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THE EFFECT OF ß$_2$-ADRENOCEPTOR AGONISTS AND STEROIDS ON INDUCED AIRWAY INFLAMMATION AND BRONCHIAL RESPONSIVENESS

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To Tore, Linnéa and Sofia

Abstract

Acute exposure of healthy subjects in a swine barn induces an intense airway inflammation and increased bronchial responsiveness. Dust collected in swine houses is a potent stimulus for release of pro-inflammatory cytokines from cells *in vitro*. The main aim of this thesis was to elucidate the effects of long-acting ß$_2$-agonists and glucocorticosteroids on inflammatory mechanisms *in vivo* and *in vitro* using organic dust as pro-inflammatory stimulus.

In the first study, formoterol and salmeterol were shown to induce enhancement of IL-6 and IL-8 release from non-stimulated primary bronchial epithelial cells (PBEC) and A549 cells *in vitro*. The β_2-agonists also added to the effect of organic dust. This enhanced release was blocked by a β-blocker in PBEC, but not in A549 cells. The results indicate different mechanisms of β_2-agonists action in bronchial and alveolar epithelial cells, and that A549 cells do not possess functional β_2-adrenoceptors.

In the second study formoterol was shown to add to the dust-induced IL-6, but not IL-8 release from PBEC. Budesonide attenuated the release of both cytokines in a dose-response manner. This inhibiting effect was sustained but not reinforced by formoterol. No synergistic effect between formoterol and budesonide was found.

In the third study the effect of formoterol and budesonide on chemokine/cytokine release, chemokine receptor expression and chemotaxis in isolated human neutrophils *in vitro* was evaluated. Formoterol enhanced and budesonide inhibited IL-6, IL-8, and GRO-α release from LPS-stimulated neutrophils. Formoterol upregulated both CXCR1 and CXCR2 expression, whereas budesonide upregulated the expression of CXCR2 only. Despite the effects on chemokine release and drug-induced up-regulation of chemokine receptors, no influence on neutrophil chemotaxis could be demonstrated by the β_2-agonist or the glucocorticosteroid.

In the fourth study, 12 healthy subjects were exposed to organic dust in a swine barn. In this cross-over designed study, we found that one single dose of salmeterol partially protected against the increased responsiveness to methacholine. Salmeterol did not influence the inflammatory response to dust exposure. One week pre-treatment with fluticasone or ibuprofen had no effect on the airway responses and did not alter the effect of salmeterol. In addition, a retrospective analysis of pooled data from four previous studies was performed. We concluded that exposure leads to an enhancement of bronchial responsiveness to a certain maximal level which is similar in all subjects, and almost totally unrelated to pre-exposure level of bronchial responsiveness.

In conclusion, although β_2-agonists and glucocorticosteroids influence the release of pro-inflammatory cytokines/chemokines and up-regulate chemokine receptors *in vitro*, these drugs did not influence the investigated inflammatory parameters *in vivo*. As the increase in bronchial responsiveness following organic dust exposure is strongly related to pre-exposure bronchial responsiveness, interventions altering bronchial responsiveness have to be compared between groups with similar pre-challenge bronchial responsiveness or in a cross-over design. No additive/synergistic effects between β_2-agonists and steroids were found.

List of Publications

I. Strandberg K, Palmberg L, Larsson K.
”Effect of formoterol and salmeterol on IL-6 and IL-8 release in airway epithelial cells.”
Respiratory Medicine 2007; 101:1132-1139

II. Strandberg K, Palmberg L, Larsson K.
”Effect of budesonide and formoterol on IL-6 and IL-8 release from primary bronchial epithelial cells.”
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III. Strandberg K, Blidberg K, Sahlander K, Palmberg L, Larsson K.
“Effect of formoterol and budesonide on chemokine release, chemokine receptor expression and chemotaxis in human neutrophils.”
Submitted.

