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Effects of glucocorticoids or β_2-agonists on inflammatory responses induced by organic dust *in vitro* and *in vivo*

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När man är en björn med Mycket Liten Hjärna och Tänker Ut Saker,
upptäcker man ibland att en Idé som verkade vara riktigt Idéaktig inne i hjärnan,
är helt annorlunda när den kommer ut i det fria och andra människor ser på.

Nalle Puh

to my family, with love

List of Original Papers

This thesis is based on the following papers, which will be referred to by their Roman numerals. Permission to reproduce the articles has kindly been granted by Blackwell Publishing, Elsevier and the European Respiratory Society Journals Ltd.

I. Ek A, Larsson K, Siljerud S, Palmberg L.
"Fluticasone and budesonide inhibit cytokine release in human lung epithelial cells and alveolar macrophages."
Allergy 1999; 54(7): 691-9.

II. Lidén J, Ek A, Palmberg L, Okret S, Larsson K.
"Organic dust activates NF-κB in lung epithelial cells."
Respir Med 2003;97(8):882-92.

III. Ek A, Palmberg L, Larsson K.
"The effect of fluticasone on the airway inflammatory response to organic dust."
Eur Respir J 2004; 24:1-7: *in press*.

IV. Ek A, Palmberg L, Larsson K.
"Influence of fluticasone and salmeterol on airway effects of inhaled organic dust; an *in vivo* and *ex vivo* study."
Clin Exp Immunol 2000;121(1):11-6.

V. Ek A, Palmberg L, Sundblad B-M, Larsson K.
"Salmeterol has no effect on the increased bronchial responsiveness caused by organic dust."
Submitted

Abbreviations

AP-1	Activator protein-1
b.i.d.	Twice daily
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
ELISA	Enzyme-linked immunosorbent assay
FEV_1	Forced expiratory volume in one second
IL	Interleukin
IκB	Inhibitory protein -κB
LPS	Lipopolysaccharide
LUC	Luciferase
NF-κB	Nuclear factor-κB
ODTS	Organic dust toxic syndrome
$PC_{20}FEV_1$	Cumulative provocation concentration of methacholine causing a 20% decrease in FEV_1
$PD_{20}FEV_1$	Cumulative provocation dose of methacholine causing a 20% decrease in FEV_1
PDTC	Pyrrolidinedithiocarbamate
PEF	Peak expiratory flow
SD	Standard deviation
SEM	Standard error of the mean
TNF	Tumor necrosis factor
VC	Vital capacity

Contents

1 Introduction

1.1 Background

Three hours of exposure to organic dust in a swine barn causes an acute inflammatory response in both upper and lower airways of healthy subjects and an increase in bronchial responsiveness to methacholine (Larsson et al., 1997; Larsson et al., 1994a; Larsson et al., 1994b; Malmberg et al., 1993; Muller-Suur et al., 1997; Wang et al., 1997; Wang et al., 1996; Wang et al., 1998; Zhiping et al., 1996). The reaction is characterized by an influx of inflammatory cells into the airways, cell activation and cytokine/mediator release as demonstrated in a number of studies at our laboratory (Larsson et al., 1999; Muller-Suur et al., 2000; Palmberg et al., 1998; Wang et al., 1999). This reaction is known as Organic Dust Toxic Syndrome (ODTS), characterized by flu-like symptoms following exposure to organic dust (Seifert et al., 2003). The duration of symptoms is often less than 24 hours.

In the present thesis, the acute inflammatory response following swine house dust exposure is used as a model for studies of inflammatory mechanisms involved in innate immunity. The inflammatory profile in chronic obstructive pulmonary disease (COPD), severe asthma, or during asthma exacerbation shows similarities with the acute inflammatory response in healthy subjects following exposure in a swine barn. Glucocorticoids and β_2-agonists are the most frequently used drugs to treat inflammation in bronchial asthma and COPD. The inflammatory disease mechanisms and the precise mechanisms underlying the beneficial action of glucocorticoids and β_2-agonists in these context are still not fully understood.

In this thesis, we have studied the effects of glucocorticoids or β_2-agonists on the inflammatory response following exposure to swine house dust both *in vitro* and *in vivo*. The aim was to study features of the inflammatory mechanisms during the acute inflammatory response induced by exposure in a swine barn and the mechanisms by which this innate inflammatory reaction may be controlled by drugs known to interact with airway inflammatory conditions.

1.2 Inflammatory responses induced by organic dust exposure

1.2.1 Organic dust toxic syndrome

Organic dust toxic syndrome (ODTS), a condition following heavy organic dust exposure, is associated with fever, cough, malaise, chest-tightness, dyspnea, headache, chills, nausea and muscle pain (Von Essen et al., 1990). The symptoms usually disappear within 1-2 days. ODTS is observed among farmers at work. The mechanism of this pathological condition is a non-specific immune response but the exact mechanism of toxicity is not known (Von Essen et al., 1990).

This condition appears to be quite common among swine confinement workers. Workers in swine confinement buildings are exposed to high levels of organic dust and to gases. Dust levels ranging from 1.7 to 21 mg/m^3 have been reported in swine farms with corresponding endotoxin concentrations ranging from less than 0.1 to 1.9 μg/m^3 (for review see (Larsson,

2001)). Swine confinement workers have an increased frequency of airway symptoms such as cough, phlegm, wheezing and shortness of breath and have higher prevalence of pulmonary disorders, such as chronic bronchitis, than non-farmers and than other farmers (Zejda et al., 1993) (Larsson, 2001). It has been demonstrated that also healthy pig farmers have signs of airway inflammation (Larsson et al., 1992).

Healthy, previously non-exposed, individuals exposed to organic dust in a swine house for three hours develop ODTS with an intense airway inflammation and an increase in bronchial responsiveness to methacholine. The inflammatory response is characterized by a massive influx of inflammatory cells, into the upper and lower airways. The cell increase consists mainly of neutrophilic granulocytes which increase about 20-fold and 70-75-fold in nasal and bronchoalveolar lavage fluid, respectively (Larsson et al., 1997; Larsson et al., 1994b). There is also a significant increase of alveolar macrophages, lymphocytes and eosinophils in bronchoalveolar lavage fluid (Larsson et al., 1997) and an increase of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-1β, IL-6 and IL-8 in bronchoalveolar and nasal lavage fluid after exposure (Larsson et al., 1997; Wang et al., 1997). Exposure also causes systemic effects such as increase in acute phase proteins (CRP, orosomucoid and haptoglobin) and increase in serum IL-6 and TNF-α (Larsson et al., 1994b; Wang et al., 1996).

The exact mechanisms causing this condition and the components or combination of the organic dust that elicit specific effects are still not clear. Swine dust consists of a complex mixture of micro-organisms, fungal spores, hay, animal feed and animal products. Bacterial endotoxin (lipopolysaccharids, LPS), Gram positive bacteria and their products, including peptidoglycans, and (1-3) β-D-glucan from mould might contribute or be important in mediating the response to organic dust (Larsson et al., 1999; Palmberg et al., 1998).

1.3 The innate immunity

The innate immune system is a universal and ancient form of first line host defence against invading microbial pathogens. Collectively, it consists of many interacting systems, including epithelial barriers (skin and mucosal epithelium), antimicrobial peptides (for example defensins) and circulating effector cells (neutrophils, mononuclear phagocytes and natural killer (NK) cells). Immunology in recent year expresses renewed interest in innate immunity because of its regulatory role on the adaptive immune response.

1.3.1 The inflammatory response

Invading micro-organisms or tissue injury induce an inflammatory response which plays an important role in health and disease. This response is rapidly initiated by the innate immune system and does usually not require the participation of the adaptive immune system. The response to organic dust mainly involves the innate, non-specific immunity.

Inflammation is a general term used to describe the many diverse processes that tissues employ in response to infections by pathogens and injuries. Classically, the cardinal signs of the inflammatory reaction are redness, swelling, pain, heat and reduced function (Kuby, 1994). These signs are characteristic for the initial phase of inflammation, termed the acute inflammatory response.

The inflammatory response comprises a complex sequence of events. The systemic reaction is manifested as fever, leukocytosis, increase of several cytokines, activation of

cyclooxygenase and lipoxygenase pathways as well as clotting, complement activation, kinin-forming cascades and acute phase protein synthesis in the liver. The local inflammatory reaction is characterized by an initial increase in blood flow to the site of injury and enhanced vascular permeability to plasma proteins and water, leading to oedema formation. At the site of injury granulocytes from the peripheral blood infiltrate and accumulate. The aim is to destroy, dilute or wall-off both the injurious agent and the injured tissue. The accumulation and subsequent activation of leukocytes are crucial events in this reaction (Dempsey et al., 2003; Kuby, 1994).

The inflammatory response is critical for host defence, helping to clear infection. It often disappears within a few days, is occasionally fatal as in septic shock or is gradually transformed into a chronic inflammatory disease. Excessive inflammation may result in lung injury and in different inflammatory diseases.

1.3.2 Cells

The epithelium has a number of critical functions; these include a structural barrier against exogenous pathogens, regulation of lung fluid balance, metabolism and/or mucociliary clearance of inhaled agents, attraction and activation of inflammatory cells in response to injury and the regulation of airway smooth muscle function via secretion of numerous mediators (Davies et al., 1992; Knight et al., 2003). Thus, epithelial cells play an active role in initiating and modulating airway inflammation.

The airway macrophages have also since long been recognized as important for the first line of defence against inhaled airborne constituents (Holian et al., 1990). Alveolar macrophages reside predominantly within the bronchoalveolar air spaces and are easily accessible by lung lavage. They have the ability to migrate to sites of inflammation. Alveolar macrophages possess a high phagocytic and microbicidal potential. Upon activation, alveolar macrophages may release reactive oxygen intermediates, nitric oxide, lysosomal enzymes, interferon, complement and a wide variety of inflammatory cytokines, chemokines and mediators including prostaglandins and leukotrienes (Lohmann-Matthes et al., 1994). They may also initiate or modulate the activities of other immune cells.

Neutrophils (polymorphonuclear leukocytes, PMNs) are the most abundant circulating leukocytes. They are characterized by the presence of both a multi-lobed nucleus and cytoplasmic granules. They can rapidly mobilize at the onset of an infection. Following exposure and triggering, adhesion molecules promotes neutrophil adherence and subsequent diapedesis and transmigration in response to chemokines and other chemotactic factors (Adams et al., 1994; MacNee et al., 1993). The lifespan of the mature circulating neutrophil is estimated to be around 7-10 hours before migrating into the tissue where they have a 3-days life span (Kuby, 1994; Moulding et al., 1998). The defensive role of the neutrophil is to kill and eliminate micro-organisms by mechanisms which include phagocytosis, the respiratory burst and the release of cytotoxic peptides and proteins (Gompertz et al., 2000; Witko-Sarsat et al., 2000). Neutrophils play an important role in the inflammatory process. Persistent activity of neutrophils also contributes to tissue destruction due to production of proteases and reactive oxygen metabolites. Through this mechanism, the protective role of these cells may turn into a deleterious action targeting the host itself.

Other cells such as mast cells, T-lymphocytes, endothelial cells, fibroblasts and eosinophils (which are the characteristic inflammatory cell in bronchial asthma) may also contribute to the inflammatory process and have important roles in inflammatory responses.

1.3.3 Cytokines and inflammatory mediators

The cellular influx to inflammatory sites is mediated by a plethora of mediator substances supporting and dispersing inflammation. These mediators are found in the serum or tissue fluids, and are released by degranulating cells, secreted by inflammatory cells upon activation or secreted by activated epithelial or endothelial cells at the site of inflammation. They serve as muscle-active and oedema-promoting substances, chemotaxins and cellular activators and inducers of all kinds of effector cells.

Cytokines are multifunctional mediator molecules of low molecular mass (<50 kDa) which are extremely biologically active at nano- to picomolar concentrations (Kuby, 1994). The most important pro-inflammatory cytokines involved in starting and developing the inflammatory response are TNF-α, IL-1α, IL-1β, IL-6, IL-8 and IFN-γ. These cytokines either act as endogenous pyrogens (IL-1, IL-6, TNF-α), up-regulate the synthesis of secondary mediators and pro-inflammatory cytokines by both macrophages and mesenchymal cells (including fibroblasts, epithelial and endothelial cells), stimulate the production of acute phase proteins, or attract inflammatory cells (IL-8).

TNF-α is a multifunctional cytokine produced by many cell types, mainly macrophages/monocytes but also by mast cells, endothelial cells and epithelial cells in response to inflammation. TNF-α can be rapidly up-regulated upon a stimulus, but also rapidly degraded. TNF-α elicits a broad spectrum of cellular responses including lymphocyte and leukocyte activation, cell proliferation and migration, fever, acute-phase response, differentiation and apoptosis (Baud et al., 2001; Thomas, 2001).

IL-6 is a circulating key pro-inflammatory cytokine known to be secreted from a number of different cells including macrophages, fibroblasts, lymphocytes, epithelial and endothelial cells. IL-6 is also a multifunctional cytokine which is an important factor in the immune and haematopoietic system and it is the major mediator in the hepatic acute phase response (Heinrich et al., 1990; Kishimoto, 1989). IL-6 expression and secretion is induced by IL-1 and TNF-α. In addition, IL-6 has several anti-inflammatory activities including suppression of the pro-inflammatory cytokines TNF-α and IL-1 (Barton, 1997).

