic patients perceiving stress [92-94]. It has been suggested that NGF could have important functions in the stress-mediated immune response, such as allergic asthma [86]. This is based on the effects of NGF on the nervous and endocrine systems in response to stress, such as feedback up-regulation of the HPA axis [95] and up-regulation of neuropeptides in the sensory nerves that may enhance AHR [96].

1.9 LEUKOTRIENES

The leukotrienes (LT) are a family of fatty acid derivates that play important roles in a variety of allergic and inflammatory reactions [97]. Leukotrienes are divided into two classes, cystenyl-leukotrienes (cys-LT) and LTB₄, that is a potent leukocyte chemoattractant [98]. LTB₄ promotes adhesion of leukocytes to the endothelium,

followed by migration into the tissue [99]. LTB₄ is mainly produced by neutrophils, but also by eosinophils, macrophages, mast cells and B lymphocytes [100]. LTB₄ has also been shown to stimulate the IL-5 production by T cells [101], suggesting that LTB₄ has immune-regulatory properties relevant to inflammation in asthma.

1.10 MURINE MODELS OF AIRWAY ALLERGY

To obtain information on several aspects of allergic airway inflammation, *in vivo* animal models have been used for several years. The most common used model is the one where mice are subjected to ovalbumin (OVA) sensitization and OVA challenge. The animal models mimic many, but not all of the human manifestations of asthma, such as IgE-dependent eosinophilia, Th2-mediated recruitment of tissue eosinophils to the airways, activation of bone marrow eosinophilopoiesis, airway mucus secretion and AHR [e.g., Ref. 20, 28, 33, 102-105]. Other findings in the allergic mice are increased expression and levels of immune-regulatory molecules, such as chemokines and cytokines in the lung tissue and BALF [e.g., Ref. 20, 28, 33, 106]. In combination with modulations of the HPA axis, e.g. stress, the work with allergic mice may provide information on links, or lack of links, between the neuroimmunological interactions and the development of airway inflammation.

2 AIMS

- 1) To investigate the effects of endogenous glucocorticoids on allergic inflammation in both upper and lower airways and bone marrow in mice.

- 2) To study effects of pharmacological or physiological doses of glucocorticoids, given at different time points, on inflammatory cells, such as eosinophils, lymphocytes, neutrophils and macrophages in a murine model of allergic airway inflammation.

- 3) To analyze the timing-dependent effects of restraint stress on airway inflammation in a murine model, and to determine whether these effects are glucocorticoid-dependent.

- 4) To determine the stress effects on airway nerve growth factor levels in a model of allergic inflammation and also in non-allergic animals, and to determine whether these effects are glucocorticoid-dependent.

3 MATERIAL AND METHODS

3.1 ANIMALS

These studies were approved by the Stockholm South local committee on ethics of animal experiments. All mice used in these studies were 6-8 weeks old male BALB/c mice, obtained from B& K Universal AB (Sollentuna, Sweden). They were kept in a room with a 12-hour light-dark cycle and provided with food and water *ad libitum*.

3.2 ALLERGEN PROVOCATION

3.2.1 Sensitization

Mice were sensitized two (I) or three (II, III, IV) times, day 0 and 5 (I) or 0, 7 and 14 (II, III, IV) by intra peritoneal injection (i.p.) of 10 µg ovalbumin (OVA) (Sigma-Aldrich) adsorbed to alum (aluminium hydroxide, Sigma-Aldrich) in PBS. Control mice were sensitized with only alum in PBS.

3.2.2 Allergen exposure/ Airway challenge

Airway challenge was performed in the mornings on day 18, 19 and 20 (I) or 27, 28 and 29 (II, III, IV) by intra nasal (i.n.) administration of 100 µg OVA suspended in PBS under slight CO₂ anaesthesia of the mice (*Figure 4*).



Figure 4: Intra nasal OVA- challenge in CO₂ anaesthetized mice.

3.3 TREATMENT/ STRESS PROCEDURES

3.3.1 GCs inhibition (I, III, IV)

To inhibit the release of endogenous GCs, mice were treated with an i.p. injection of the corticosterone synthesis inhibitor metyrapone (ME) (30 mg/ kg) (Aldrich Chemical Company Inc.) 12 hours before the first, and two hours before every OVA challenge. Peripheral GCs effects were inhibited with the GCs receptor antagonist RU 486 (RU)

(Roussel UCLAF), administered as an i.p. injection of 20 mg/kg RU 486 dissolved in 0.5% Caboxymethyl cellulose in saline 12 hours before first, and 1.5 h before every OVA challenge. In study III, ME (100 mg/kg) was given before the stress to a group of mice that were stressed before OVA challenge, also in study IV, ME (100 mg/kg) was given prior to stress to inhibit endogenous GCs.

3.3.2 Dexamethasone treatment (II)

Mice were treated with a low dose of dexamethasone (Sigma), 1 µg/ kg in PBS, corresponding to a physiological level of GC or a dose more commonly used in these types of studies, 500 µg/ kg in PBS. The mice were treated at different time points close to challenge, two hours before, immediately before or seven hours after each challenge.

3.3.3 Stress procedures (III and IV)

Restraint was used to provoke stress in the mice. The mice were not physically squeezed or compressed. This type of stress is believed to approximate a stressful experience that is psychological in nature due to the feeling of confinement on the animal [107]. Restraint stress was carried out by placing the animals in well ventilated plastic tubes. In the tube, the mouse could move from prone to supine position, but not turn head to tail. Tubes were placed in horizontal position and animals stayed in the tubes for two hours during each stress period. In study III the mice were stressed before, after or both before and after each challenge and in study IV after each challenge.

3.4 SAMPLE COLLECTION, PROCESSING AND ANALYSIS

All mice were sacrificed with a lethal dose of CO₂ (I) or a lethal i.p. injection of sodium pentobarbiturate (II-IV)(Apoteksbolaget) 24 hours after the last OVA challenge.

3.4.1 Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed through the tracheal cannula by instillation of 0.4 ml PBS.