IV. Strandberg K, Ek A, Palmberg L, Larsson K.
”Fluticasone and ibuprofen do not add to the effect of salmeterol on organic dust-induced airway inflammation and bronchial hyper-responsiveness”.
Journal of Internal Medicine 2008; 264:83-94

Contents

List of abbreviations

β_2AR	**β_2-adrenoceptor**
cAMP	**cyclic adenosine monophosphate**
COPD	**chronic obstructive pulmonary disease**
COX	**cyclo-oxygenase**
CRP	**C-reactive protein**
ELISA	**enzyme-linked immunosorbent assay**
ENA-78	**epithelial-derived neutrophil activator**
ERK	**extracellular signal-regulated kinase**
FEV	**forced expiratory volume**
FEV1	**forced expiratory volume in one second**
G-CSF	**granulocyte colony-stimulating factor**
GM-CSF	**granulocyte-macrophage colony-stimulating factor**
GPCR	**G-protein coupled receptor**
GR	**glucocorticosteroid receptor**
GRE	**glucocorticoid response element**
GRK	**G protein-receptor kinase**
GRO-α	**growth-related oncogene-α**
GS	**glucocorticosteroid**
ICS	**inhaled corticosteroids**
IL	**interleukin**
LPS	**lipopolysaccharide**
MAP	**mitogen-activated protein**
M-CSF	**macrophage colony-stimulating factor**
MIP-1α	**macrophage inflammatory protein 1α**
NF-κB	**nuclear factor-κB**
NO	**nitric oxide**
NSAID	**non-steroidal anti-inflammatory drug**
ODTS	**organic dust toxic syndrome**
PAMP	**pathogen-associated molecular pattern**
$PC_{20}FEV_1$	**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**
PEF	**peak expiratory flow**
PGE_2	**prostaglandin E_2**
PKA	**protein kinase A**
RANTES	**regulated upon activation normal T cell expressed and secreted**
SEM	**standard error of the mean**
TLR	**toll-like receptor**
TNF	**tumour necrosis factor**
VAS	**visual analogue scale**
VC	**vital capacity**

Man har inte roligare än man gör sig

Introduction

The airways and lungs are exposed to a complex mixture of particles and gases in the surrounding air, some being detrimental for the cells and tissues. Airway inflammation is associated with some of the most common diseases in the western countries. The prevalence of asthma in developed countries is approximately 10% in adults and even higher in children. The global prevalence of chronic obstructive pulmonary disease (COPD) is approximately 10% among individuals over the age of 40 years (1). Obstructive lung diseases are characterized by airway obstruction that is variable and reversible in asthma but is progressive and irreversible in COPD. There is an overlap in symptoms between asthma and COPD, but the features of inflammation differ between these diseases, with different inflammatory cells and mediators being involved. The characteristic cell type in asthma is eosinophils, while COPD is characterized by neutrophils and macrophages. The distinction between the diseases becomes less clear in patients with severe asthma, in asthmatic subjects who smoke and during acute exacerbations - conditions in which the inflammation is more dominated by neutrophil granulocytes.

Anti-inflammatory therapy is central in the treatment of asthma and COPD. Glucocorticosteroids and long-acting β_2-agonists are the most commonly used drugs in asthma treatment, and have been shown to be beneficial for patients with COPD as well. A better understanding of the mechanisms by which these drugs influence airway inflammation is of importance for the development of new therapeutic strategies.

Massive exposure to organic dust, especially when contaminated with large amounts of micro-organisms, often leads to a self-limiting, flu-like condition called organic dust toxic syndrome, ODTS (2). The symptoms start a few hours after exposure and subside after 1-2 days. Work-related variable airflow limitation may occur with occupational exposure to organic dust, and some work-related obstructive disorders have been classified as COPD or asthma-like disorder without completely fitting into these categories (3).

Acute exposure of healthy, previously non-exposed subjects in a swine barn induces an intense, non-allergic airway inflammation, increased bronchial responsiveness, and ODTS with systemic effects. Dust collected in swine houses has also been shown to be a potent stimulus for release of pro-inflammatory cytokines in human airway epithelial cells and alveolar macrophages *in vitro*. This kind of exposure has been used in our research group as an experiment model for studies of airway inflammation, and the inflammatory response has been the subject for pharmacological interventions *in vitro* and i*n vivo*.