Stimulated airway epithelial cells and alveolar macrophages can secrete IL-8, which is an important mediator of airway neutrophil chemotaxis and has neutrophil activating properties (Hebert et al., 1993). Recruited neutrophils may further amplify neutrophilic airway inflammation via the generation of additional IL-8 (Gainet et al., 1998).

1.3.4 Innate immune recognition

A basic concept for the innate immune system is to recognize and detect microbial invaders. Pathogen-associated molecular patterns (PAMPs), unique for micro-organisms and distinguished from the host, are detected by a range of different recognition molecules. Soluble pattern recognition molecules include lysozyme and complement proteins (C1q, C3a and C5a) and pattern recognition receptors include CD14 and the Toll-like receptors (TLR) (Palaniyar et al., 2002).

Two receptors which are likely to be activated by organic dust from a swine house are the TLR2, TLR4 and TLR9 which are implicated in the recognition of peptidoglycan, lipopolysaccharide (LPS) and bacterial DNA, respectively (Liu et al., 2001; Takeuchi et al., 1999). Upon activation, they transduce signals for NF-κB activation.

1.3.5 NF-κB

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that regulates the expression of many inflammatory and immune genes. Many of these genes are induced in inflammatory and structural cells and play an important role in the inflammatory process.

Several related proteins have been characterized that belong to the Rel family of proteins. NF-κB was originally identified as a heterodimer consisting of two subunits, p65 (RelA) and p50 (NF-κB1) but a variety of other forms may also occur. p65 has potent transactivation domains which are critical in transcriptional activation, whereas p50 is mainly a DNA-binding subunit and a relatively poor transactivator (Siebenlist et al., 1994). NF-κB is present in an inactive state in the cytoplasm, sequestered by the inhibitory protein (IκB), of which several isoforms exist, the most abundant being IκB-α. Activation by extracellular signals induces phosphorylation and ubiquitinylation of IκB-α by specific IκB kinases (IKK), leading to rapid degradation, and thus release of NF-κB (figure 1)(Baldwin, 1996). As a result, NF-κB translocates in to the nucleus and bind to NF-κB response elements in the promoter region of many inflammatory and immune genes. Persistent NF-κB activation can in turn induce the synthesis of IκB-α, which terminates the NF-κB response, explaining its transient nature (Beg et al., 1993).

Many different agents that activate the NF-κB signalling system are a consequence of inflammation and infection. Many different bacteria and bacterial products (such as LPS), viruses, cytokines (such as IL-1, TNF-α), physical stress and oxidative stress activate NF-κB (Siebenlist et al., 1994). The activation and nuclear translocation of NF-κB have been associated with increased transcription of a number of different genes, including those coding for chemokines (IL-8), cytokines (IL-1, IL-2, IL-6, TNF-α and IL-12), enzymes, immune receptors and adhesion molecules (Caamano et al., 2002). These mediators are required for the ability of inflammatory cells to migrate into areas where NF-κB is being activated. Thus, NF-κB is an important component of the innate immune response to invading micro-organisms.

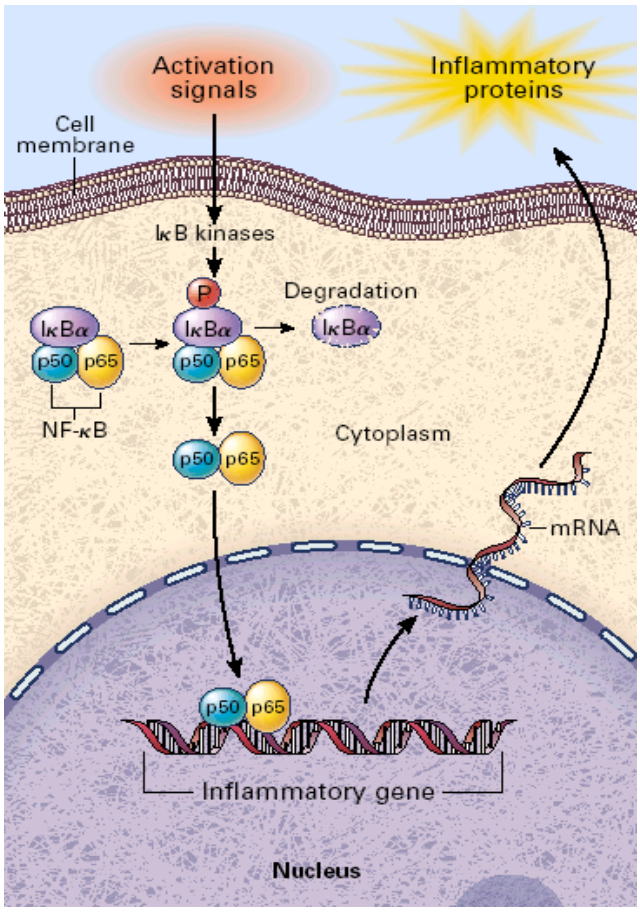

Figure 1. Activation of the transcription factor NF-κB (Barnes et al., 1997). Reprinted from Barnes PJ and Karin, M N Engl J Med, 336(15), 1066-71. Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. Copyright © 1997, with permission from Massachusetts Medical Society. All rights reserved.

1.4 Bronchial responsiveness

Airway or bronchial responsiveness describes the tendency of the airway to constrict to stimuli, such as spasmogenic chemical mediators or physical stimuli (O'Byrne et al., 2000). Bronchial responsiveness is measured as the change in airway calibre after inhalation of a bronchoconstrictor agent. A variety of different stimuli are being used to measure bronchial responsiveness, including "direct" and "indirect" stimuli. Direct stimuli act direct on airway smooth muscle receptors and include methacholine, histamine, cysteinyl leukotrienes and prostaglandin D_2. Indirect stimuli act on inflammatory cells, epithelial cells and nerves and include exercise, inhaled hyper- and hypotonic saline, cold/dry air and adenosine (Joos et al., 2003).

Airway hyperresponsiveness is one of the cardinal features of asthma. Inhalation of ozone induces hyperresponsiveness to methacholine in healthy subjects and an increased neutrophilic influx into the airways (Seltzer et al., 1986). Viral respiratory infections induce airway hyperresponsiveness in asthmatic and healthy subjects (Busse, 1994). Thus, bronchial hyperresponsiveness and airway inflammation seem to be related. However, it is not clear whether the inflammatory reaction causes bronchial hyperresponsiveness or if these two findings are parallel phenomena.

A number of different mechanisms likely interact to cause airway hyperresponsiveness and probably differ in normal and asthmatic subjects (Lotvall et al., 1998; O'Byrne & Inman, 2000). The mechanisms responsible for hyperresponsiveness are unknown but may involve airway epithelial damage, thickening of basement membrane and airway wall, release of mediators with the capacity to cause bronchial smooth muscle contraction, oedema and exudation of plasma.

1.5 Pharmacological intervention

Acute inflammation is a natural response for the organism to defend itself from harmful micro-organisms and other toxic agents. Sometimes in certain diseases this response turns towards the own body and preventing excessive injury can be important in protecting the organism. At that point glucocorticoids or other anti-inflammatory agents can be useful in the treatment of the disease. However, it is important to emphasize that inflammation is a natural defending process and that there is a balance between protection and injury.

1.5.1 Glucocorticoids

Glucocorticoids are widely used as an anti-inflammatory agent and are currently the most effective anti-asthma therapy (Barnes, 1998). The anti-inflammatory action of glucocorticoids is mediated by a glucocorticoid receptor which is localized in the cytoplasm in most cell types. Steroids are lipophilic and cross the cell membrane rapidly and enter the cytoplasm where it binds to the glucocorticoid receptor. The inactive glucocorticoid receptor is bound to a protein complex that includes two molecules of heat shock protein 90 (hsp90) and once the glucocorticoid binds to the receptor, the heat shock proteins dissociate which activates the glucocorticoid-receptor complex allowing it to rapidly translocate into the nucleus (Brattsand et al., 1994). The glucocorticoid-receptor complex binds to specific DNA sequences of the glucocorticoid response elements (GREs), and regulates the transcription of target genes which can either be repressed (inflammatory genes) or induced (anti-inflammatory genes; table 1) (Schleimer, 1993). The glucocorticoid receptor can also interact with activated transcription factors such as nuclear factor-κB (NF-κB) or activator protein-1 (AP-1) to inhibit the expression of multiple inflammatory genes (Adcock, 2001). Post-transcriptional mechanisms have also been suggested to contribute to the action of glucocorticoids, such as destabilize or stabilize specific mRNAs (Umland et al., 2002).

Table 1. Glucocorticoid regulated genes (Adcock et al., 2003).

Increased transcription
Lipoprotein-1/annexin-1 (phospholipase A_2 inhibitor)
β_2-adrenoceptor
Secretory leukocyte inhibitory protein (SLPI)
Clara cell protein (CC10, phospholipase A_2 inhibitor)
IL-1 receptor antagonist
IL-1R2 (decoy receptor)
IκBα (inhibitor of NF-αB)
MKP-1 (MAPK phosphatase)
CD163 (scavenger receptor)
Decreased transcription
Cytokines (IL-1, 2, 3, 4, 5, 6, 9, 11, 12, 13, 16, 17, 18, TNFα, GM-CSF, SCF)
Chemokines (IL-8, RANTES, MIP-1α, MCP-1, MCP-3, MCP-4, eotaxin)
Inducible nitric oxide synthase (iNOS)
Inducible cyclo-oxygenase (COX-2)
Endothelin-1
NK1 receptors, NK2 receptors
Adhesion molecules (ICAM-1, E-selectin)
Cytoplasmic phospholipase A_2 ($cPLA_2$)

CD163, cluster differentiation 163; GM-CSF, granulocyte macrophage-cell stimulating factor; SCF, stem cell factor; RANTES, Regulated upon activation normal T-cell expressed and secreted; MIPIα, macrophage inflammatory protein-1α; MCP, monocyte chemoattractant protein; NK, neurokinin; ICAM-I, intercellular adhesion molecule I.

Thus, glucocorticoids inhibit the expression of several key proteins including pro-inflammatory cytokines involved in the control of inflammation. Glucocorticoids may have direct inhibitory effects on many of the cells involved in airway inflammation, including macrophages, T-lymphocytes, eosinophils and airway epithelial cells. Inhaled glucocorticoids decrease the number and activation status of most inflammatory cells in the bronchus, including mast cells, eosinophils, T-lymphocytes and dendritic cells. In addition to their suppressive effects on inflammatory cells, glucocorticoids may also inhibit plasma exudation and mucus secretion in inflamed airways (Barnes, 1998; van der Velden, 1998).

Inhaled glucocorticoid therapy is the most effective therapy for patients with asthma with significant effects on symptoms, exacerbations lung function and bronchial hyperresponsiveness (Barnes, 1995b). Glucocorticoids have, however, no effect on the progression of COPD. Although glucocorticoids have been used for a long period of time, the precise mechanism of action is still not completely understood.

1.5.2 β_2-Agonists

Inhaled selective ß$_2$-agonists are the most widely used treatment for the acute relief of asthma symptoms. In patients with asthma, β-adrenoceptor agonists cause bronchodilation and reduced responsiveness to a number of bronchoconstrictor stimuli (bronchoprotection). The major action of β-adrenoceptor agonists in asthma is the functional antagonism on airway smooth muscles leading to relaxation.

The ß$_2$-agonists bind to the ß$_2$-adrenoceptor, causing activation of the receptor and subsequent stimulation of adenylyl cyclase with the formation of cyclic adenosine monophosphate (cAMP). CyclicAMP acts as a second messenger and relaxes the bronchial smooth muscle in part by protein kinase A -activation (Barnes, 1995a; Johnson, 1998).

Long-term use of ß$_2$-agonists is associated with tolerance to the bronchoprotective effect due to desensitization of the ß$_2$-adrenoceptor. Reduced responsiveness of receptors as a consequence of chronic stimulation by an agonist is a general biological phenomenon and β_2-adrenoceptors are no exception. Stimulation of the β_2-adrenoceptor leads to homologous desensitization via three steps; uncoupling from the stimulatory G protein, sequestration (internalisation) into the cell and destabilization of the β_2-adrenoceptors mRNA (Lohse, 1993; Nijkamp et al., 1992).

2 Aims of the Thesis

The general aim of the thesis was

- to study how glucocorticoids or β_2-agonists interfere with the acute non-aquired inflammatory response exemplified by swine house dust exposure *in vitro* and *in vivo.*

Specific aims were:

- to study the *in vitro* effect and time-kinetics of two different glucocorticoids, fluticasone propionate or budesonide, on the swine house dust- or LPS- mediated release of pro-inflammatory cytokines from epithelial cells and alveolar macrophages, respectively.

- to investigate whether the transcription factor NF-κB is involved in the signal transduction of swine house dust-mediated cytokine release from epithelial cells.
- to study the effect of glucocorticoids on the activation of NF-κB.

- to study the effect of inhaled and intra-nasally administered fluticasone, on the airway inflammatory response and bronchial responsiveness to methacholine in healthy subjects following exposure to organic dust in a swine barn.
- to study the effect of exposure and medication with fluticasone or salmeterol on the capability of alveolar macrophages to release cytokines *ex vivo*.
- to study the effect of a long-acting β_2-agonist, salmeterol (regular treatment or single dose) on the increased bronchial responsiveness to methacholine in healthy subjects following exposure in a swine barn.
- to study whether salmeterol influences the inflammatory response induced by exposure in a swine barn in healthy subjects.