BAL was examined for inflammatory cells and soluble mediators in the airway lumen. Samples were centrifuged at 1200 rpm in an Eppendorf centrifuge for 10 minutes at 4°. Supernatant was aspirated and the cells were resuspended in PBS. BAL supernatant was later used for LTB₄ (II) or NGF analysis (IV).

BALF was centrifuged in a cytospin (Shandon Cytospin 2) at 450 rpm for 5 minutes. Slides were let air-dry, stained with May-Grünwald Giemsa (MGG) and Luxol fast Blue (LFB). A differential cell count was performed on the MGG stained slides and an eosinophil count on the LFB slides, counting 300 leukocytes under light microscopy vision in a blinded manner.

3.4.1.1 Leukotriene B₄ analysis (Study II)

The levels of LTB₄ were analyzed in BAL supernatant using LTB₄ Biotrak enzymeimmunoassay system (Amersham Biosciences) according to the manufacturer's instructions. In brief, the BAL supernatant was incubated with anti-leukotriene B₄ serum in a microwell plate pre-coated with ant-rabbit IgG. Competing LTB₄ coupled to peroxidase was added, followed by substrate addition and the optical density was determined at 450 nm. Detection range for LTB₄ was 0.3 to 40 pg/well. All measurements were performed in duplicates.

3.4.1.2 Nerve Growth Factor (NGF) (study IV)

The BAL supernatant was analyzed for NGF by a commercially available enzyme-linked immunosorbent assay (ELISA) (Promega) according to the manufacturer's instructions. In brief, BAL supernatant was incubated on a microtiter plate coated with polyclonal anti-NGF. After addition of monoclonal Rat anti-NGF, visualization was performed using HRP conjugated anti-Rat IgG, followed by substrate addition. The optical density of the enzyme substrate reaction was determined at 450 nm. The detection range for NGF was 2.0 to 250 pg/ml. All measurements were performed in duplicates. Cross-reactivity with other neurotrophic factors is less than 3% at 10ng/ml according to the manufacturer.

3.4.2 Bone marrow

Bone marrow (BM) cells were collected by flushing 1 ml PBS from the end-opened right femur. Bone marrow was collected for determination of total amount of eosinophils. BM was centrifuged in a cytospin (Shandon Cytospin 2) at 450 rpm for 5 minutes. Slides were let air-dry, stained with May-Grünwald Giemsa (MGG) and Luxol fast Blue (LFB). A differential cell count was performed on the MGG stained slides and an eosinophil count on the LFB slides, counting 300 leukocytes under light microscopy vision in a blinded manner.

3.4.3 Lung tissue

One lobe of the lung was put in 4% buffered formaldehyde (study I-III) and the other in Stefaninis fixation (study II-III).

The formaldehyde fixed lung lobes were embedded in paraffin, sectioned and stained with Haematoxylin-Eosin (HE) (study I-III). The Stefanini fixed lung lobes were rinsed in Hank's balanced salt solution embedded in OCT medium and frozen in liquid nitrogen (study II-III). The lung lobes were sectioned and stained for eosinophilic peroxidase (EPO). Histological findings such as inflammatory cell infiltration in HE slides and amount of EPO positive cells on EPO slides were semi quantitatively graded from 0 (no infiltration/ cells) to 3+ (abundant) in an blinded manner.

3.4.4 Nasal tissue

The nose complex was removed, trimmed and fixed in 4% buffered formaldehyde (II, III) or embedded in OCT medium and snap frozen in liquid nitrogen (I). Immunohistochemistry was used for semi quantitative grading of CD3, CD4 and CD8 positive T-cells in the nasal mucosa. For details on antibodies and staining procedures, see study I. In brief, the Nasal complex was sectioned coronary, fixed in ice cold acetone and processed for immunohistochemistry with CD3, CD4, and CD8 antibodies and the immunoreactive cells were detected by incubation with secondary antibodies conjugated with biotin. Bound secondary antibodies were visualized with DAB substrate kit (Vector lab) and the slides were counterstained with Mayer's hematoxylin (Histolab). The number of positive cells were graded from 0 to 3+ (0= no positive cells to 3+= abundant).

In addition, a histopathological examination of the nasal mucosa was performed in study I-III. A semi quantitative grading system was employed regarding: (a) ciliary loss, (b) inflammatory cells in the mucosa (II-III), (c) secretion, (d) number of goblet cells (I) and (e) eosinophils (I- II). Each parameter was estimated over a three- graded scale, ranging from 0 corresponding to "no increase" to 3 corresponding to "pronounced increase".

The nasal complex was decalcified in 24% ethylenediaminetetratic acid at 37⁰C for approximately one month (study II-III). After dehydration in ethanol and paraffin embedding the whole mounted complex was sectioned, stained with HE (study I-III) and Alcian blue-periodic acid-Shiff (study II-III) for morphological studies and alkaline Congo Red (study II) technique for eosinophil detection.

3.5 STATISTICAL ANALYSIS

Data were presented as a mean± standard error of the mean (SEM) or medians with 25-75th percentiles. Statistical analyses used in this thesis include; Kolmogorov-Smirnov normality test (I-IV), Student's t-test (I), Mann-Whitney U-test (I), two-way analysis of variance (ANOVA), Kruskal-Wallis ANOVA followed by Man-Whitney U-test (III) and one-way ANOVA followed by a post hoc Fisher Least Significant Test (IV). Correlations were made with Pearson's correlation. Difference was considered significant at $p \leq 0.05$. The analysis in study I was performed with a standard statistical software package (Microsoft Office, USA). Analyses in study II-IV were performed using Statistica (StatSoft Inc, USA).

4 RESULTS AND DISCUSSION

4.1 EFFECTS OF OVA-SENSITIZATION AND OVA-CHALLENGE (I-IV)

OVA-challenge in OVA-sensitized animals led to a higher degree of airway inflammation as compared to PBS-sensitized mice. In the BALF, OVA-sensitization and OVA-challenge increased the levels of total leukocytes and the proportion of eosinophils compared to PBS-sensitized animals. Lung tissue (I-III) in the OVA-sensitized animals displayed increased inflammatory cell influx, mainly eosinophils demonstrated as eosinophil peroxidase (EPO) positive cells, in the perivascular and peribronchial areas. The lungs also demonstrated epithelial shedding.