The main aims of the studies forming the basis of the present thesis, were to elucidate the effects of long-acting β_2-agonists and glucocorticosteroids on inflammatory mechanisms *in vivo* and *in vitro* with focus on airway epithelial cells, neutrophil granulocytes, and bronchial responsiveness.

Background

The inflammatory process

Inflammation is an expression of the host's attempt to localize and eliminate metabolically altered cells, foreign particles, microorganisms, or antigens. Its cardinal signs are redness (rubor), heat (calor), swelling (tumor), and pain (dolor), often accompanied by loss of function (functio laesa). It is a protective action of the organism to remove the injurious stimuli as well as initiate the healing process for the tissue. Inflammation can be classified either as *acute* or *chronic*. The hallmarks of acute inflammation include accumulation of fluid and plasma components in the affected tissue, intravascular stimulation of platelets, and the presence of polymorphonuclear leukocytes. Histamine, kinins, and various other substances, like cytokines and chemokines, mediate the inflammatory process.

Cells participating in airway inflammation

Airway epithelial cells

The adult human bronchial tree is covered with a continuous single layer of epithelial cells. The airway epithelium is pseudostratified in the large airways, becoming columnar and cuboidal in the small airways. The epithelium penetrates the submucosa at intervals, forming ducts leading to glands consisting of serous and mucous cells (4). In the large airways (1st to 5th ramification), the major cell types are ciliated cells, undifferentiated columnar cells, goblet cells, and basal cells. In the small airways (6th to 23rd ramification), the proportion of ciliated cells is higher, and the secretory cells shift from goblet cells to Clara cells. After 23 branches, the airway epithelium merges with the alveolar epithelium (5). The alveolar ducts and the alveoli are lined with type I and type II alveolar cells. Type I alveolar cells are squamous in shape and cover about 95% of the alveolar surface. They form a thin barrier for gas exchange, and have also been suggested to have a potential role in defending the alveolus against oxidative stress (6). Type II cells secrete pulmonary surfactant which decreases the surface tension of the alveolar surface, allowing the alveoli to expand during inspiration, and preventing their collapse during expiration. Type II alveolar cells are able to differentiate into both type I and type II alveolar cells (7, 8).

Airway epithelial cells constitute a complex physical barrier that defends against exposures to potentially harmful inhaled substances and microbial pathogens. They regulate both innate and adaptive immunity through production of functional molecules and via physical interactions with cells of the immune system (9). Epithelial cells play important roles in host defense, inflammation, and regulation of immune responses, and epithelial activation is a characteristic of asthma, rhinitis, chronic rhinosinusitis, chronic obstructive pulmonary disease, and other airway diseases. Airway epithelial cells actively participate in the inflammatory response in asthma by secreting cytokines, reactive oxygen metabolites, and other mediators that regulate infiltrating inflammatory cells such as lymphocytes, eosinophils, and mast cells (10).

Monocytes circulate in the blood, and migrate into the tissues where they differentiate into *macrophages.* Monocytes entering the lungs differentiate into alveolar macrophages, and account for the majority of inflammatory cells recovered by bronchial alveolar lavage (11). The alveolar macrophages are exposed to a large variety of particles in the inhaled air. The

name macrophage means large phagocyte, and like the neutrophil, it is well equipped for phagocytosis. Macrophages are generally the first phagocytic cell to sense an invading microorganism and secrete the cytokines that recruit neutrophils and other leukocytes into the infected area.

Neutrophil granulocytes, once called microphages, are effector cells of innate immunity that are rapidly mobilized to enter sites of infection. The neutrophil is the most abundant of all leukocytes and is specialized in the capture, engulfment and killing of microorganisms. Activated neutrophils release tissue damaging enzymes such as neutrophil elastase, reactive oxygen compounds, cytokines and lipid mediators (12). Acute airway inflammation characterized by the presence of neutrophils is a prominent feature of many lung disorders including bronchitis, cystic fibrosis, bronchiectasis, and exacerbations of bronchial asthma (13). In addition to their phagocytic activity, neutrophils also act as secretory cells able to release preformed proteins stored in the cytoplasmic granules as well as toxic oxygen radicals (14). It is known that the neutrophil is capable of contributing to immune and inflammatory responses through the generation and release of cytokines such as IL-1, IL-6 and tumour necrosis factor (TNF); chemokines such as IL-8 (CXCL8) and macrophage inflammatory protein 1α (MIP-1α; CCL3); leukotriene B4 (LTB4) which is a potent chemoattractant for neutrophils; growth factors, such as macrophage colony-stimulating factor (M-CSF) and granulocyte colony-stimulating factor (G-CSF). Neutrophil elastase and reactive oxygen species induce mucus hypersecretion, a prominent feature in severe asthma and COPD (11).