3 Materials and Methods

The methods are briefly summarised below and detailed descriptions of methods are provided in paper I-V.

3.1 In vitro studies

3.1.1 Cells

(Study I, II and IV)
For *in vitro* studies the human pulmonary epithelial carcinoma cell line A549 (American Type Culture Collection, Rockville, Maryland, USA) was used (study I and II). Alveolar macrophages were obtained by bronchoalveolar lavage of healthy subjects and the cells were cultured both for *in vitro* studies and *ex vivo* studies (study I and IV).

3.1.2 Swine dust extract

(Study I and II)
Swine dust was collected in a swine house, on surfaces located approximately 1.2 m above the floor. The dust was dissolved in culture medium supplemented with penicillin and streptomycin. A stock solution was prepared which was thoroughly mixed and put in an ultrasound bath for 10 minutes.

3.1.3 Measuring effect of glucocorticoids

(Study I)
Epithelial cells were stimulated with swine house dust (100 μg/ml) for 24 hours and alveolar macrophages were stimulated with LPS (100 μg/ml) for 8 hours. Budesonide or fluticasone propionate (10^{-13} to 10^{-6} M) were co-incubated with swine dust or LPS and added before or after the stimuli at different time points. Cell supernatants were frozen until analysed for cytokine content (IL-6, IL-8 and TNF-α).

3.1.4 Measurements of NF-κB activity

(Study II)
Epithelial cells were transfected with reporter plasmids from the human IL-6 promoter (containing NF-κB binding site) or reporter plasmids containing 3 copies of the NF-κB binding site (3xNF-κB enhancer region), fused to the luciferase (LUC) reporter gene. For control experiments, respective reporter plasmid with mutated NF-κB binding sites, fused to the luciferase (LUC) reporter gene, were used. Transfection was performed by incubating the cells with Lipofectamine and reporter plasmids for 3 hours in serum-free medium. Then fresh culture medium was added to the wells, resulting in a final concentration of 10% FCS, to incubate for another 20 hours until the experiment was performed. Experiments using co-transfection with CMV-IκBα expression vector (0, 5 or 20 ng) were also performed.

The transfected cells were stimulated for 24 hours with swine house dust (100, 200 or 400 μg/ml) or TNF-α (200 U/ml) and in some experiments co-incubated with pyrrolidinedithiocarbamate (PDTC; 100 μM), fluticasone propionate (10^{-11} to 10^{-8} M) or dexamethasone (10^{-6} M). The cells were harvested and luciferase assay performed with the GeneGlow kit (Bio-Orbit, Turku, Finland). By measuring enzymatic activity of the luciferase protein, the activity of the reporter plasmid was revealed. For some experiments, the supernatants were collected and frozen until IL-6 and IL-8 concentrations were measured. In addition, NF-κB or C/EBPβ DNA binding in nuclear extracts from swine dust-stimulated (0 or 200 μg/ml, 30 minutes) epithelial cells was measured using the electromobility shift assay.

3.1.5 Main questions

Study I.
The dose-response effect and time-kinetics of fluticasone propionate and budesonide on the cytokine response from swine dust-stimulated epithelial cells and from LPS-stimulated alveolar macrophages were evaluated.

Study II.
The main aim was to evaluate whether NF-κB is involved in the signal transduction of swine dust-mediated IL-6 and IL-8 release from epithelial cells. The swine dust-induced reporter gene activities of the IL-6 promoter and the 3x NF-κB enhancer region, in parallel with the IL-6 and IL-8 release, were investigated. Furthermore, these parameters were also measured when inhibiting NF-κB activation, through addition of the chemical NF-κB blocking agent PDTC or through increased expression of IκBα or through mutating the NF-κB binding sites. Additionally, swine dust-induced NF-κB and C/EBPβ DNA binding was evaluated. Another aim was to investigate whether glucocorticoids influenced the dust-mediated activation of NF-κB and cytokine response.

3.2 Human studies *in vivo* and *ex vivo*

3.2.1 Subjects

The studies were approved by the local ethics committees and all subjects gave their informed consent to participate in the studies. Three separate trials were performed involving healthy subjects (table 2).

Table 2. Description of the trials and the participating subjects.

Trial	No. of subjects (men)	Mean age (range)	Exposure#	Type of study	Groups	Results in Study
1	24 (19)	27 (21-47)	600-900 pigs	Randomized, single-blind	2 weeks of treatment Placebo (n=8) Fluticasone (n=8)[1] Salmeterol (n=8)[2]	III, IV, V
2	12 (5)	24 (18-32)	300 pigs	Randomized, single-blind	One single dose Placebo (n=6) Salmeterol (n=6)[3]	V
3	8 (2)	37 (24-52)	no	Randomized, single-blind, cross-over	One single dose Placebo or salmeterol[4]	V

Exposure to organic dust involves a three hours stay in a swine confinement building.
[1]Two weeks treatment with fluticasone 500 μg *b.i.d.* for inhalation and 100 μg once daily intranasally.
[2] Salmeterol inhalation 50 μg *b.i.d.* for two weeks.
[3]One single dose salmeterol (100 μg) inhaled one hour before the start of the exposure.
[4] One single dose salmeterol (100 μg) inhaled 2 or 8 hours before methacholine provocation.

3.2.2 Designs and main questions

Study III.
The effect of 2 weeks treatment with fluticasone propionate (500 μg *b.i.d.* for inhalation and 100 μg once daily intra-nasally) on the increased bronchial responsiveness to methacholine, the systemic response, the upper airway inflammatory response and symptoms, induced in healthy subjects following exposure to organic dust, was evaluated. Nasal lavage and serum samples were obtained before and after treatment and exposure in a swine house. Lung function tests and bronchial methacholine provocation were performed before the 2 weeks treatment period and 7 hours after the start of the exposure (approximately 8 hours after the last dosing).

Study IV.
The effect of 2 weeks inhalation of fluticasone propionate (500 μg *b.i.d.* for inhalation), salmeterol (50 μg *b.i.d.*) or placebo on the increase in cellular and cytokine concentration in the lower airways following exposure was evaluated. Additionally, we investigated the effect of exposure of healthy individuals to organic dust in a swine barn, on the capability of alveolar macrophages to release cytokines *ex vivo*. Bronchoalveolar lavage was performed before the 2 week treatment period with inhaled placebo, fluticasone or salmeterol and 24 hours after exposure in the swine house. The influence of treatment on the alveolar macrophage function was also evaluated.

Study V.
We investigated the effect of salmeterol, after two weeks treatment (50 μg *b.i.d.*) and after one single dose (100 μg) on the increased bronchial responsiveness to methacholine induced in healthy subjects exposed in a swine barn. A control study was performed to find out whether a single dose inhaled salmeterol (100 μg) attenuated the bronchial response to methacholine in healthy unexposed subjects. Lung function measurements and a bronchial methacholine

provocation were performed before medication (approximately 2-3 weeks before exposure) and 7 hours after the start of the exposure (approximately 8 hours after last dosing).

3.3 Methods

Methods are briefly summarized below, for details see individual manuscripts.

3.3.1 Nasal lavage

(Study III)
Nasal lavage (NAL) was performed before the start of medication, after 2 weeks of treatment (1 hour before exposure) and 7 hours after the start of the exposure. A nasal lavage method procedure described by Bascom and Pipkorn (Bascom et al., 1988; Pipkorn et al., 1988) was used with minor modifications (see study IV). In each nostril 5 ml 0.9% NaCl was instilled and withheld for 10 seconds and then expelled and collected. The volume was measured, the cells were counted and the fluid was frozen for later analyses.

3.3.2 Bronchoalveolar lavage

(Study I and IV)
Bronchoscopy was performed with a flexible fibreoptic bronchoscope under local anaesthesia. A total of 250 ml sterile saline solution was used. The lavage fluid was collected, volume measured, cells counted, cell viability determined and fluid frozen for later analyses. Before *in vitro* experiments, cells were added onto culture plates and allowed to adhere for 2 hours at 37^0C, in culture medium containing 5% fetal calf serum and in the presence of 5% CO_2. The non-adherent cells were then removed by washing with serum-free medium.

3.3.3 Peripheral blood and symptoms

(Study III and V)
Blood samples were allowed to coagulate at room temperature for 1 hour before centrifugation (1550 g for 10 min). Following exposure in swine houses, symptoms (shivering, headache, malaise, muscle pain and nausea) were assessed using a questionnaire. The symptoms were graded according to a severity scale (1= no symptoms, 5= severe symptoms). Only 4 or 5 were classified as clinically relevant. Oral temperature was measured with an electronic mouth thermometer (Teflo, Sweden).

3.3.4 Analyses of inflammatory mediators

(Study I, II, III, IV and V)
The cell culture supernatants, nasal lavage fluid, bronchoalveolar lavage fluid and serum were dispensed in several aliquots, kept at -70°C and underwent only one freeze-thaw cycle before assay. IL-6, IL-8, TNF-α, albumin, α_2-macroglobulin, GM-CSF and RANTES were all quantified by different enzyme-linked immunosorbent assays (ELISA) either using commercial high sensitive sandwich enzyme immunoassay kits (QuantikineTM R&D Systems, Europe Ltd, UK) or commercially available antibody pairs, standards and controls (R&D Systems Europe, Abingdon, UK) together with an enzyme amplified detection system when necessary (see each article). Analysis of LTE_4 (Study IV) was performed with an enzyme immunoassay

(EIA) procedure. For all analyses, duplicates were measured and an intra-assay coefficient of variation of <10% and an inter-assay coefficient of variation <20% was accepted. Absorbance was read using a Thermomax 250 reader (Molecular Devices, Sunnyvale, CA, USA).

3.3.5 Lung function and bronchial responsiveness

(Study III and V)
Lung function (FEV_1 and VC) was measured using a wedge spirometer (Vitalograph®, Medical Instrumentation, Buckingham, U.K.) according to the recommendations of the American Thoracic Society (1995). Local reference values were used (Hedenstrom et al., 1985; Hedenstrom et al., 1986). A mini-Wright peak flow meter (Clement Clarke Ltd, London, UK) was used to measure peak expiratory flow (PEF).

Bronchial responsiveness was assessed by a methacholine challenge, described in detail previously (Malmberg et al., 1991). Inhalation of the diluent was followed by inhalation of doubling concentrations of methacholine, starting at 0.5 mg/ml up to 32 mg/ml or to 64 mg/ml or until FEV_1 decreased by 20%. The results were expressed as the concentration or cumulative dose causing a 20% decrease in FEV_1 ($PC_{20}FEV_1$ and $PD_{20}FEV_1$, respectively) and as the dose-response slope (percent FEV_1-decrease as a function of the cumulated dose of methacholine calculated with linear regression (Chinn, 1998; Chinn et al., 1993))

3.3.6 Exposure and dust measurements

(Study III, IV and V)
Exposure to organic dust involves a three hours stay in a swine confinement building during which pigs are weighed, a procedure leading to dust agitation. At each occasion, the individuals carried equipment to sample inhalable (<10 μm) and respirable (<5 μm) dust. Inhalable dust (IOM inhalable dust sampler, SKC Ltd, Blandford, England) and respirable dust (plastic cyclone samplers, Casella London Limited, Bedford MK42 7JY, England) were sampled, weighed and analyzed for endotoxin (*Limulus amebocyte assay*, QCL-1000, Endotoxin, BioWhittaker, Walkersville, USA).

3.3.7 Statistics

Results are presented as mean and SEM or as median (25^{th}-75^{th} percentile). For comparisons both parametric and non-parametric tests were used. Corrections for multiple comparisons have been performed when appropriate. Statistical calculations are described in detail in each paper.

4 Results and Discussion

4.1 Effects of glucocorticoids on organic dust-induced responses

4.1.1 Glucocorticoids: cytokine release *in vitro*

(Study I)
In swine-dust stimulated epithelial cells and in LPS-stimulated alveolar macrophages, simultaneous incubation of the stimulus and fluticasone or budesonide inhibited the increase in IL-6 and IL-8 release from epithelial cells and IL-6, IL-8 and TNF-α release from alveolar macrophages in a dose-dependent manner (fig. 2). At the highest concentration (10^{-9}-10^{-8}M), fluticasone inhibited the swine dust (or LPS)-induced release of these cytokines by at least 75%, with the exception of IL-8 release from alveolar macrophages that was inhibited by 33%.

Fluticasone was about 10 times more potent than budesonide in inhibiting cytokine release from epithelial cells and alveolar macrophages. This might be explained by the fact that fluticasone is about 300-fold more lipophilic than budesonide and has higher affinity for the glucocorticoid receptor (Johnson, 1995).

We also showed that it is important to consider the stimulating effect of the diluent in which the drugs are solved, in addition to the stimuli itself. We dissolved the drugs in 99.5% ethanol or dimethylacetamid. High concentrations of these diluents (0.05% ethanol corresponding to a budesonide concentration of 10^{-5}M and 0.0001 % dimethylacetamid corresponding to a fluticasone concentration of 10^{-8}M) in combination with either swine house dust or LPS, enhanced the IL-6 and IL-8 release significantly from A549 epithelial cells and alveolar macrophages. The diluents, at these concentrations, did not significantly enhance the IL-6, IL-8 or TNF-α response to LPS in human alveolar macrophages but may do that at higher concentrations.