Bone marrow showed increased eosinophilia in response to OVA-sensitization and OVA-challenge compared to PBS-sensitization.

The findings of increased eosinophils in BALF and BM, and inflammatory cells in the lung tissue have also been shown in many other studies using OVA-sensitization and OVA-challenge, despite that the protocols for sensitization and challenge may vary [e.g., Ref. 104, 108, 109] [e.g., Ref. 33, 20].

4.2 INFLUENCE OF ENDOGENOUS GLUCOCORTICIDS (I)

It has been suggested that endogenous GC can reduce inflammation in allergic reactions in both humans and animals [50, 51, 54]. These studies have investigated either the peripheral (GR) or central (corticosterone synthesis) inhibition of endogenous GC. The focus of the study in paper I was to investigate the effects of endogenous GCs in allergic airway inflammation. In order to investigate the effects, we inhibited the corticosterone synthesis with the GC synthesis inhibitor ME, and the GCs-receptor with the antagonist RU 486, or we used a combination of these drugs in OVA-sensitized and OVA-challenged mice.

4.2.1 Effects on airway inflammation

In the lung tissue, we found an increase in inflammatory cell infiltration in the GC-inhibited groups, but not in the OVA-sensitized group. There were no differences between the effects of the inhibition of release or receptor blockage. Thus, the endogenous GCs may protect from allergic airway inflammation in the lungs and this is supported by early studies showing that exogenously administered GCs have protective effects in the lung tissue in allergic airway inflammation [110].

4.2.2 Effects on bone marrow

Inhibiting the release of GCs with ME before the allergen challenge resulted in increased eosinophilia in the BM compared to eosinophil amounts in the untreated OVA-sensitized and -challenged group. The combination of GC synthesis inhibition and GR-blockade further enhanced the allergen-induced BM eosinophilia (*Figure 5*). This indicates that the allergen-induced eosinophilia in the BM may be protected by endogenous GCs release.

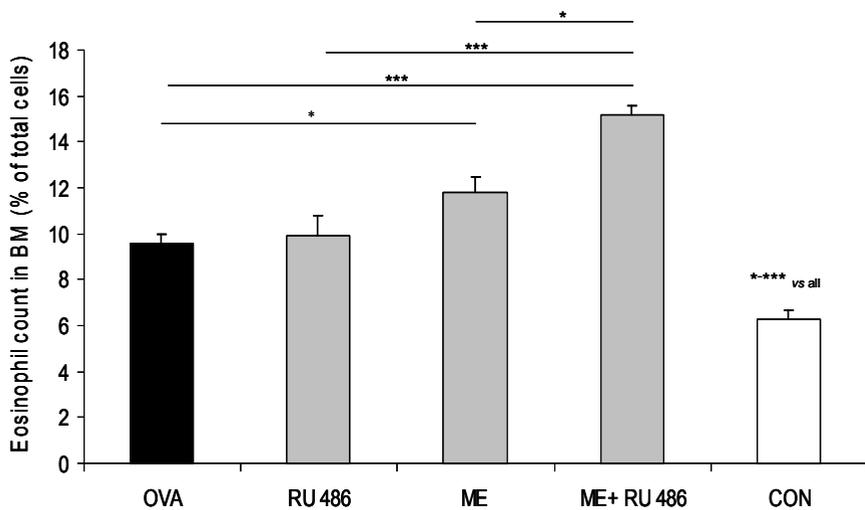


Figure 5: Bone marrow Eosinophil count as % of total cells in OVA-sensitized and -challenged Balb/C mice. The mice were treated with RU 486 (20 mg/kg), metyrapone (ME) (30 mg/kg) or a combination of both RU 486 and ME before each OVA-challenge. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Results are shown as Mean \pm SE. Figure reproduced from paper I.

4.2.3 Effects on the nasal mucosa

In the nasal tissue, we detected an increase of CD3+ T lymphocytes in the group treated with the combination of ME and RU 486. Of these CD4+ and CD8+ cells that were amplified in these animals, the CD4+ cells were increased compared to CD8+ cells. The proportion of CD4+ to CD8+ cells was increased in the groups with GC synthesis and the combination of synthesis/ GR inhibition. This may indicate that an enhancement of the allergic inflammatory response develops when the endogenous GCs effects are inhibited at the levels of both GC release and GR sensitivity [111]. These results are in line with the concept that patients with atopic asthma show a defective HPA-axis response and a predominant activation of the Th2-like T-cell population that has been found to be relatively resistant to corticosteroid [31, 112]. A concomitant increase of lymphocytes could be an early cellular marker of allergic inflammation, and is consistent with the theory that various lymphocyte phenotypes act as regulators of the subsequent eosinophilia.

4.3 TIMING EFFECTS OF LOW AND PHARMACOLOGICAL DOSES OF DEXAMETHASONE (II)

In the clinical setting, there has been a debate regarding the anti-inflammatory mechanisms of GCs given as pre- or post-treatment in relation to allergen exposure or exacerbations [33, 51]. It has also been demonstrated that in patients with moderate asthma, the inflammation could be adequately suppressed with a low dose of

corticosteroid, and at exacerbations a temporary addition of inhaled steroid was effective in controlling the asthma [113, 114]. The focus in study II was to perform a closer analysis of the timing-dependency of the effects of a very low GC dose on eosinophilic airway inflammation and compare those to the effects of a classical pharmacological, 500 times higher dose.