Eosinophils are multifunctional leukocytes involved in the defense against helminth worms and other intestinal parasites. Apart from being involved in inflammation induced by parasites, they are also involved in inflammatory processes in allergic diseases. In response to stimuli, such as helminth or allergen exposure, they are recruited from the circulation to sites of inflammation. Eosinophils have been implicated in innate immunity by being an early source of cytokines, e.g. IL-4 (15). Lymphocytic inflammation enriched by esoinophils is characteristic of the bronchi in mild asthma, and also of the bronchi and luminal secretions in fatal asthma (16). In response to stimuli, eosinophils in the blood are selectively retained at bronchial microvascular surfaces by TNF-induced and IL-4-induced upregulation of adhesive molecules. They then migrate to the surface epithelium where they cross it, in response to eosinophil chemoattractants such as eotaxin, macrophage/monocyte chemotactic protein 4, RANTES (regulated upon activation normal T cell expressed and secreted, or CCL5), and cysteinyl leukotrienes.

Lymphocytes can be divided into bursa derived *B cells*, thymus derived *T cells* and *natural killer* (*NK*) cells, and are involved in the defence against pathogens. For B cells the cell-surface receptors for pathogens are immunoglobulins, whereas those of T cells are known as T cell receptors (TCR). The sole function of B cells is to produce antibodies. B cells generally require help from activated T cells to mature into antibody-secreting plasma cells. Thymus is the principal organ where the T cells mature. Helper T cells (effector T cells or T_H cells) have CD4 glycoproteins on the cell surface. The general function of CD4 T cells is to help other cells of the immune system to respond to extracellular sources of infection, such as bacteria, by secreting cytokines that regulate or assist in the immune response. This is carried out of two subclasses of CD4 T cells – $T_H1$and T_H2 cells. T_H1 cells activate tissue macrophages and T_H2 cells are involved mainly in B cell differentiation and production of antibodies. Cytotoxic T cells have CD8 glycoprotein on the cell surface and their main function is to kill cells that have become infected with a virus or some intracellular pathogen, and to kill tumour cells. NK cells are cytotoxic lymphocytes providing innate immunity, and target virus infected and transformed cells. They migrate from the blood into infected tissues in response to inflammatory cytokines.

Cytokines

Cytokines are small protein mediators acting as inter-cellular communicators. The action of cytokines may be autocrine (act on the producing cell type), paracrine (act on other cells nearby the producing cell type), and endocrine (secreted into the blood stream and affect distant cells). Cytokines are critical to the development and functioning of both the innate and adaptive immune response, although not limited to just the immune system. Some of them are proinflammatory, e.g. IL-1, TNF, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF. These cytokines contribute to leukocytosis by inducing release of neutrophils into the blood from bone marrow and by inducing the production of cytokines, including IL-6 IL-8, RANTES, GM-CSF and TNF, from a variety of cells (17). IL-1, IL-6 and TNF have systemic effects and may cause a rise in body temperature and induce the production of acute-phase proteins in the liver, e.g. C-reactive protein (CRP).