Jusko studied the pharmacokinetics and receptor-mediated pharmacodynamics of glucocorticoids and concluded that the slow onset of biological responses to glucocorticoids is not caused by pharmacokinetic factors, but by the time needed for cell movement, mediator suppression or mRNA and protein synthesis (Jusko, 1990; Jusko, 1995). We found that the glucocorticoid-induced inhibition of cytokines from both lung epithelial cells and alveolar macrophages was not enhanced by pre-incubation of the glucocorticoid for different periods of times before adding either swine house dust or LPS. Furthermore, adding the glucocorticoids after the stimuli still resulted in a significant inhibitory effect. Conclusions from our time-kinetic results are that the suppression of cytokine release from these cells by glucocorticoid starts rapidly and might thus be a direct non-gene-mediated effect.

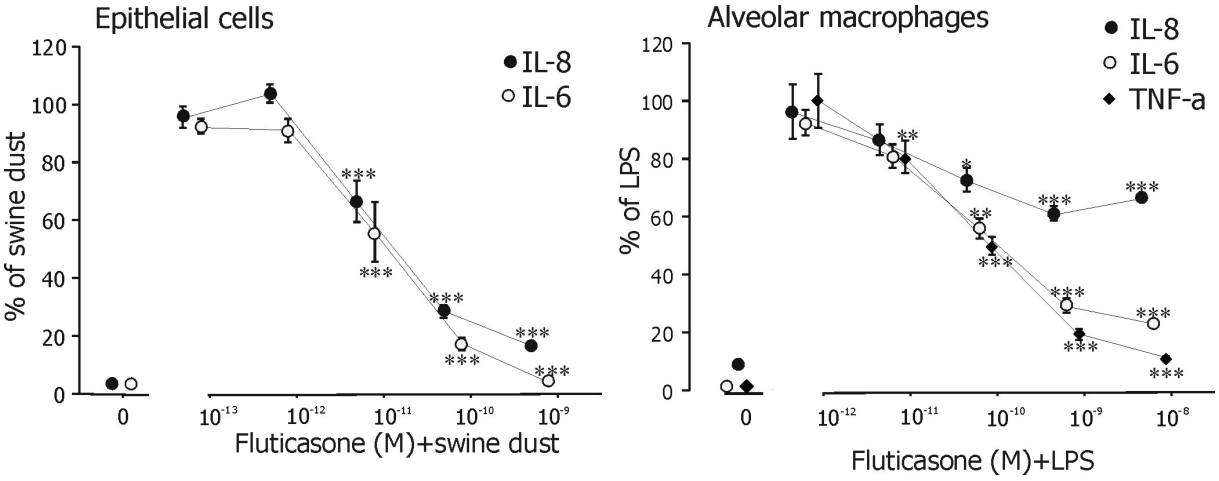

Figure 2. Results from Study I. Effect of fluticasone propionate incubation, in combination with swine house dust (100 μg/ml; 24 hours) or LPS (100 μg/ml; 8 hours), on the release of IL-6 and IL-8 from A549 epithelial cells or on the release of IL-6, IL-8 and TNF-α from human alveolar macrophages respectively. Results are presented as percent of cytokine release induced by swine house dust or LPS only (mean ± SEM). *:p<0.05; **:p<0.01; ***:0<0.001 versus swine dust or LPS respectively.

4.1.2 NF-κB activation *in vitro*

(Study II)

We have used several different approaches to show that NF-κB was activated in lung epithelial cells after swine house dust stimulation and that NF-κB is involved in the dust-mediated IL-6 and IL-8 release. Swine dust increases reporter gene activities of the IL-6 promoter and the 3x NF-κB enhancer region, in parallel with IL-6 and IL-8 release, and an intact NF-κB binding site was required. By adding the NF-κB blocking agent PDTC or by increasing expression of IκBα, inhibition of reporter gene activities of the IL-6 promoter and the 3x NF-κB enhancer region was demonstrated. Additionally, swine dust induced NF-κB DNA binding, composed of the NFκB1 and RelA proteins, and induced a small increase of C/EBPβ DNA binding.

4.1.3 Glucocorticoids: NF-κB activation *in vitro*

(Study II)

Fluticasone, in a dose-dependent manner, and dexamethasone, at one tested dose, inhibited the swine house dust-induced activation of the reporter gene containing 3x the NF-κB enhancer region.

We also confirmed previous results that fluticasone inhibited IL-6 and IL-8 from epithelial cells in a dose-dependent manner. The conditions for these two studies were slightly different. In Study I, the culture medium contained no FCS and the cells were not pre-transfected while in study II, the culture medium contained 10% FCS and the cells were pre-transfected. In both studies we showed that swine house dust-induced IL-6 and IL-8 release from epithelial cells are inhibited to almost basal, pre-exposure levels, by fluticasone at 10^{-9}M. Thus, transfected and non-transfected cells behaved similar in respect of cytokine inhibition.

In parallel with the inhibition of cytokine release by fluticasone (10^{-9} M), the NF-κB reporter gene activity was significantly inhibited, but not to basal levels. We also found that incubation with the NF-κB blocking agent PDTC together with swine house dust inhibited the reporter gene activities of the 3x NF-κB enhancer region and of the dust-induced IL-6 and IL-8 release to basal levels. Increasing the levels of IκBα, inhibited the swine house dust-induced IL-6 promoter reporter gene activity. These results taken together demonstrated that inhibition of IL-6 and IL-8 from epithelial cells by fluticasone is, in part, explained by inhibition of NF-κB activation.

Both the IL-6 and the IL-8 genes contain transcriptional control element motifs for NF-κB, AP-1 in the promoter region (Kishimoto, 1989; Roebuck, 1999). Many anti-inflammatory effects of glucocorticoids have been linked to their ability to inhibit the activation of NF-κB (McKay et al., 1999). Several mechanisms have been described for the antagonistic effect of glucocorticoids on NF-κB. Through protein-protein interaction, the activated glucocorticoid receptor can repress NF-κB activation and/or function. This interaction can occur either by blocking the access of NF-κB to its DNA site or by forming a complex with NF-κB (either in the cytoplasm or in the nucleus) which loses DNA capacity and thus preventing NF-κB-modulated transcription. In addition, the activated receptor may indirectly inhibit the function of NF-κB by inducing the synthesis of the NF-κB inhibitor, IκB (Barnes & Karin, 1997). In addition, the activated glucocorticoid receptor may compete with NF-κB for nuclear co-activators, thereby reducing and inhibiting activation by NF-κB (Almawi et al., 2002). Direct interaction between the activated glucocorticoid receptor and NF-κB seems to be important in repression of NF-κB activity by glucocorticoids in A549 cells (Wissink et al., 1998).

Newton et al showed that 50-100% depression of inflammatory genes in A549 cells by dexamethasone did not induce repression via changes of NF-κB expression (Newton et al., 1998). Thus, inhibition of NF-κB-dependent transcription might not itself account for the full suppressive effect of glucocorticoids in A549 cells. It is not clear how to interpret that swine dust-induced IL-6 and IL-8 release was inhibited to basal levels while the NF-κB activity was not. The glucocorticoid-mediated inhibition of the swine dust-induced IL-6 and IL-8 release might, in addition to repression of NF-κB activity, also involve interaction with other transcription factors such as AP-1 (Barnes et al., 1998). It could also interact with proteins involved in other signalling pathways (Wikstrom, 2003), post-transcriptional mechanisms (such as modulation of mRNA stability (Chang et al., 2001)) or the direct binding of the glucocorticoid receptor to DNA in the promoter region of the gene resulting in repression (Ray et al., 1990).

4.1.4 Glucocorticoids: nasal effects

(Study III)

Compared to placebo, intra-nasally administered fluticasone almost totally inhibited the plasma protein leakage, assessed as albumin (67 kDa) and α_2-macroglobulin (725 kDa) concentrations in nasal lavage fluid (P=0.02 and P=0.06 respectively), and attenuated the nasal IL-8, TNF-α and LTE_4 response to organic dust (P=0.02, P=0.03 and P=0.07 respectively). However, there were no significant differences between the placebo and fluticasone group regarding the increased cell content in nasal lavage (P=0.3).

At present it is not clear whether glucocorticoids inhibit microvascular leakage via a direct effect on endothelial cells or via a reduction of inflammatory mediators that increase vascular leakage (Persson et al., 1993). We found a relationship between post-exposure increases in albumin and α_2-macroglobulin (Rho =0.79, P=0.003), albumin and IL-8 (Rho=0.90, P=0.0007), albumin and TNF-α (Rho=0.84, P=0.004) and between albumin and LTE_4 (Rho =0.92, P=0.004). Both IL-8 and LTE_4 may induce plasma exudation (Rampart et al., 1989; Woodward et al., 1983). It is thus possible that the IL-8 and LTE_4 released after exposure contributed to the increased plasma exudation into the nasal cavity and that the inhibition by fluticasone of the induced IL-8 and LTE_4 release into the nose might have contributed to the inhibition of plasma leakage.

4.1.5 Glucocorticoids: symptoms and systemic effects

(Study III)
Five out of eight participants in the placebo group and two out of seven participants in the fluticasone group experienced symptoms (grade 4-5) following exposure.

The increased serum IL-6 and oral temperature following exposure in the swine barn, was significantly lower in the subjects treated with fluticasone compared to placebo (figure 3). It is well known that IL-6 is an endogenous circulating pyrogen, responsible for induction of fever during infection and inflammation. In a previous study the body temperature of 38 subjects, at 7 hours after the start of exposure in a swine barn, correlated significantly with maximal serum IL-6 levels after exposure (Wang, 1997). Our *in vitro* data are in agreement with the finding of lower IL-6 levels following exposure in the subjects who were treated with glucocorticoids. The inhibition of IL-6 production by fluticasone might therefore have contributed to the attenuated increase in post-exposure body temperature in the fluticasone treated group.

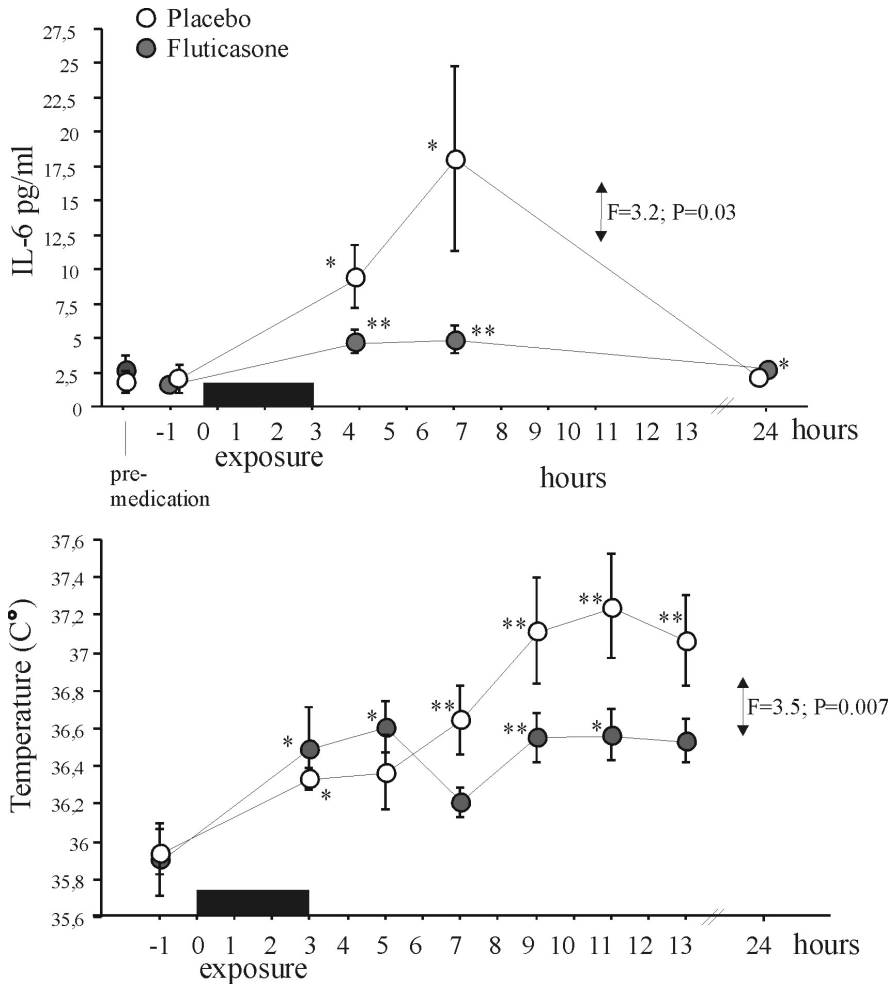

Figure 3. IL-6 concentrations in serum and oral temperature in healthy subjects before and after exposure in a swine house. Subjects were treated with placebo (n=8) or fluticasone (n=7) for 2 weeks prior to exposure. Mean ± SEM. * P<0.05, ** P<0.01 and *** P<0.001 compared with pre-exposure values. The increase in IL-6 and body temperature differed significantly between the fluticasone group and the placebo group (ANOVA F=3.2; P=0.03 and F=3.5; P=0.007 respectively).

4.1.6 Glucocorticoids: inflammatory airway effects

Compared to placebo, there were no effects of fluticasone treatment on the organic dust-induced increase in cell content and cytokine responses in bronchoalveolar lavage compared to placebo (study IV). Neither was there an effect on the slight decrease in lung function or the increased bronchial responsiveness to methacholine following exposure (study III).