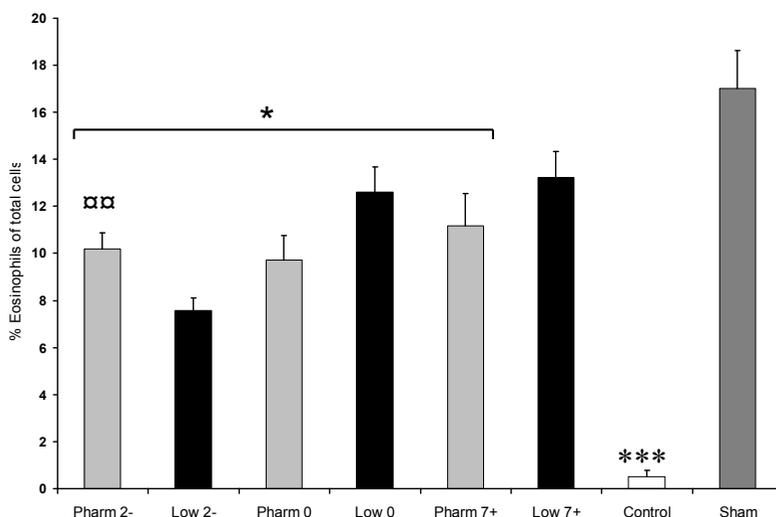


Figure 6 : Eosinophil count (%) of total cells in BALF in OVA sensitized and challenged mice. Groups were treated with a low (Low) (1µg/kg) or pharmacological (Pharm) (500µg/kg) dose of DEX and at three separate time points. * $p < 0.05$, compared with the sham-treated group, ☐☐ $p < 0.01$ compared with the low-dose administered 2 hours before challenge. Figure reproduced from paper II.

4.3.1 Effects on airway inflammation

Low dose dexamethasone (DEX) treatment administered two hours before or at the OVA-challenge gave a significant decrease of BALF eosinophils compared to what was demonstrated in the sham-treated mice (Figure 6). All treatments with the pharmacological dose of DEX gave decreased eosinophilia compared to sham treated mice and were not dependent on the time of administration. The low dose had a better effect as compared to the pharmacological dose if given before the OVA-challenge, an effect not seen when DEX was administered after challenge. In the low dose group treated two hours before challenge, an inhibition of 55% of the BALF eosinophils compared to sham treated animals was demonstrated. One explanation for the lack of effects of the pharmacological dose administered early may be that it caused a negative feedback on the HPA-axis [54]. The lack of effect could also be due to an induction of a relative insensitivity to GCs in the initiated cell compartments [54, 115].

We also observed a corresponding pattern in lung histopathology scores and amount of lung EPO positive cells as demonstrated in BALF eosinophilia (Figure 7), where the low dose given two hours before challenge had the most prominent effect on the

inflammation. These results indicate that a very low dose of GCs might be sufficient for inhibition of eosinophilic airway inflammation, although the timing of the low dose is important.

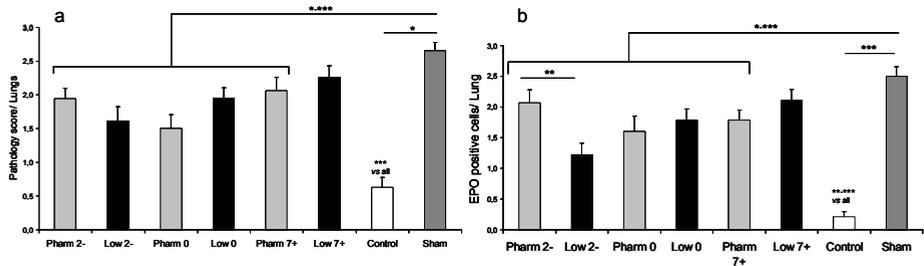


Figure 7: Semi quantitative grading of lung histopathology (a) and the number of eosinophil peroxidase positive (EPO) cells in lung tissue (b). Groups were treated with a low ($1\mu\text{g}/\text{kg}$) or pharmacological (Pharm) ($500\mu\text{g}/\text{kg}$) dose of DEX and at three separate time points.

4.3.2 Effects on nasal mucosa

Total inflammatory scores (inflammatory cells, ciliary loss, epithelial deformation) and eosinophils were increased in the sham-treated OVA-sensitized and OVA-challenged mice compared to PBS-sensitized controls (Figure 8). Concerning the nasal mucosa, only minor, non-significant effects of the DEX treatments were found. However, the general pattern of the effects of the treatment on total inflammatory scores and eosinophils were similar to that for the inflammatory scores demonstrated in the lungs. Since the nose is in the first line of defense, it may not be as sensitive to allergens as the lung tissue and in our animal model, nasal inflammation might also be too weak to detect any differences between differently treated groups.

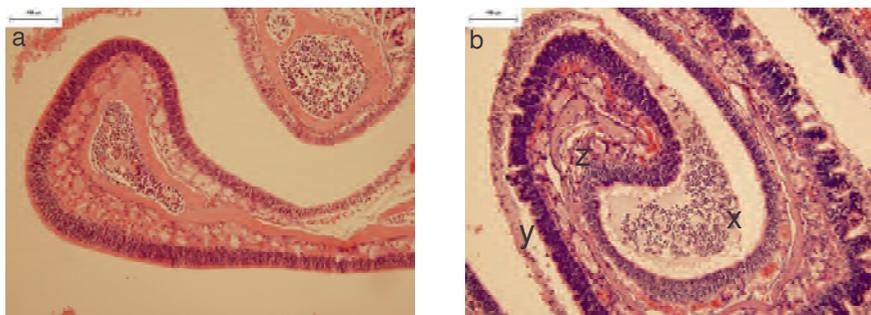


Figure 8: Pictures of nasal mucosa from healthy (a) and allergic (b) mouse. The allergic mouse displayed increased (z) inflammatory cell influx, (x) intra luminal secretion with inflammatory cells, and (y) ciliary loss.

4.3.3 Effects on LTB₄

The levels of LTB₄ in BALF increased significantly in the sham-treated mice compared to the control group. The levels of LTB₄ decreased when treated with DEX two hours before or at allergen challenge. In the animals treated after allergen challenge, the LTB₄ levels increased compared to all other groups, including sham treated animals. The lack of effect on LTB₄ levels when treated after challenge, indicates a relative lack of effect via DEX inhibition on late cellular activation.

4.3.4 Effects on bone marrow

In the bone marrow, eosinophils decreased in the groups treated at challenge or 7 hours after allergen challenge compared to what was found in the sham-treated animals. There was a slight difference in BM eosinophils between the low and pharmacological dose groups, although this was not significant, when GC was administered two hours before allergen challenge (*Figure 9*). These results indicate that the GCs effect on allergen-induced BM eosinophilia seems more dependent on timing for administration than given dose.