Chemokines

Chemokines are small protein mediators that act as chemoattractants for inflammatory cells. There are more than 50 distinct chemokines, broadly classified into C, CC, CXC, and CX3C subgroups based on the arrangement of conserved cysteines located near the amino terminus (N-terminus) of the protein. In the CXC chemokines the first two cysteine residues have an amino acid in between, and in the CC chemokines the cysteine residues are adjacent. There is also a functional classification according to which the chemokines are classified as being inflammatory and inducible, or homeostatic and constitutive (18, 19). The structural classification has a general, not absolute, functional correlate in that CC chemokines target monocytes, T-cells, eosinophils, and basophils, whereas CXC chemokines target neutrophils (20-22). Chemokines exert their effects by interaction with seven-transmembrane domain G-protein coupled receptors in the target-cell membrane. Of the CXC chemokines, IL-8 (CXCL8, i.e. CXC ligand 8), growth-related oncogene-α (GRO-α or CXCL1) and epithelial-derived neutrophil activator (ENA-78 or CXCL5) are of particular interest with their chemoattractant and activating effects on neutrophils (17). The effects of these chemokines are mediated by two receptors, CXCR1 and CXCR2, which are expressed predominantly on neutrophils. While IL-8 binds to both CXCR1 and CXCR2, GRO-α and ENA-78 bind to CXCR2 only.

Nitric oxide

Nitric oxide (NO) is produced by several types of pulmonary cells, including inflammatory, endothelial, and airway epithelial cells (23) and may be detected in the exhaled air of animals and humans (24, 25). Different forms of lung inflammation may increase the exhaled NO levels although the relationship is far from clear as pneumonia, ciliary dyskinesia and cystic fibrosis have been shown to be associated with reduced exhaled NO (26). Smoking is associated with a reduction in exhaled NO levels, and ex-smokers exhibit lower levels of exhaled NO than never-smokers (27). Several studies have demonstrated increased levels of exhaled NO in patients with asthma (28-30) and that the levels decrease during steroid use (31). Allergic asthma, in particular, is associated with increased levels of exhaled NO, but the relation between non-allergic asthma and increased exhaled NO is not as clear. It has recently been shown, however, that both allergic and non-allergic asthma are associated with increased exhaled NO levels, but only in never-smokers (28). It has been suggested that the concentration of exhaled NO might be used to provide information about the presence of eosinophilic, corticosteroid responsive airway inflammation (26).

Innate immunity

Animals have evolved a variety of defense mechanisms against microbial infections, the first being the protective epithelial surfaces of the body. The other is the immune system, which in mammals comprises two branches, the innate immune system and the adaptive immune system. The innate immunity constitutes nonspecific resistance to infections, the word "innate" indicating existence from birth, not acquired. The adaptive immune system on the other hand, needs time, often several days, to develop a specific response to the pathogen or protein, unless the organism has been exposed to that particular agents previously. These adaptive mechanisms include specialized effector T cells and antibodies that recognize the specific pathogen when it re-enters the organism (immunological memory). Most infections are efficiently cleared by the innate immune response, which is the first line of host defense and is responsible for immediate recognition and control of microbial invasion. Pathogen-associated molecular patterns (PAMPs) are diverse products with conserved motifs. Pathogen-associated molecular patterns often have an essential role in bacterial structure, and they activate the host immune system through a process that is mediated by Toll-like receptors (TLRs). Examples of PAMPs include Gram-negative (e.g. LPS) and Gram-positive (e.g. peptidoglycan, lipopeptide) bacterial components that activate TLR4 and TLR2, respectively (32, 33). The innate immune response relies on the pattern-recognition receptors (PRRs), which are evolutionarily ancient receptors recognizing highly conserved microbial structures (9). The PRR family of Toll-like receptors has been identified as a major component of the innate immune system (34). The innate immune response includes nuclear factor-κB (NF-κB) activation and pro-inflammatory molecule production (35, 36). The innate system must be accurately regulated because inadequate responses cause increased susceptibility to infectious pathogens, whereas exaggerated responses may lead to chronic inflammation or lethal acute inflammation such as septic shock. The homeostatic balance of the innate immune system is maintained partly by pro- and anti-inflammatory cytokines (37).