In bronchoalveolar lavage fluid from placebo treated subjects, there was a significant increase in total cell count, IL-6, TNF-α, albumin and α_2-macroglobulin concentrations 24 hours after exposure in the swine barn (table 3). RANTES and GM-CSF concentrations did not significantly increase after exposure and were 7.2 (5.9-8.4) pg/ml and <1.0 (<1.0-3.2) pg/ml, respectively, before exposure and 9.4 (4.2-11.2) pg/ml and 2.4 (1.6-6.7) pg/ml, respectively, after exposure in the placebo group. Fluticasone treatment did not significantly influence any of these inflammatory mediators (table 3).

Table 3. Findings in bronchoalveolar lavage fluid from healthy subjects before 2 weeks treatment with inhaled placebo (*b.i.d.*; n=8), fluticasone (500 μg *b.i.d*; n=7.) or salmeterol (50 μg *b.i.d.*; n=8) and 24 hours after the start of the exposure to organic dust in a swine barn. Last dosing was one hour prior to exposure (part of study IV).

	Placebo		Salmeterol		Fluticasone	
	Before	After	Before	After	Before	After
Total cells ($x10^3$/ml)	109 (73-167)	424* (290-726)	117 (92-132)	343* (304-437)	131 (89-147)	274* (259-339)
IL-8 (pg/ml)	<25 (<25-57)	122 (86-174)	<25 (<25-39)	121 (67-196)	<25 (<25-<25)	262 (193-436)
IL-6 (pg/ml)	1.7 (1.3-2.0)	41.5* (25.1-95.7)	1.6 (1.1-2.1)	37.0* (20.9-60.3)	2.6 (1.5-3.0)	30.7* (28.2-40.0)
TNF-α (pg/ml)	<0.5 (<0.5-<0.5)	4.3* (3.1-7.6)	<0.5 (<0.5-<0.5)	4.5* (1.8-6.5)	<0.5 (<0.5-<0.5)	5.0 * (2.5-7.1)
Albumin (μg/ml)	52 (47-83)	112* (95-137)	42 (38-52)	80* (66-100)	59 (40-81)	101* (57-131)
α_2-Macro-globulin (μg/ml)	0.16 (0.13-0.24)	0.75* (0.53-2.47)	0.12 (0.07-0.29)	0.68* (0.54-0.79)	0.22 (0.01-0.45)	0.94 (0.40-1.49)

* P<0.05 compared with pre-exposure values. There were no significant differences between the groups. Median (25^{th}-75^{th} percentiles).

We found no effect of fluticasone treatment on the increase in methacholine-induced airway responsiveness induced by exposure in a swine barn in healthy subjects. Bronchial responsiveness to methacholine increased significantly by 3.2 (2.8-4.1) doubling concentration steps in the placebo group and by 2.5 (1.5-3.9) doubling concentration steps in the fluticasone group (P=0.4 between the groups) after exposure. In a meta-analysis by Currie et al. (Currie et al., 2003), glucocorticoid inhalation reduced methacholine-induced bronchoconstriction compared to placebo by 1.25 (95% CI 1.08 to 1.42) doubling dose/dilution shift at low/medium dose and 2.16 (95% CI 1.88 to 2.44) doubling dose/dilution shift at high-dose in asthmatic patients. However, the factors that predict improvement in bronchial hyperresponsiveness by inhaled glucocorticoids are still largely unknown and the relationship between inflammation and bronchial hyperresponsiveness is not clear. Inhalation of ozone in normal subjects causes a neutrophilic inflammatory response in the airways and an increased responsiveness to methacholine. Nightingale *et al* showed, in agreement with our findings, that budesonide inhalation neither affected sputum neutrophils nor the increased methacholine reactivity in normal subjects (Nightingale et al., 2000).

4.1.7 Glucocorticoids: alveolar macrophages *ex vivo*

(Study IV)

We found that exposure of healthy individuals to organic dust in a swine barn reduced the basal release of IL-6, IL-8 and TNF-α in alveolar macrophages *ex vivo* (figure 4). Pre-exposure treatment with either salmeterol or fluticasone *in vivo* did not influence this capability. There was a weak tendency of fluticasone to counteract the reduced alveolar macrophage basal function; however, with no significant differences between the groups (Figure 4). There were no significant differences between cytokine releases in LPS-stimulated alveolar macrophages *ex vivo* before compared to after exposure. There might be a reduced capability of the alveolar

macrophages to release TNF-α following LPS stimulation after exposure in a swine barn (placebo P=0.09). The clinical relevance of the reduced alveolar macrophage function is not clear.

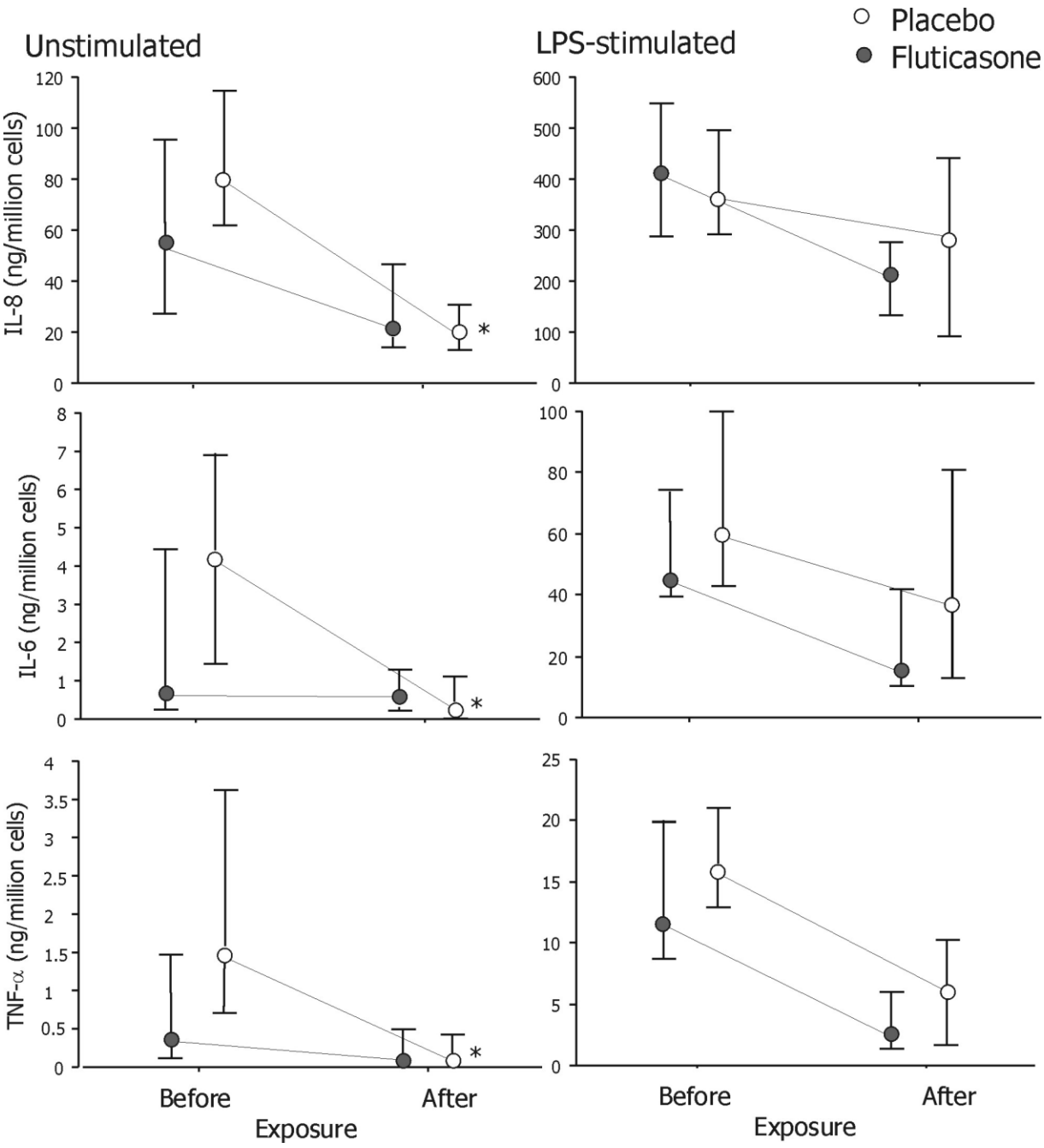

Figure 4. Results from study IV. Cytokine release from un-stimulated (24 hours in culture medium) or LPS-stimulated (100 μg/ml for 24 hours) alveolar macrophages *in vitro* obtained from healthy subjects before a 2 week treatment period with either inhaled placebo (n=8) or fluticasone (n=7; one subject had an airway infection at the time of exposure and was therefore excluded) and 24 hours after exposure in a swine house. The cells from each subject were cultured in duplicates and the mean value of the two measurements was used for statistical calculations. Results are presented as median and 25^{th} to 75^{th} percentiles. *P<0.05 compared to pre-exposure (Wilcoxon Signed Rank test). There were no significant differences between the groups (Mann-Whitney U-test).

4.2 Effects of β_2-agonists on organic dust-induced responses

Salmeterol is a highly lipophilic, partial long-acting ß$_2$-agonist providing bronchodilation for at least 12 hours (Johnson, 1998; Johnson et al., 1993; Lotvall et al., 1993).

4.2.1 β_2-Agonists: inflammatory responses

(Study IV and unpublished results)
We found no significant effects of inhaled salmeterol (50 μg *b.i.d.*) on the inflammatory response to organic dust in either serum or bronchoalveolar lavage fluid (performed 24 hours after exposure). Serum IL-6 concentrations increased to a maximum of 10.7 (3.3-21.4) pg/ml in the placebo group and 12.5 (5.0-24.4) pg/ml in the salmeterol group with no significant difference between the groups (table 4). The inflammatory mediators measured in bronchoalveolar lavage fluid (table 3) increased after exposure in both the salmeterol group and the placebo group with no significant differences between the groups.

Table 4. IL-6 concentrations (pg/ml) in serum from healthy subjects before 2 weeks treatment with either inhaled placebo (n=8) or salmeterol (50 μg *b.i.d.*; n=8), one hour before, and 4, 7 and 24 hours after the start of the exposure in a swine barn.

	Before	-1 hour	+4 hours	+7 hours	+ 24 hours
Placebo	1.1	1.0	9.1 *	12.4 *	1.8
	(0.8-1.5)	(0.8-1.4)	(4.9-11.8)	(6.4-22.6)	(1.2-3.4)
Salmeterol	1.1	2.2	13.2 *	9.4 *	2.0
	(0.8-2.8)	(0.9-3.9)	(7.5-30.8)	(7.1-36.5)	(1.4-3.7)

P<0.05 compared with values obtained one hour before exposure. Differences between the groups (P=0.6;Mann-Whitney). Median (25^{th}-75^{th} percentiles).

Salmeterol inhalation had no effect on the increased plasma leakage, reflected by increase in albumin and α_2-macroglobulin concentrations in bronchoalveolar lavage fluid, following exposure (table 3). There are, however, several animal data showing that β_2-agonists have the capacity to reduce extravasation of plasma in animal airways (Tokuyama et al., 1991; Whelan et al., 1992) but the clinical relevance of this effect in the treatment of asthma is unclear (Barnes, 2002; Persson, 1993). Greiff *et al* have found that inhaled formoterol reduced the increase in plasma proteins in sputum induced by inhaled histamine in normal subjects, indicating that therapeutic doses of inhaled long acting β_2-agonists inhibit plasma exudation (Greiff et al., 1998). The lack of effect of salmeterol on vascular permeability in our study might have been influenced by tachyphylaxis.

There are also data reporting mast cell inhibitory effects of β_2-agonists (Wallin et al., 1999). Other findings indicate that β_2-agonists may cause reduction in both the number and activation of neutrophils (Reid et al., 2003). There are also data on inhibitory effects of salmeterol on allergen induced increases in sputum eosinophils in asthmatic subjects (Dente et al., 1999). However, the relevance of these effects for the control of airway inflammation in asthma is not clear (Howarth et al., 2000). We were not able to demonstrate that salmeterol influenced the marked increase of neutrophils or IL-8 in bronchoalveolar lavage fluid following exposure.

4.2.2 β_2-Agonists: bronchial responsiveness

(Study V)
We found that neither two weeks treatment with inhaled salmeterol nor salmeterol inhaled as a single dose, prior to exposure in a swine barn, altered the increased bronchial responsiveness to methacholine in healthy subjects. The bronchial responsiveness to methacholine in subjects receiving salmeterol for two weeks (n=8) increased significantly by 2.6 (1.4 – 3.7) doubling concentration steps following exposure. This increase did not significantly differ from the placebo group which increased by 3.2 (2.8-4.1) doubling concentration steps (n=8; figure 5). The bronchial responsiveness to methacholine in subjects receiving one single dose of salmeterol (100 μg; n=6) prior to exposure increased by >1.7 (0.4-2.4) doubling concentration steps and did not significantly differ from subjects receiving placebo (n=6), which increased by 3.3 (2.9-4.4) doubling concentration steps (figure 5). The explanation to the lack of bronchoprotective effect of salmeterol against the organic dust-induced increased bronchial responsiveness is not clear. In healthy non-exposed individuals, bronchial responsiveness to methacholine was attenuated by 1.2 (0.8-1.7) doubling concentration steps 8 hours after inhalation of one dose salmeterol (100 μg) compared to inhalation with placebo (figure 6).