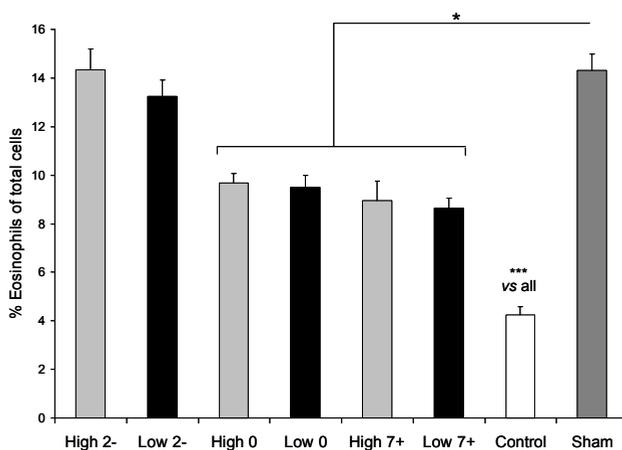


Figure 9: Bone marrow eosinophils in OVA-sensitized and OVA-challenged mice. Groups were treated with low or pharmacological dose of DEX 2 hours, just before or 7 hours after challenge.

4.4 TIMING-DEPENDENT EFFECTS OF GCs ON RESTRAINT STRESS (III)

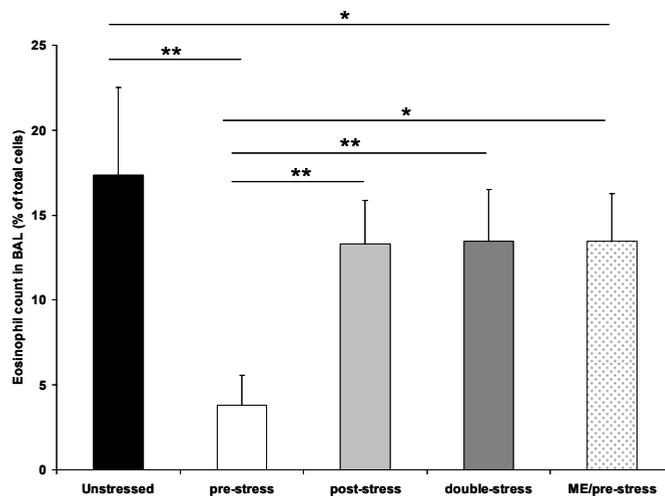
Acute stress has been shown to improve allergen-induced airway obstruction [3] and dyspnea in severe asthmatic women [4], whereas chronic stress is known to aggravate the allergic inflammation [46] and increase the number of exacerbations [2].

The aim with this study was to investigate the effects of alterations in timing of restraint stress (RST) on the allergic airway inflammation in both upper and lower airways. An

animal model was developed in which an allergic airway inflammation and stress exposure were combined.

4.4.1 Restraint stress and lower airway inflammation

OVA-sensitized mice subjected to RST prior to the OVA-challenge displayed a significant decrease of BALF eosinophils and lymphocytes compared to unstressed mice. When RST was applied after allergen challenge or as a double stress both before and after challenge a small, but not significant inhibition of BALF eosinophilia was seen. Stress before allergen challenge inhibited BALF eosinophilia stronger compared to the other stressed groups (*Figure 10*). In addition, in the lungs pre-challenge stress decreased the inflammatory scores as compared to in unstressed animals. The decreased levels of eosinophils in the airways may depend on prevention of eosinophil progenitor expansion and terminal differentiation [32], due to increased levels of endogenous GCs, as it has previously been demonstrated that restraint stress increases the corticosterone levels 10-fold [116].



*Figure 10: Bronchoalveolar lavage fluid (BAL) eosinophil count as (%) of total cells in OVA sensitized and -challenged Balb/C mice. Mice in the metyrapone (ME)/pre-stress group were treated with ME (100 mg/kg) before exposure to a two hour restraint stress according to protocol (Figure 1). *: $p < 0.05$, **: $p < 0.01$. Results are shown as Mean \pm SEM. Figure reproduced from paper III.*

To investigate whether the effects of RST were dependent on endogenous corticosterone release, mice were treated with metyrapone (ME) before the pre-challenge stress. ME treatment inhibited the decrease in BALF eosinophils seen in response to a pre-challenge stress, and thus it might be speculated that the stress effects on airway eosinophilia are corticosterone-dependent. Thus, the inhibition of the inflammatory response is suggested to be a result of increased endogenous GC levels.

In the lung tissue pre-challenge stress seemed to reduce allergic inflammation, as shown by the decrease in inflammatory scores. In the lungs, ME also inhibited the stress-evoked increased inflammation. Pre-challenge stress had no effect on the amount of eosinophil peroxidase positive cells in lung tissue.

4.4.2 Restraint stress and upper airway inflammation

In the nasal tissue, pre-challenge stress decreased the inflammatory cell influx, mucus secretion and ciliary loss as compared to the unstressed animals. We found that the nasal pathology followed the same pattern as in lung tissue and BALF. The findings in the nose indicate that we have an ongoing inflammation and remodeling process in the nasal tissue (*Figure 11*).

The stress evoked changes were only partly reversed with corticosterone inhibition by ME treatment. Thus, the mechanism for stress-evoked inhibition of nasal pathology is suggested to be less dependent on GCs. Instead, it may be regulated by other mechanisms, such as catecholamines and corticotrophin-releasing factor (CRF).