Organic dust from pig farms and the inflammatory response

Workers in animal confinement facilities, and hog confinement workers in particular, are at increased risk for the development of respiratory diseases (38-40). Acute exposure in swine confinement facilities causes airway inflammation characterized by neutrophilia with an associated increase of proinflammatory cytokines, including IL-6 and IL-8 (41-43). Dust from swine confinement buildings is primarily composed of feed and bedding material, but it also contains epithelial cells from the swines (dander), faecal material including micro-organisms, predominantly bacteria of both Gram negative and Gram positive genera (44-46). Endotoxins are soluble lipopolysaccharide (LPS) fragments from the outer membrane of Gram negative bacteria (47) and are known to be one of the ingredients in swine house dust, though not the only agents responsible for the biological effects. The endotoxin level in swine dust is low (22 ng/100 μg dust), i.e. only 0.02% of the dust consist of endotoxin. Still the dust at a concentration of 100 μg/ml is 5-8 times more potent than LPS at a concentration of 100 μg/ml as a stimulus for IL-8 release from A549 cells and normal bronchial epithelial cells *in vitro* (48). Gram positive bacteria and soluble components from the bacteria are also potent stimuli for IL-6 and IL-8 release from A549 cells and human alveolar macrophages (49). Exposure to organic dust, both *in vivo* and *in vitro*, from swine houses induces production and release of a number of pro-inflammatory cytokines, including IL-1β and TNF (43, 50, 51). Bacteria, including endotoxins and other microbial components present in the swine dust, activate the

innate immunity by inducing the production of antimicrobial peptides and inflammatory mediators including arachidonic acid metabolites, TNF and IL-8 (52). Organic dust from swine confinement buildings activates NF-κB which mediates secretion of IL-6 and IL-8 in A549 cells (53).

Swine confinement workers have an increased prevalence of airway symptoms such as cough, phlegm, wheezing and shortness of breath and have higher prevalence of chronic bronchitis than non-farmers and farmers not working with swines (54). It has been demonstrated that also healthy pig farmers have signs of airway inflammation (55). Exposure of healthy subjects in swine confinement buildings induces an intense airway inflammation and increased bronchial responsiveness to direct stimuli such as methacholine (43, 56-58) and histamine (59). Exposure also leads to systemic effects such as fever, chills, increased serum levels of acute phase proteins and blood leukocytosis (50, 57, 60, 61). Following three hours of exposure in a swine barn the levels of pro-inflammatory cytokines (TNF, IL-1, IL-6 and IL-8) are elevated in nasal and bronchoalveolar lavage fluids, and the concentration of TNF and IL-6 increases in peripheral blood (43, 50). It has been shown that swine farmers respond to a lesser extent than do healthy controls to acute exposure to inhaled organic material suggesting adaptive mechanisms in chronically exposed individuals (61, 62). Dust collected in swine houses has been shown to activate human airway epithelial cells and alveolar macrophages and is a very potent stimulus for cytokine release in these cells *in vitro* (48, 63-65).

Pharmacology

G-protein coupled receptors

The super-family of G-protein coupled receptors (GPCRs) is one of the largest groups of proteins in vertebrate species. More than 800 GPCR sequences in the human genome have been identified (66). G-protein coupled receptors participate in a diversity of important physiological functions and are targets for many modern drugs. The main structural characteristic of GPCRs is seven hydrophobic transmembrane-spanning regions of about 25-35 consecutive amino acid residues. The name GPCRs indicates that these receptors interact with G-proteins but this has, however, not yet been demonstrated for most of the proteins classified as GPCRs. G-protein coupled receptors have many alternative signalling pathways, interacting with a number of other proteins such as arrestins and kinases. "Seven transmembrane (7TM) receptors" would perhaps be a more correct term for this super-family, but the GPCR terminology is more established (66, 67). Both chemokine receptors and β-adrenoceptors belong to the GPCRs.

Chemokine receptors

The nomenclature of chemokine receptors is based on the chemokine class they bind. CXCR1, 2, 3, 4, and 5 bind CXC chemokines; CCR1 through CCR9 bind CC chemokines; XCR1 binds the C chemokine; and CX3CR1 binds the CX3C chemokine, fractalcine or neurotactin (68). CXCR1 and CXCR2 are expressed mainly on neutrophils, and both mobilize intracellular calcium as a second messenger (69). Both CXCR1 and CXCR2 are of importance for neutrophil chemotaxis, but only CXCR1 mediates cytotoxic and cross-regulatory signals (70). Like other G-protein coupled receptors, CXCR1 and CXCR2 are rapidly desensitized following agonist-mediated signalling (15, 32).