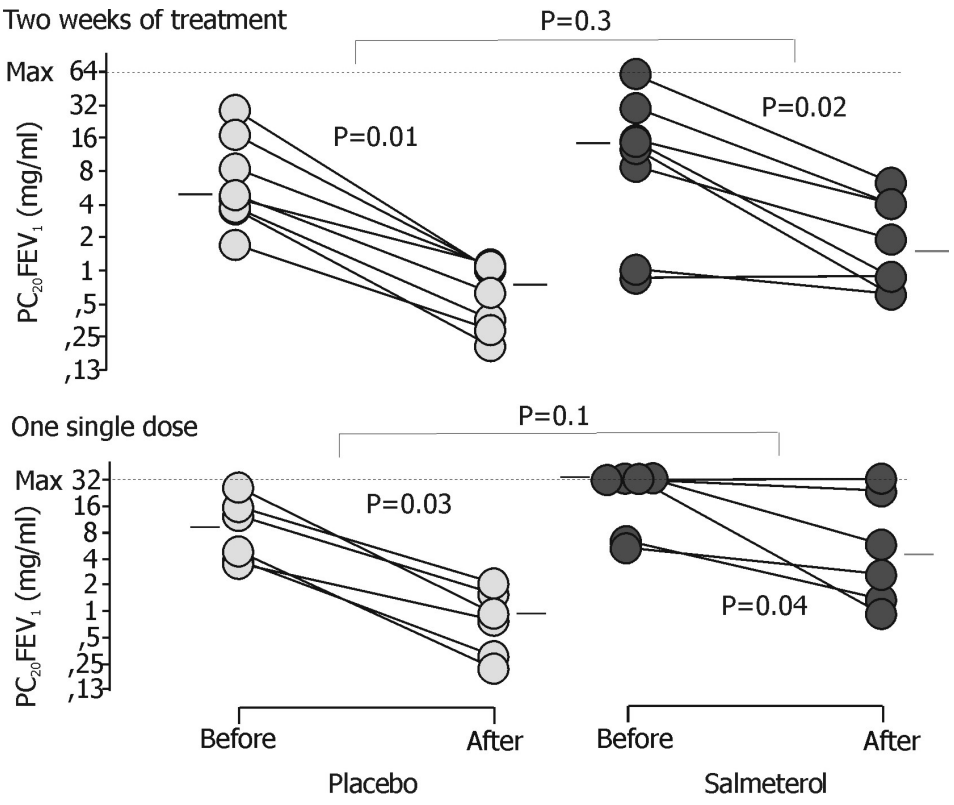

Figure 5. Results from Study V. Bronchial responsiveness to methacholine ($PC_{20}FEV_1$) in healthy subjects, before medication and after exposure in a swine barn, treated either two weeks with inhaled placebo (n=8) or salmeterol (50 μg *b.i.d.*, n=8; upper panel) or with one dose of placebo (n=6) or salmeterol (100 μg; n=6; lower panel) Horizontal lines indicate median values and horizontal dashed line represents the highest inhaled concentration of methacholine (64 mg/ml). One subject did not attain a 20% decrease in FEV_1 after exposure when the maximum concentration was reached, but had a >15% FEV_1-decline. No significant difference between the groups. P-values indicate pre- and post-exposure comparisons and comparisons between the groups.

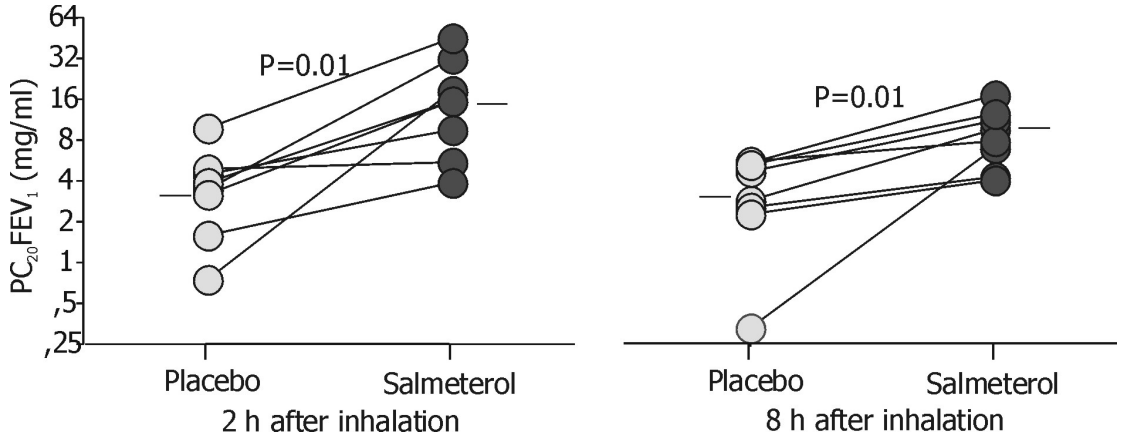

Figure 6. Results from Study V; the $PC_{20}FEV_1$ of eight healthy subjects after inhaling one dose of placebo or salmeterol (100 μg) 2 hours or 8 hours before the methacholine provocation. Horizontal lines indicate median values. P-values indicate the $PC_{20}FEV_1$ differences between salmeterol and placebo inhalations. There were no significant differences between the increase of $PC_{20}FEV_1$ at 2 or 8 hours.

It is known that regular inhalation of a β_2-agonist induces tachyphylaxis and reduction of the bronchoprotective effect against bronchoconstrictor stimuli. Several studies have investigated this issue and shown that regular treatment with salmeterol, or other β_2-agonists, induces tolerance to the bronchoprotective effects against methacholine, exercise or allergen (Bhagat et al., 1995; Cheung et al., 1992; Giannini et al., 1996; January et al., 1998; O'Connor et al., 1992; Ramage et al., 1994). Additionally, the tolerance to the bronchoprotective effect of salmeterol against methacholine induced bronchoconstriction occurs rapidly and is found already 12 hours after starting a twice daily treatment (Drotar et al., 1998). Therefore, it would be possible that 2 weeks of treatment with salmeterol induced β-adrenoceptor tachyphylaxis which might, at least in part, explain the reason for the lack of effect of 2 weeks salmeterol treatment on the increased responsiveness to methacholine induced by exposure in a swine barn. However, since neither a single dose of salmeterol nor 2 weeks treatment altered the increased bronchial responsiveness following exposure and since the effect was similar, it is unlikely that the lack of effect of salmeterol after two weeks of treatment is explained by development of β_2-adrenoceptor tachyphylaxis.

Our finding that one single dose salmeterol attenuated the bronchial responsiveness in healthy non-exposed subjects together with the above described results, indicates that exposure to organic dust has altered the airway response to β_2-agonists. One explanation to this observation may be that pro-inflammatory cytokines released in response to exposure in the swine house, have direct effects on airway smooth muscle cells that reduce the ability to relax in response to a β_2-agonist. There are an increasing number of studies describing the mechanism by which IL-1β and TNF-α induce heterologous desensitization of β_2-adrenoceptors leading to decreased responsiveness of the β_2-adrenoceptor through a mechanism involving COX-2 and PGE_2 formation (Hakonarson et al., 1996; Koto et al., 1996; Laporte et al., 2000; Laporte et al., 1998; Moore et al., 2001; Pang et al., 1998; Pang et al., 1997; Shore, 2002) (figure 7). Pro-inflammatory cytokines (IL-1β and TNF-α) and LPS have been shown to induce the expression of COX-2 which appears to require the activation of p38 in addition to NF-κB (Steer et al., 2003). COX-2 in turn, increases PGE_2 release, resulting in

increased cAMP formation leading to PKA activation, which results in heterologous desensitization of the β_2-adrenoceptor.

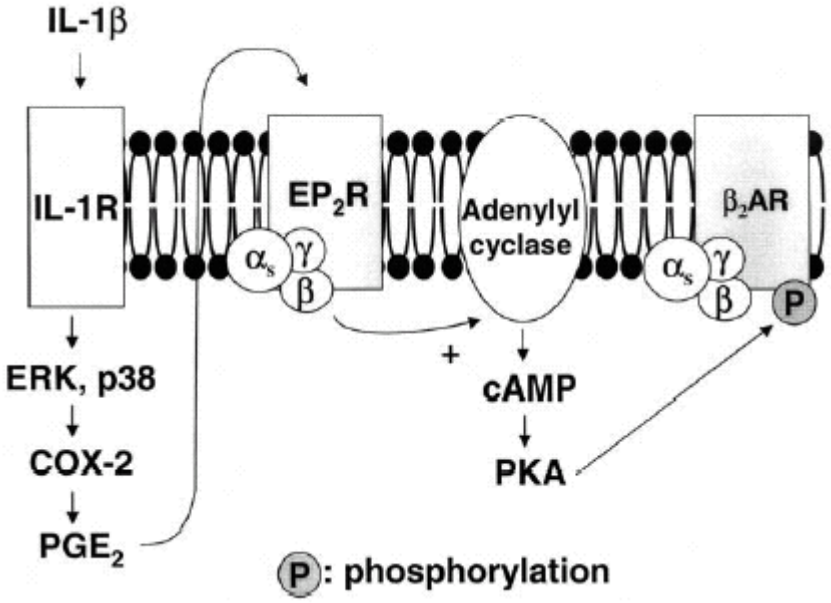

Figure 7. Mechanism of heterologous desensitization of β_2-adrenergic receptors. Reprinted from Respir Physiol Neurobiol, 137, Shore S.A. and Moore P.E., Regulation of beta-adrenergic responses in airway smooth muscle, 179-195. ©Copyright (2003), with permission from Elsevier. IL-1β leads to extracellular signal-regulated kinase (ERK) and p38 dependent cyclooxygenase-2 (COX-2) expression and prostaglandin E_2 (PGE_2) release. PGE_2 acts on E prostanoid 2 (EP_2) receptors coupled to stimulatory G protein (Gs) leading to cyclic AMP (cAMP) formation, and protein kinase A (PKA) activation. PKA phosphorylates the β_2-adrenoceptor, uncoupling it from Gs (Shore et al., 2003).

Previous studies have reported significantly increased levels of IL-1α and IL-1β in nasal and bronchoalveolar lavage fluid of healthy subjects after exposure in a swine house (Wang et al., 1997). Furthermore, concentrations of IL-1β in peripheral blood are also increased after exposure (Wang et al., 1998). We found elevated TNF-α concentrations in both bronchoalveolar and nasal lavage fluids following organic dust exposure (table 3). In a previous study TNF-α levels in serum significantly increased following exposure (Wang et al., 1996). Therefore, it is not unreasonable to assume that there have been elevated levels of both TNF-α and IL-1β in the lower airways prior to the methacholine provocation (7 hours after exposure) and that these cytokines may have influenced the β_2-adrenoceptors on airway smooth muscle cells.

We found a relation between TNF-α in serum and the increase in bronchial responsiveness to methacholine after, compared to before, exposure in the subjects who received two weeks of salmeterol treatment (figure 8). This relationship was not found in the subjects receiving placebo and has not been shown in previous studies of healthy untreated subjects following exposure in a swine barn. Additionally, the almost total inhibition of the organic dust-induced TNF-α release in bronchoalveolar lavage by cromoglycate without influence on bronchial

responsiveness speaks against a central role of this cytokine in the development of increased bronchial responsiveness following exposure (Larsson et al., 2001). Thus, TNF-α in serum does not seem to have influenced bronchial responsiveness itself but might have influenced effects by the β_2-agonist on bronchial responsiveness.

It could be argued that post-exposure TNF-α levels in serum does not reflect the lower airway reaction to organic dust exposure. However, we found a relation between increase of TNF-α in serum (4 and 7 hours after exposure) and bronchoalveolar lavage fluid (24 hours after exposure) in both groups together (n=16; Rho=0.49; P=0.06 at 4 hours and Rho=0.69; P=0.008 at 7 hours). In the subjects receiving salmeterol for two weeks there was a relationship between granulocytes in bronchoalveolar lavage fluid following exposure and the increase in bronchial responsiveness (n=8; Rho=0.76; P=0.04); this relationship was not found in the placebo group (n=8; Rho=0.17; P=0.44; figure 8). It is also previously shown that post-exposure levels of TNF-α in bronchoalveolar lavage fluid was significantly correlated with the influx of granulocytes into the lower airways (Wang, 1997). Thus, these findings indicate that TNF-α has influenced the cellular response in the lower airway and this might have influenced β_2-adrenoceptors on airway smooth muscles leading to decreased responsiveness.