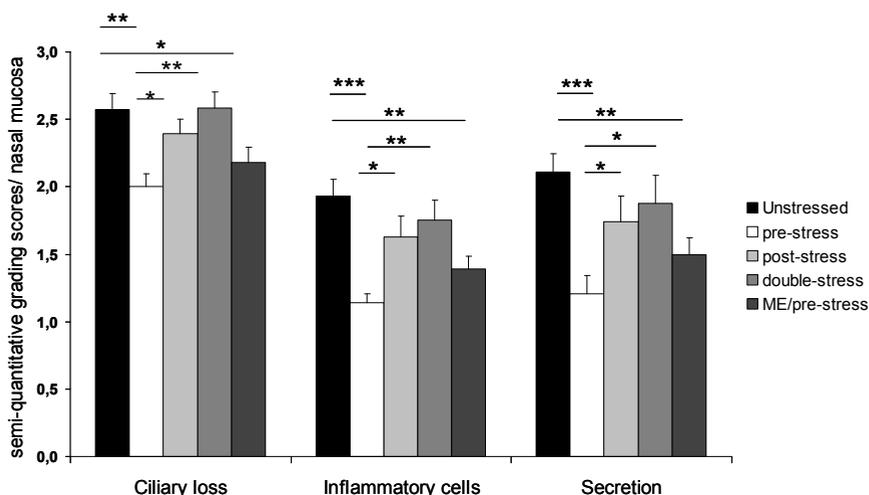


Figure 11: Scores from semi quantitative grading of nasal mucosa pathology. Groups were exposed to a single or double two hour restraint stress. Mice in the ME/pre-stress group were treated with 100 mg/kg ME prior to a pre-challenge stress. Figure reproduced from paper III.

4.5 STRESS EFFECTS ON AIRWAY NGF LEVELS (IV)

NGF has been shown to increase in response to stress [92, 94] and it has also been found to be increased in patients with allergic rhinitis and asthma [76-78].

The focus in the study (IV) was to investigate if acute restraint stress could regulate levels of NGF and inflammatory cells in the airways during a mild allergic airway inflammation. A similar model as in study III, with both airway inflammation and stress was used.

4.5.1 Stress effects

Acute restraint stress increased the levels of NGF in BALF in non-sensitized animals (*Figure 12*). Thus, we show for the first time that NGF increases locally in the lungs in response to stress. The increase could be due to increased production or release in structural cells, such as fibroblasts, airway epithelial cells inflammatory cells or neurons, since these cell types have been suggested to be sources of NGF in the airways [77, 86, 89-91] or it could be due to a systemic increase of NGF. Stress had no effects on BALF neutrophils, lymphocytes, macrophages or BM eosinophils.

In order to investigate the relationship between inflammatory cells and NGF, Pearson's correlation analysis was performed but no correlation between NGF and the amount of cells could be shown.

As in study III, metyrapone (ME) was used to investigate whether endogenous GCs would influence the NGF levels or the inflammatory cells in response to stress. In stressed mice treated with ME, the levels of NGF were significantly lower compared to NGF levels in the stressed, sham-treated mice, indicating that the stress-induced increase of airway NGF was dependent on GCs-synthesis.

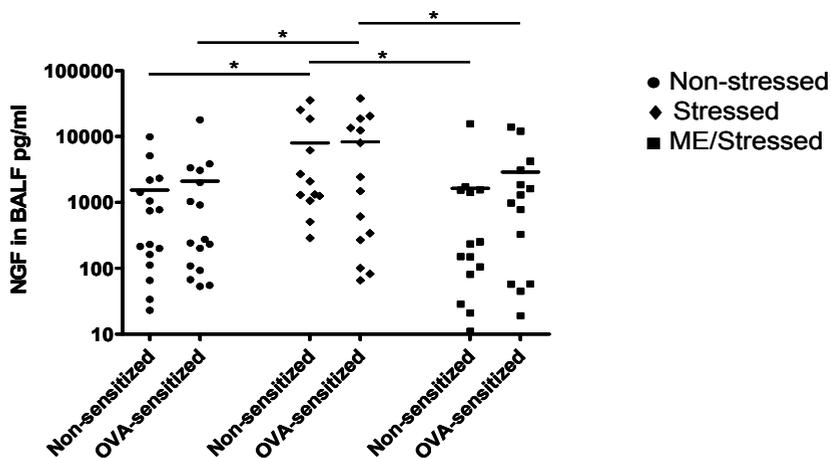


Figure 12: Levels of NGF in BAL. BALB/c mice were exposed to stress, ME and/or OVA-challenge for three consecutive days. Figure reproduced from paper IV.

4.5.2 Effect of allergen challenge

Even though we induced a significant increase in BALF eosinophils by allergen challenge, although moderate, no increase in BALF NGF levels was evident in response to allergen challenge. This is in contrast to other studies [80, 87] where increased levels of NGF in response to the allergen challenge have been demonstrated. This might be explained by the fact that we, in this study, utilized a milder airway inflammation model than used in previously mentioned studies, where it also has been suggested that the levels of NGF may correlate to the grade of inflammation [76]. The BM showed an increase of eosinophils in response to airway allergen challenge. The neutrophils, lymphocytes and macrophages in BALF remained unaltered.

4.5.3 Effects of stress and airway inflammation

The levels of NGF increased in response to stress also in allergic mice. Since NGF did not increase in response to allergen provocation alone but in response to stress in both allergic and non-allergic condition, it may be suggested that restraint stress may be a more powerful regulator of NGF than the allergic provocation in this model. The proportion of eosinophils in BALF decreases in allergic stressed animals, compared to non-stressed allergic animals (*Figure 13*). Decreased levels of eosinophils in response to stress have been shown previously [108, 117]. It is also in line with the results from study III, although, in study III we did not find any significant decrease of the eosinophils in response to a restraint stress when applied after the allergen challenge. This might be related to the milder inflammation in study IV, which may be more susceptible to stress-evoked immune-suppression. Stress had no effects in allergic mice on BALF neutrophils, lymphocytes, macrophages or BM eosinophils.

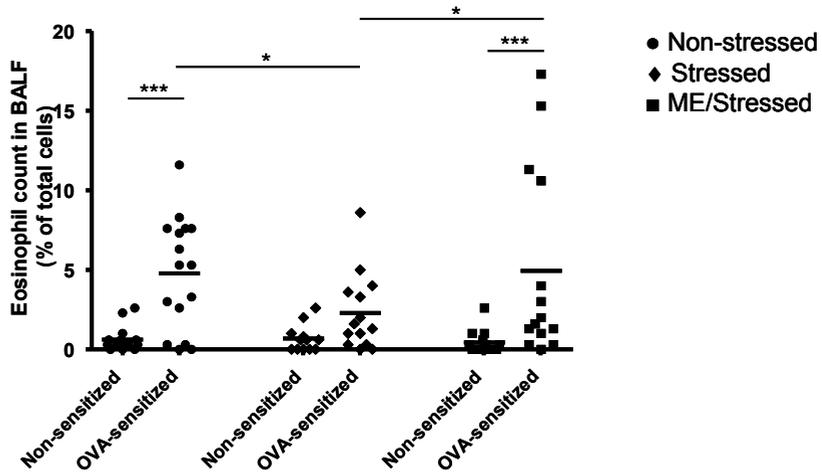


Figure 13: Proportion of eosinophils in BALF in mice exposed to stress, ME and/or OVA-challenge for three consecutive days, Figure reproduced from paper IV.