β_2-Adrenoceptors

Bronchial tone is partly regulated by vagal pathways of the parasympathetic system. Muscarinic receptors can be demonstrated on the smooth muscle and the submucousal glands; stimulation causing contraction of the former and secretion from the latter. There is no sympathetic innervation of the bronchial smooth muscle and adrenergic effects are mediated by circulating catecholamines. Adrenaline is the endogenous β_2-agonist which acts as a hormone, reaching the tissue via the blood stream from the adrenal glands where it is produced. β-Adrenoceptors have been subdivided into three distinct groups: β_1, β_2, and β_3, classically identified in cardiac, airway smooth muscle, and adipose tissue respectively. β_2-Adrenoceptors are widely distributed throughout the human respiratory system and are present on a variety of cells, such as smooth muscle cells, alveolar type II cells, mast cells, lung epithelial and endothelial cells.

Binding of a β-agonist to the β_2-adrenergic receptor results in activation of adenylyl cyclase, through a G-protein coupled mechanism. Adenylyl cyclase causes formation of the second messenger cyclic adenosine monophosphate (cAMP), which activates protein kinase A (PKA). Protein kinase A phosphorylates a number of target proteins resulting in smooth muscle relaxation. The activation of PKA also serves as an important negative feed-back loop since PKA directly phosphorylates the β_2-adrenoceptor protein, which uncouples the receptor from its G protein, thereby terminating signalling events. Stimulation of airway β_2-adrenoceptors induces relaxation of airway smooth muscle and interacts with epithelial cells by increasing ciliary beat frequency and affecting ion transport across the cells by opening apical ion channels (71). β_2-Agonists also regulate cytokine release and have been shown to enhance release of IL-8 from bronchial epithelial cells (72, 73) and airway smooth muscle cells (74).

Desensitization of β-adrenoceptors

It is often found that the effect of a drug gradually diminishes when it is given continuously or repeatedly. Desensitization and tachyphylaxis are synonymous terms used to describe this phenomenon. The term tolerance is conventionally used to describe a more gradual decrease in responsiveness to a drug, taking days or weeks to develop, but the distinction is not sharp.. Decreased responsiveness (desensitization) of the β-receptor (as well as of other GPCRs) is mediated by three overlapping pathways: phosphorylation of the receptor with subsequent *uncoupling* from signal transduction, *internalization* of cell-surface receptors, and *down-regulation* of the production of new receptors. Uncoupling and internalization occur almost immediately, from seconds to minutes, whereas down-regulation is a more long-term event (hours) (75). The term *homologous* (agonist-specific) *desensitization* indicates that when a receptor is activated by the agonist, this mechanism desensitizes the subsequent response of the same receptor only. This means that homologous desensitization of the β_2-adrenoceptor occurs in response to β-agonists. *Heterologous* (agonist-nonspecific) *desensitization* indicates that stimulation by one agonist attenuates the response to multiple distinct agonists operating through different receptor types (76).

Agonist stimulation of the β_2-adrenoceptor triggers almost immediate phosphorylation of the third intracellular loop and in the carboxy-terminal tail of the β_2-adrenoceptor. The β_2-adrenoceptor is phosphorylated either by the second messenger (i.e. cAMP) dependant PKA or by G-protein receptor kinase (GRK) 2 and GRK3 (75). Receptor phosphorylation by GRKs requires that the receptor is occupied by the agonist, i.e this mechanism is strictly homologous

(76). The activation of any other receptor that also utilizes cAMP as a second messenger may result in heterologous desensitization of the β_2-adrenoceptor since cAMP formation results in PKA activation and thereby phosphorylation and uncoupling of the receptor from the G-protein (75).

Inflammatory cytokines, such as IL-1β and TNF, attenuate the ability of cultured cells to relax in response to β-agonists, an effect that has been claimed to involve cyclo-oxygenase (COX)-2 (75, 77). According to this hypothesis COX-2 increases prostaglandin E_2 (PGE_2) release, resulting in increased cAMP formation which in turn leads to PKA activation and thereby phosphorylation of the β_2-adrenoceptor. (75). (Figure 1). Thus, cytokines may induce heterologous desensitization of the β_2-adrenoceptor.