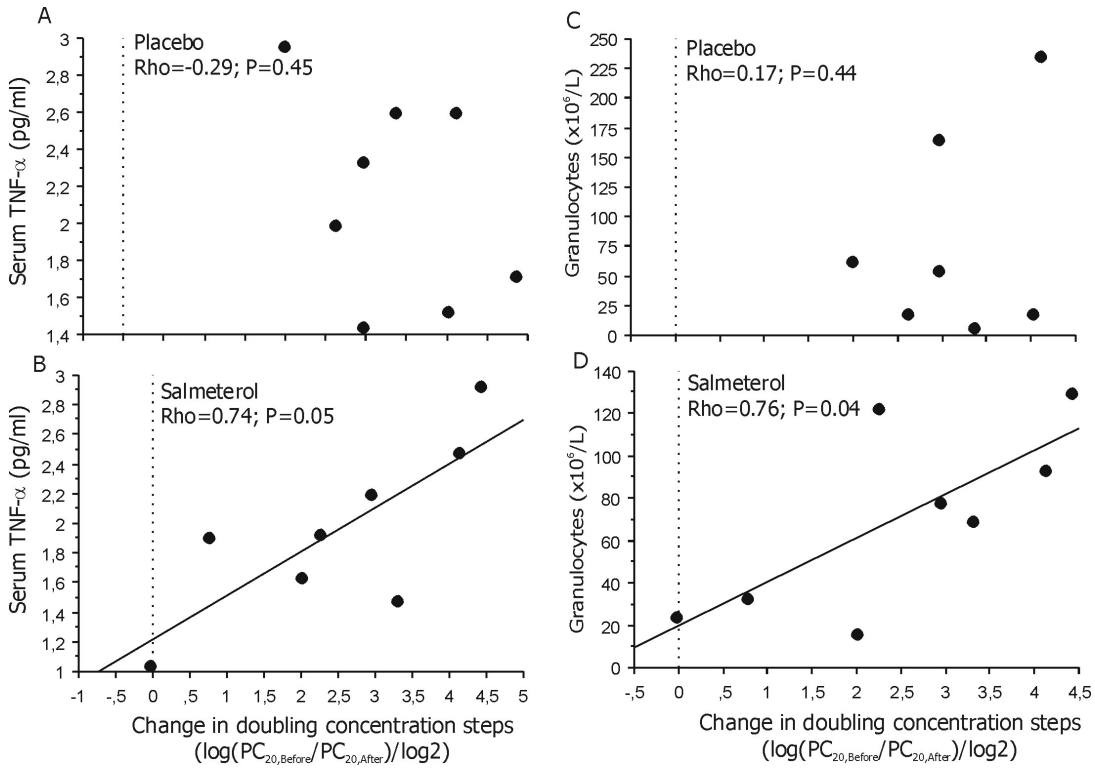

Figure 8. Co-variance between the increase in bronchial responsiveness to methacholine (doubling concentrations steps) following exposure and serum TNF-α concentrations (4 hours after exposure) and the increase in granulocytes in bronchoalveolar lavage fluid (difference between pre- and 24 hours post-exposure) in the placebo group (A and C, respectively) and in the salmeterol group (B and D, respectively).

4.3 Exposure measurements

Table 5. Concentration of inhalable and respirable dust and endotoxin in the different trials.

Trial	Exposure	Inhalable dust (mg/m^3)	Inhalable endotoxin (ng/m^3)	Respirable dust (mg/m^3)	Respirable endotoxin (ng/m^3)	Results in Study
1	600-900 pigs	25.4 (21.2-34.7)	733 (402-1068)	1.02 (0.76-1.27)	32 (13-56)	III, IV, V
2	300 pigs	11.5 (10.4-18.3)	236 (123-281)	0.68 (0.55-0.93)	41 (28-44)	V

The exposure levels were higher in trial one, probably due to the larger number of pigs in the swine barn (table 5). There were no significant differences in exposure between the groups in either trial.

5 General Discussion

5.1 Glucocorticoids

We found that fluticasone treatment attenuated the nasal and systemic inflammatory responses induced by exposure in a swine barn *in vivo*. These results were confirmed by our *in vitro* data. However, there were no effects of fluticasone treatment on the elevated inflammatory mediators found in bronchoalveolar lavage fluid or on cytokine release from alveolar macrophages *ex vivo* or on the increased bronchial responsiveness following exposure. There might be several explanations to this discrepancy.

Our *in vitro* data demonstrate that glucocorticoids are potent inhibitors of swine house dust and LPS-induced cytokine release from lung epithelial cells and human alveolar macrophages. Inhalation of fluticasone or budesonide (one single dose of 1-1.6 mg) has previously been shown to result in a steroid concentration of approximately 5 nmol/kg in central lung tissue and the concentration was about 3-4 times lower in peripheral lung tissue in patients who underwent surgery due to lung cancer (Esmailpour et al., 1997; Van den Bosch et al., 1993). In healthy subjects with no airway obstruction inhalation of the drug might yield a more favourable lung deposition. It is not unreasonable to assume that the glucocorticoid concentration is approximately 1nM in the airway lining fluid during regular inhalation. Since we found that fluticasone inhibited cytokine release from both lung epithelial cells and alveolar macrophages significantly at 1 nM, and some cytokines were almost totally inhibited at this concentration, this effect might be relevant also *in vivo*.

However, the concentration of the deposited drug might still not have been enough to elicit the same effects in the lower airways as we found *in vitro*. Intra-nasally administered fluticasone has probably resulted in higher concentrations of the drug in the nasal cavity compared to the concentrations in peripheral airways following inhalation. Thus, the difference in glucocorticoid concentration may explain the discrepancy between the findings in nasal and bronchoalveolar lavage fluids.

On the other hand, the findings of a lower increase in IL-6 levels in serum in the fluticasone group and that post-exposure IL-6 levels in nasal lavage fluid was not significantly attenuated by treatment, indicate that IL-6 concentrations in the airways have been reduced by fluticasone. Other *in vitro* studies at our laboratory have shown that swine dust induced mRNA IL-6 at an early time point and the expression levels of IL-6 reduced with time over a 24 hour period (Burvall et al., 2004). Thus, these findings indicate that the steroid mediated effect on airway cytokine release is found at an early phase of the inflammatory response to exposure and that the inflammatory reaction is less intense after 24 hours (at the time of the bronchoalveolar lavage) in both the placebo and fluticasone group, resulting in less difference between the groups. This might be an additional explanation for the finding of inhibitory effects of fluticasone on swine dust-induced IL-6 release *in vitro* and in serum *in vivo* but no effect of fluticasone on IL-6 levels in bronchoalveolar lavage fluid.

Another explanation for the discrepancy between the *in vivo* and *in vitro* results might be that the cell types from where the cytokines originate differ. Epithelial cells and alveolar macrophages are important sources of the cytokines and they are most likely the initial

activators of the inflammatory response. However, the inflammatory cells and phagocytic cells recruited from the peripheral blood into the airways also cause release of cytokines. The main feature of the inflammatory response to organic dust inhalation was a massive accumulation of neutrophilic granulocytes in the airways. After exposure there is an 70-75 fold increase in neutrophils, a doubling of alveolar macrophages and the increase in lymphocytes was about three-fold in bronchoalveolar lavage fluid (Larsson et al., 1994b). Eosinophils are also significantly increased after exposure in bronchoalveolar lavage fluid, although the proportion of the total cell concentration is very low (<0.5%) (Larsson et al., 1994b). Thus, in Study IV, the increase of granulocytes in bronchoalveolar lavage fluid after exposure consisted mainly of neutrophils (from 3.7% before exposure to 34% of the total cell count after exposure). In blood there is a three fold increase of granulocytes (Muller-Suur et al., 1997). These results implicate that neutrophils are important contributors to the cytokine content and inflammatory response after organic dust inhalation.

Neutrophils are important sources of pro-inflammatory cytokines, including IL-8 and TNF-α (Cassatella et al., 1997; Scapini et al., 2000) and glucocorticoids inhibit IL-8 release from neutrophils *in vitro* (Irakam et al., 2002). However, it has also been shown that glucocorticoids increase neutrophil survival by reducing apoptosis (Haslett, 1999; Meagher et al., 1996; Zhang et al., 2001). Glucocorticoids exert weak or no inhibitory effect on neutrophilic inflammatory responses (Cox, 1998; Sampson, 2000) such as the mobilisation of neutrophils in COPD (Gizycki et al., 2002; Keatings et al., 1997), a condition which is characterized by an increased number of neutrophils (Lacoste et al., 1993). There is a considerable heterogeneity of the asthma phenotype and many subjects may exhibit an airway disease characterized by neutrophilia rather than eosinophilia (Douwes et al., 2002; Gibson et al., 2001). Elevated levels of neutrophils have been measured in the airways of subjects with acute severe asthma and during exacerbations (Fahy et al., 1995; Jatakanon et al., 1999; Norzila et al., 2000). There are data suggesting that glucocorticoids are less effective in attenuating airway inflammation in asthma patients with high levels of neutrophils (Gauvreau et al., 2002; Inoue et al., 1999). These data suggest that glucocorticoids may have limited effects on neutrophils and that may explain the lack of effect of fluticasone on the organic dust-induced neutrophilic influx into the upper and lower airways (assessed as nasal and bronchoalveolar lavage) in the present study. Additionally, since neutrophils are not major producers of IL-6, it is possible that the effect of fluticasone on serum IL-6 levels reflects influences of fluticasone on other airway cells than neutrophils.

We found that fluticasone inhibited IL-8 release from LPS-stimulated human alveolar macrophages *in vitro* by maximal 33%. Dexamethasone inhibited IL-8 release in stimulated alveolar macrophages from smokers by maximally 25% and had no effect on IL-8 release in stimulated alveolar macrophages from patients with COPD (Culpitt et al., 2003). In another study, dexamethasone suppressed IL-8 release by 64% in non-smokers and by 29% in smokers (Ito et al., 2001). Thus, there might be a limited effect of glucocorticoids on IL-8 release which might be more pronounced after exposure. This could contribute to the understanding of the lack of effect of fluticasone on the cytokine and neutrophilic response to organic dust in the lower airways.

In previous studies at our laboratory, no relationship between the bronchial responsiveness to methacholine and the inflammatory response, measured as change in concentration of cells and mediators (cytokines, leukotrienes and PGD_2) following exposure to swine dust, was found. Sodium cromoglycate significantly reduced the organic dust-induced increase in

neutrophils, IL-6, TNF-α and myeloperoxidase in bronchoalveolar lavage fluid but did not affect the increased bronchial responsiveness to methacholine (Larsson et al., 2001). Administration of zileuton, a leukotriene synthesis inhibitor, also failed to affect the increase in bronchial responsiveness following exposure in a swine barn (Larsson et al., 2002). Additionally, wearing respiratory protection device dramatically decreased the inflammatory cells in blood and mediators in nasal lavage fluid but did only influence the increased bronchial responsiveness to a minor extent following exposure in a swine barn (Palmberg et al., 2004). It has been shown that eosinophilic inflammation is associated with increased methacholine responsiveness (PC_{20}) whereas neutrophilic inflammation is not (Woodruff et al., 2001). The data suggest that neutrophils might not be involved in the bronchial hyperreactivity observed in asthma. In most studies, but not in all (Nielsen et al., 2000), glucocorticoid treatment reduces airway hyperresponsiveness to methacholine in asthmatics (Barnes, 1990; Overbeek et al., 1996; Reynolds et al., 2002; Van Schoor et al., 2002). We have showed that fluticasone treatment does not affect the increased bronchial responsiveness to methacholine induced by organic dust inhalation in healthy subjects. Thus, there does not seem to be a direct relationship between the neutrophilic inflammation and the increased bronchial responsiveness to methacholine after exposure in a swine barn.

NF-κB plays a central role in inflammation and knowledge of its mode of action is necessary for molecular understanding of inflammatory diseases. It is believed that NF-κB plays a major role in the onset and maintenance of the asthmatic inflammation. It has been shown that the levels of pro-inflammatory cytokines, such as IL-1β, TNF-α, IL-6 and IL-8 are elevated in asthmatic airways (Broide et al., 1992; Marini et al., 1992). In addition, expression of p65, as well as increased NF-κB DNA binding is found in biopsies and induced sputum from asthmatic subjects (Hart et al., 1998). Our results indicate that acute phase transcription factors such as NF-κB also may play an important role during the acute response to organic dust inhalation.

Several mechanisms by which glucocorticoids act might be important for the inhibition of swine house dust-induced cytokine release, including repression of NF-κB activity, as shown in Study II. Understanding of the mode of action of glucocorticoids is of importance in inhibiting the transcription of inflammatory genes without having negative side effects. It is believed that anti-inflammatory effects to a major extent are mediated via transrepression, while many side effects are due to transactivation (Barnes, 1998). This has led to a search for novel glucocorticoids that are more active in transrepression than transactivation (Schacke et al., 2004).

Taken together, glucocorticoids may exert anti-inflammatory effects on the organic dust induced inflammatory response. The major effects of fluticasone *in vivo* on the organic dust-induced inflammatory response are inhibition of cytokine release and plasma leakage into the nose and attenuation of the fever response and the systemic IL-6 response. These effects might in part be caused by glucocorticoid-mediated inhibition of pro-inflammatory cytokine release from epithelial cells and alveolar macrophages. Furthermore, inhibition of NF-κB activation by fluticasone seems to be a possible mechanism through which pro-inflammatory cytokines are inhibited. On the other hand, glucocorticoids had no effect on the cell- and cytokine inflammatory response in the lower airways. This discrepancy might be due to low glucocorticoid concentration, to methodological time differences or weak inhibitory effects of glucocorticoids on neutrophilic responses. Additionally, glucocorticoids had no effect on

bronchial responsiveness and there does not seem to be a direct relation between bronchial responsiveness and the inflammatory response following exposure in the swine barn.

5.2 β_2-Agonists

Although *in vitro* data indicate that β_2-agonists may have anti- (Barnes, 1999) or pro-inflammatory properties (Kavelaars et al., 1997; Linden, 1996), we did not find any significant effects of salmeterol treatment on the inflammatory response induced by exposure in a swine barn. Our results are consistent with a number of studies which suggest that salmeterol has no or small effects on airway inflammation in asthma (Howarth et al., 2000; Li et al., 1999; Lindqvist et al., 2003; Roberts et al., 1999). Bronchoalveolar lavage studies have not shown any significant influence of salmeterol on airway inflammatory cells in patients with stable asthma (Gardiner et al., 1994; Kraft et al., 1997). Thus, there are still no clear data to support that salmeterol exerts any clinically significant anti-inflammatory effects in man.