Also, in allergic mice, ME treatment prevented a stress-induced increase in NGF in BALF. ME prevented also the stress-evoked decrease of allergen induced eosinophilia in BALF. Thus, ME treatment abolished the stress-induced changes in the levels of BALF NGF and eosinophils indicating a corticosterone-dependent regulation.

5 GENERAL DISCUSSION

A role for stress in asthma and allergic diseases has been proposed, and both immune-enhancing and -suppressive effects by stress have been suggested [65, 68]. In humans, psychological stress has been shown to aggravate several factors characteristic for asthma, including the extent and numbers of exacerbations [2] and the degree of the allergic inflammation [45, 46]. Stress has also been shown to have positive effects on the asthmatic reactions by improving the allergen-induced airway obstruction and dyspnea in severe asthma [3, 4].

In these studies, mouse models of allergic airway inflammation have been used. These types of models are suggested to be valuable in evaluating the dynamics of certain phenomena, or a specific trait of the human disease [118]. We mainly used the model to investigate the effects of GCs on regulation of the eosinophils.

Sensitization and intra-nasal challenge with OVA induced an eosinophilic inflammation, both in BAL and lung tissue, in all studies, although the effect was milder in the fourth study (IV). We could also demonstrate an allergen-induced activation of the bone marrow, as shown by increased levels of eosinophils. Thus, one possibility for the increase of airway eosinophilia in the airways is increased egression from the bone marrow, which is in agreement with previous studies in mice [119].

In our first study, we demonstrated that inhibition of the GCs synthesis increased the inflammation in the bone marrow and in combination with GR inhibition the increment was further enhanced. In nasal mucosa, an increase of CD4⁺ cells was demonstrated when both GCs release and peripheral GC receptors were inhibited. It has previously been demonstrated that CD4⁺ T-cells play an important role in the induction of allergen-induced nasal inflammation, thus the endogenous GCs may protect from an allergic nasal inflammation [111].

The airway inflammation may induce an activation of the HPA axis by specific cytokines (e.g. IL-1, IL-6), and thus, induce an increase of cortisol to downregulate the inflammatory response [56, 120]. In atopic individuals a malfunctional HPA axis regulation could be a key regulator for developing asthma or rhinitis. It has been demonstrated that infants of an allergic parent had a flattened circadian cortisol rhythm [121] and several studies have reported that children with allergic asthma have a blunted HPA axis response and in response to stress they have showed reduced cortisol release. [reviewed in Ref. 56] Thus, a flattened or decreased cortisol release could up-regulate an allergic inflammation or may be a risk factor for development of airway allergies.

The inflammatory process can up-regulate the HPA axis function, e.g. through activation of the proinflammatory cytokines IL-1 and IL-6. Inappropriately normal cortisol levels have been found in other inflammatory diseases, such as in rheumatoid arthritis (RA) when cytokine levels are highly increased [122]. In those patients, an inadequate regulatory interaction between the HPA axis and adrenal glands, may lead to decreased levels of ACTH, but normal levels of cortisol. This could be due to activation of the adrenal glands by mediators other than ACTH, e.g., IL-6 is known to

activate the adrenal glands directly and increase the cortisol levels. Although, the alterations of HPA-regulation might be stronger in RA patients, the same phenomenon is likely to occur also in patients with other inflammatory diseases, such as allergic airway inflammations.

When the inflammation persists the HPA axis response may decrease in robustness, and the effects of the endogenous GCs diminish, probably through downregulation in GR sensitivity. It has also been shown that when stimulus for the inflammation is withdrawn, the HPA axis is readjusted rather quickly to the basal level and this indicates that it is in the peripheral tissues most of the unresponsiveness occurs [122]. This unresponsiveness may be the result of either repeated GCs exposure (repeated stress) or upregulated inflammation in various inflammatory compartments.

In the second (II) study, allergic mice were treated with an extremely low dose of a synthetic steroid, dexamethasone, in order to mimic increased endogenous GCs. In the third (III) study we used restraint stress to induce increase of the endogenous corticosterone levels [123-125]. In both studies, application of stress or steroid before the allergen provocation resulted in reduced inflammation in the airways. Thus, endogenous GCs were demonstrated to have a protective quality in both upper and lower eosinophilic airway inflammation by reducing the eosinophilic infiltration in the lung tissue and BALF and also the histopathological changes in the nasal mucosa. Varied timing of increased endogenous GCs provoked differential effects on the eosinophilic inflammation, where an early timing of GCs attributes to the most prominent reduction of the inflammation within the airways, indicating that an already ongoing inflammation is more difficult to reduce.

In the clinical settings for asthma and rhinitis treatments, the dosages of steroids are much higher and it is not possible to start the treatment before the first episode of allergic asthma or rhinitis. Although it may be possible to find some stage of the disease where the treatment could have a more pronounced effect, such as immediately after exacerbations. The pharmacological dose we used in the second study gave less inhibitory effects on the inflammation than the low dose, when administered before allergen provocation, and the pharmacological dose was not as dependent on the timing as the lower dose.

The finding from our first study, where the inhibition of endogenous GCs enhances the eosinophilia within the bone marrow, is in line with other studies indicating that exogenous administered GCs inhibit the eosinophils [32, 126]. A steroid treatment may influence the BM eosinophilia in several ways; a decrease in eosinopoietic mediators such as cytokines could reduce the terminal differentiation of eosinophils in the BM, or could increase the release of mature eosinophils to the peripheral tissue, which both cause a decrease in eosinophilia [32]. An increase of endogenous GCs, induced with a short restraint stress, did not affect bone marrow eosinophils. This could be due to the fact that an eventual effects on bone marrow eosinophilia could have occurred earlier than the time for evaluation, and that an alteration in bone marrow eosinophil number may have been restored by eosinophil progenitor expansion [30].