Many different factors have been suggested to be involved in airway hyperresponsiveness. Separate mechanisms are probably responsible for the underlying hyperresponsiveness in asthmatic patients and in normal individuals (Lotvall et al., 1998; O'Byrne & Inman, 2000). However, the mechanism of the increased bronchial responsiveness in healthy subjects following exposure in a swine barn might have similarities with the transient worsening of asthma control. It is known that β_2-agonists fail to relieve symptoms in patients during acute asthma exacerbations induced by airway infections (Rebuck et al., 1971; Reddel et al., 1999). Respiratory tract infection is a common cause of acute asthma exacerbations in children and adults. In most of these episodes the infection is caused by respiratory viruses but bacterial pathogens are also recognized as causative agents (Lieberman et al., 2003). Airway neutrophilia and increased levels of pro-inflammatory cytokines such as IL-1, IL-8 and TNF-α are associated with both bacterial and viral inflammation causing exacerbations of asthma (Gern, 2004; Mizgerd, 2002). The mechanism behind this impaired β_2-adrenoceptor function might thus be similar to what we have found following exposure to organic dust. In agreement with the previous discussion (5.2.2), the pro-inflammatory cytokines IL-1β and TNF-α might have a role in the pathogenesis of attenuated β-adrenoceptor-responses.

5.3 Future perspectives

Current evidence suggest that the combination of inhaled corticosteroids and long acting β_2-agonists is the most effective means of controlling asthma and used in combination they are more effective than either drug alone. There might thus be additive or synergistic benefits of the combination of glucocorticoids together with β_2-agonists. Although glucocorticoids have a number of actions in the inflamed airway, an additional important way in which glucocorticoids may be effective in asthma is through direct effects on smooth muscle through inhibition of cytokine-induced hyporesponsiveness to β_2-agonists (Adcock et al., 2002; Moore et al., 1999). Furthermore, combining a β_2-agonist with a COX-2 inhibitor might more directly inhibit this mechanism. Thus, further studies using the acute inflammatory response following swine house dust exposure as a model, may increase the understanding of the inflammatory response and provide new targets for pharmacological intervention when treating chronic airways inflammation.

6 Conclusions

- Glucocorticoids are potent inhibitors of swine house dust-induced cytokine release from lung epithelial cells (IL-6 and IL-8) and of LPS-induced cytokine release (IL-6, IL-8 and TNF-α) from human alveolar macrophages *in vitro* at concentrations likely to be found in the airways during inhalation therapy. The onset of the cytokine suppression by glucocorticoids *in vitro* was rapid and might thus be a direct, non-gene mediated, effect.

- Swine house dust stimulation of lung epithelial cells activated the transcription factor NF-κB, involved in mediating the release of IL-6 and IL-8. The mechanisms by which glucocorticoids suppress swine house dust-induced cytokine release from epithelial cells *in vitro* include repression of NF-κB activity.

- Fluticasone attenuated the inflammatory response in the upper airways, attenuated the fever response and attenuated the systemic IL-6 response induced in healthy subjects following exposure in a swine barn. However, fluticasone did not affect the cell- and cytokine inflammatory response in the lower airways. This discrepancy might be due to differences in steroid concentration, to methodological time differences or that glucocorticoids have only weak or no inhibitory effects on neutrophilic responses.

- Inhalation of fluticasone attenuated the IL-6 serum response and the increase in body temperature following exposure in a swine barn. There might be a causal relationship between glucocorticoid-induced attenuation of serum IL-6 and body temperature increases.

- Intra-nasal administered fluticasone attenuated plasma leakage, assessed as albumin concentrations, and attenuated the IL-8 and TNF-α concentrations in nasal lavage fluid following exposure in a swine barn. The inhibition of plasma leakage by glucocorticoids might reflect the ability of the drug to inhibit inflammatory mediators such as IL-8 and LTE_4, which have been shown to influence vascular leakage.

- Fluticasone inhalation had no effect on the increased bronchial responsiveness to methacholine following exposure. There might be no direct relationship between the inflammation and the increased bronchial responsiveness in healthy subjects following exposure in a swine barn.

- Exposure of healthy individuals in a swine barn reduced the capability of alveolar macrophages to release cytokines *ex vivo* indicating that alveolar macrophages have a reduced function after exposure. Pre-exposure treatment with either salmeterol or fluticasone did not influence this capability.

- Salmeterol inhalation had no effect on the inflammatory responses to inhaled organic dust supporting the theory that salmeterol has no or little influence on airway inflammation in human.

- Salmeterol inhalation did not alter the increased responsiveness to methacholine following exposure, neither when inhaled regularly during 2 weeks prior to exposure nor when administered as a single dose prior to exposure. However, salmeterol caused a decreased responsiveness to methacholine in healthy unexposed subjects 2 and 8 hours after inhalation. This indicates that the lack of protective effect of salmeterol was not due to homologous β_2-adrenoceptor desensitization but rather that exposure to organic material may have altered the airway response to β_2-agonists. Our hypothesis is that pro-inflammatory cytokines, such as IL-1β and TNF-α, have influenced β_2-adrenoceptors on airway smooth muscles leading to decreased β_2-adrenergic responses.

7 Summary

Exposure of healthy subjects in a swine barn causes an acute inflammatory response in the airways and an increase in bronchial responsiveness to methacholine. The aim of this thesis was to study the mechanisms by which glucocorticoids or β_2-agonists interfere with the inflammatory response following swine house dust-exposure both *in vitro* and *in vivo*.

In the first study, the effect of fluticasone or budesonide on cytokine response in swine house dust-stimulated A549 lung epithelial cells (IL-6 and IL-8) and in LPS-stimulated human alveolar macrophages (IL-6, IL-8 and TNF-α) was evaluated. The glucocorticoids caused a dose-response inhibition of the cytokines and potent effects were found at concentrations that are likely to be found in the airways during inhalation therapy. Furthermore, the onset of the cytokine suppression by glucocorticoids *in vitro* was rapid.

In the second study, the involvement of NF-κB on swine house dust-mediated IL-6 and IL-8 release from A549 lung epithelial cells and the influence of glucocorticoids was evaluated. By interfering with the NF-κB pathway at different levels, it was demonstrated that swine dust stimulation activated NF-κB and cytokine release. In addition, it was shown that the glucocorticoid-mediated cytokine inhibition might, in part, be explained by inhibition of NF-κB activation.

In the third study, the effect of treatment with fluticasone (inhalation and intra-nasally) on the upper airway inflammatory response, the systemic response and the increased bronchial responsiveness to methacholine induced in healthy subjects following exposure in a swine barn, was evaluated. Intranasal fluticasone attenuated plasma leakage, assessed as albumin concentrations, and cytokine concentrations in nasal lavage fluid (IL-8 and TNF-α). Fluticasone treatment also attenuated serum IL-6 levels and body temperature increases but not the increased bronchial responsiveness following exposure.

In the fourth study, the effect of inhaled fluticasone or salmeterol on the lower airway inflammatory response induced in healthy individuals following exposure in a swine barn was evaluated. There were no effects of fluticasone or salmeterol treatment on the increase in cell concentration and cytokine release in bronchoalveolar lavage fluid following exposure. Additionally, exposure reduced the capability of alveolar macrophages to release cytokines *ex vivo* and pre-exposure treatment did not affect this capability.

Last, the effect of the long-acting β_2-agonist salmeterol, on the increased bronchial responsiveness to methacholine, induced in healthy subjects exposed in a swine barn, was investigated. Salmeterol inhalation did not alter the increased responsiveness to methacholine following exposure, neither when inhaled regularly during 2 weeks prior to exposure nor when administered as a single dose prior to exposure. However, salmeterol inhalation caused a decreased responsiveness in healthy unexposed subjects. This indicates that the lack of protective effect of salmeterol was not due to homologous β_2-adrenoceptor desensitization but rather that exposure may have altered the airway response to β_2-agonists.

In conclusion, glucocorticoids may exert anti-inflammatory effects on the organic dust induced inflammatory response. Glucocorticoid-mediated inhibition of pro-inflammatory cytokines, through inhibition of NF-κB activation, might be an important mechanism by which these effects are exerted. On the other hand, the glucocorticoid had no effect on the

inflammatory response in the lower airways or on the increased bronchial responsiveness following exposure. Salmeterol did neither influence the inflammatory response nor the increased bronchial responsiveness following exposure.

8 Sammanfattning

Friska, tidigare oexponerade individer som exponeras tre timmar i svinhusmiljö får kraftig inflammation i både övre och nedre luftvägar samt en ökad bronkiell reaktivitet mot metakolin. Syftet med denna avhandling var att studera effekten av glukokortikoider och β_2-agonister på denna akuta inflammatoriska reaktion både *in vitro* och *in vivo.*

I det första delarbetet studerades dos-respons effekten av flutikason propionate eller budesonid och tidskinetiken på cytokinfrisättningen från svinhusdamms-stimulerade A549 lungepitelceller (IL-6 och IL-8) och LPS-stimulerade humana alveolära makrofager (IL-6, IL-8 och TNFα). Glukokortikoiderna hämmade cytokinerna i ett dos-respons förhållande och en kraftig hämning påvisades vid koncentrationer som mycket väl kan uppnås i luftvägarna vid normal dosering av inhalationssteroid. Tidsstudierna tyder på att verkningsmekanismen är snabb.

I det andra delarbetet undersöktes om NF-κB är involverad vid svinhusdamms-inducerad cytokinfrisättning från A549 lungepitelceller och om glukokortikoider interagerar med denna transkriptionsfaktorn. Genom att hämma eller inaktivera NF-κB på olika sätt visades att svinhusdamm aktiverade NF-κB, som var involverad i IL-6 och IL-8 frisättningen. Resultaten visade också att glukokortikoiders hämning av cytokinfrisättning dels verkar genom interaktion med NF-κB.

I det tredje delarbetet studerades effekten av flutikason propionate (inhalerat och intranasalt) på den inflammatoriska responsen i övre luftvägar, den systemiska reaktionen och på den ökade bronkiella metakolinreaktiviteten som inducerats hos friska individer efter exponering i svinhus. Flutikason hämmade plasma läckage, mätt som albumin- och α_2-makroglobulin-koncentrationer, och ökningen av cytokinkoncentrationer i nasalsköljvätska efter exponering. Dessutom hämmades ökningen av IL-6 koncentrationer i serum och ökningen av kroppstemperaturen efter exponering, av behandlingen. Däremot hade flutikason ingen effekt på ökningen av bronkiell reaktivitet. Slutsatsen var att glukokortikoider dämpar inflammationen i övre luftvägar och påverkar den systemiska reaktionen som inducerats av exponering i svinhus utan att påverka ökningen av bronkiell reaktivitet.

I det fjärde delarbetet studerades hur salmeterol eller flutikason behandling påverkar de nedre luftvägarna och makrofagernas förmåga att frisätta cytokiner *ex vivo* efter exponering i svinhus. Varken flutikason eller salmeterol påverkade den kraftiga cell- och cytokin-koncentrationsökningen i nedre luftvägarna efter exponering. Exponeringen ledde till en reducering av den basala cytokinfrisättningen (IL-6, IL-8 och TNF-α) från alveolära makrofager *ex vivo* och denna påverkades inte av behandling.

I det sista delarbetet, undersöktes effekten av den långverkande β_2-agonisten salmeterol, på den ökade bronkiella reaktiviteten mot metakolin inducerad hos friska personer efter exponering i svinhusmiljö. Varken två veckors behandling eller en enda dos påverkade den ökade bronkiella reaktiviteten efter exponering. Hos oexponerade friska individer minskade däremot bronkiell reaktivitet mot metakolin efter inhalation av salmeterol. Resultaten indikerar att den uteblivna skyddande effekten av salmeterol efter exponering inte beror på homolog

desensitisering av β_2-adrenoceptorn utan att exponering för organiskt damm kan ha lett till en förändrad luftvägsrespons mot β_2-agonister.

Sammanfattningsvis visades att glukokortikoider har anti-inflammatoriska effekter på den inflammatoriska reaktionen som inducerats hos friska individer av exponering i svinstall. Glukokortikoid-medierad hämning av pro-inflammatoriska cytokiner, via hämning av NF-κB aktivering, kan vara en betydelsefull mekanism för dessa effekter. Däremot hade glukokortikoider ingen effekt på den nedre luftvägsinflammationen vilket kan bero på skillnader i steroidkoncentration, metodologiska tidsskillnader eller på att glukokortikoider inte påverkar neutrofila inflammatoriska förlopp. Dessutom påverkade glukokortikoiden inte bronkiell reaktivitet och det verkar inte finnas något direkt samband mellan bronkiell reaktivitet och den inflammatoriska reaktionen som inducerats hos friska individer efter exponering i svinhus. β_2-Agonisten salmeterol påverkade inte den inflammatoriska reaktionen, mätt som cell-, cytokin- och plasmaproteinkoncentrationer i bronkoalveolärsköljvätska eller serum, efter svinhusexponering. Resultaten angående den uteblivna skyddande effekten av salmeterol på den ökade bronkiella metakolinreaktiviteten efter exponering indikerar att exponeringen har förändrat β_2-receptorfunktionen och att pro-inflammatoriska cytokiner kan spela en roll vid denna mekanism.

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Life is not the days which are gone,
it is the days you remember
Pavlenko

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