The decrease of eosinophils in BALF and lungs after steroid treatment in our study is probably not exclusively the result of decreased bone marrow production of eosinophils [127]. The fall of eosinophilia in the peripheral tissues of steroid-treated individuals may also be due to apoptosis. Although, this has not been compellingly shown *in vivo* [66, 128, 129], apoptotic eosinophils are often found in the airway lumen [130]. Reduced release of eosinophils from the bone marrow and reduced tissue infiltration of eosinophils from circulation may cause a decrease in peripheral eosinophils. The mechanisms for these effects may include down regulation of mediators that stimulate eosinophil recruitment, such as IL-5. An alternative explanation could be a decreased maturation of eosinophils as a result of reduced T-lymphocytes [131, 132] and we also demonstrated reduced T-lymphocytes in BALF in response to stress.

The different effects of the GCs are probably dependent on the type of cell, its grade of activation and also on the present cell compartment. There is also the possibility that different stages of the inflammatory cascade have alternate sensitivity for the GCs. After repeated exposures to elevated endogenous or exogenous GCs, it may be proposed that an adaptation will take place; either as a physiological down-regulation of the GCs release in response to natural activation, or that a disease or pathological change decreases the levels or sensitivity of the GR in the target tissue. It has been demonstrated that asthmatic children have increased levels of GR in the leukocytes, and during stress the levels are down-regulated [133].

Few murine models of airway allergy investigate both upper and lower airways, despite all proposed connections such as anatomically close location, function, local immunity and the comorbidity of asthma and rhinitis [reviewed in Ref. 102, 134]. We could in our models demonstrate increased nasal inflammation and pathology in the allergic compared to non-allergic animals. The evidence for this was increased influx of inflammatory cells, ciliary loss, epithelial deformation and mucus secretion. We also showed that short stress applied close to the allergen challenge caused reduction in the inflammation and remodelling process seen in the nasal mucosa after allergen challenge.

The positive effects on the inflammation in the airways were partly dependent on endogenous GCs release, since these effects were inhibited with the GCs synthesis inhibitor metyrapone. Thus, the endogenous GCs can also have a protective quality in the pathological process that occurs in the nasal tissue. The mechanisms for regulation of the nasal inflammation may not only depend on GCs, but could also involve regulation through the SAM system, mainly through CA and CRH, since a remaining nasal response was present after GC-synthesis inhibition. It has been demonstrated that CA and GCs in combination have a cooperative anti-inflammatory effects and that they also enhance the cytokine profile toward a Th2-type *in vitro* [135].

We could to some extent see the same influence on the pathology scores of GCs in both upper and lower airways, although they were much weaker in the nose. The nose represents the first line of defence and has also been considered to have a high normal inflammatory level and may not be as sensitive to allergens or anti-inflammatory treatments as the lungs [136]. It has also been indicated that the nose is the primary

site for immune response and triggers the systemic reaction within the bone marrow and then onward to the lungs [102, 103]. Although, these findings arise from animal models, it is reasonable to suggest that they could be applicable in humans as well.

Periods of short stress increased local airway NGF, which was shown to be mediated by endogenous GCs release. NGF has been shown by others to be increased systemically in response to stressful stimuli [92-94]. However, our study shows for the first time that a local up-regulation within the airways occurs in response to stress. NGF may be involved in the regulation of a “third stress-axis” [137], which recently has been suggested besides the HPA and SAM axis. This axis has been suggested to regulate the peripheral neuroimmune interactions. Thus, NGF could function as an early mediator of the psychoneuroimmunological stress-response. The neuroimmune interactions can cause a neurogenic inflammation with increased activation and degranulation of the mast cells and eosinophils. The neurogenic inflammation has also been shown to induce increased levels of Th2 type of cytokines, such as IL-4 and IL-5 [138].

Although, it has been shown that NGF may correlate to the degree of inflammation [76], we did not find such a correlation in our model. However, in support of our results, it has been shown that allergen-evoked eosinophilia was unaffected when NGF-deprivation has been performed before allergen challenge in rats [139].

The precise role for the stress-induced elevation in NGF in the airways needs further investigation.

Stress, especially chronic stress, in allergic patients is today mainly considered to exert a negative influence on the allergic disease and to be associated with an up-regulation of the allergic inflammation. However, we here demonstrate that a short stress provocation, when delivered at the right time point, can alleviate allergic airway inflammation. It is not inconceivable to think that stress could improve allergic airway inflammation, since the major drugs for treatment of allergic inflammation, i.e. synthetic GCs, are based on substances developed from the natural stress hormone cortisol. The factors determining why, and at which point a beneficial influence of stress on allergic disease, changes into a harmful influence, still remain to be elucidated. Such investigations would be important, not only to prevent worsening of allergic airway disease by stress, but could also contribute to further valuable information about the mechanisms behind steroid-resistant asthma.

This thesis points to the necessity to develop different intervention strategies, depending on disease-specific pathology in the activated cellular compartments and also points to the need to base this intervention on the GC sensitivity in specific cell populations during different stages of the allergic disease.

6 CONCLUSIONS

- 1) Endogenous glucocorticoids have a regulatory effect in protecting against allergen-induced eosinophilic inflammation in the lungs and bone marrow, and also reduce the expression of CD3⁺ and CD4⁺ T-lymphocytes in the nasal mucosa.

- 2) The timing of a glucocorticoid treatment is decisive for the outcome of the allergic inflammatory response in mice in that a very low dose can be sufficient to decrease the inflammation if administered before allergen-challenge.

- 3) The effects of short stress on allergic airway inflammation in mice appear dependent on timing and can lead to inhibitory effects on allergic inflammation. The stress-evoked inhibition on allergic inflammation appears to be partly glucocorticoid-dependent.

- 4) Acute stress increases local airway nerve growth factor levels in both allergic and non-allergic animals. The up-regulation of NGF appears glucocorticoid-dependent.

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