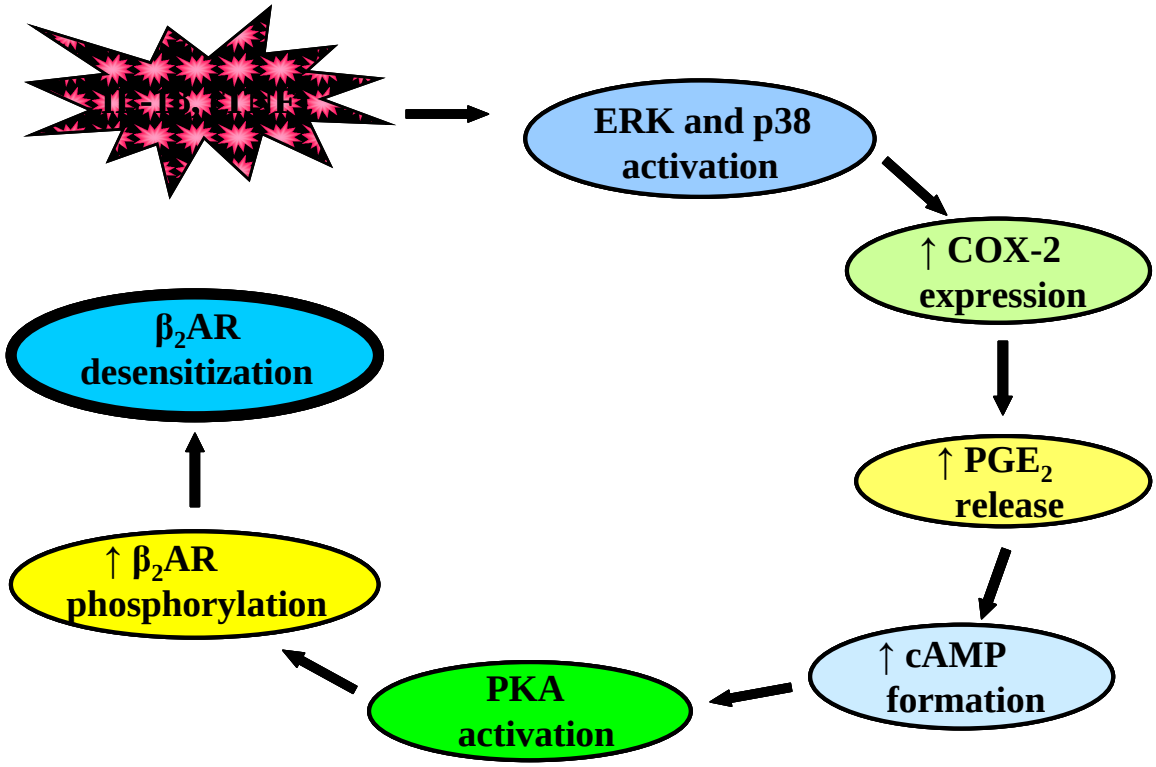

Figure 1. Heterologous desensitization. Proposed mechanism of IL-1β action on β-adrenergic responsiveness. The effect of IL-1β, and likely other pro-inflammatory cytokines as well, on COX-2 expression is dependant on activation of the ERK and p38 MAP kinases. Expression of COX-2 expression results in increased release of PGE_2. PGE_2 acts on EP_2 receptors coupled to the stimulatory G-protein leading to cAMP formation, and PKA activation. PKA phosphorylates the β_2-adrenoceptor (β_2AR) which then uncouples from the stimulatory G-protein. (Modified from Shore & Moore 2003).

β_2-agonists

β_2-Adrenoceptor agonists are effective bronchodilators, and their use is pivotal to the treatment of obstructive lung diseases. β_2-Agonists act as functional antagonists, i.e. they relax airway smooth muscle whatever the contractile stimulus is. This is of great importance in asthma where a multitude of mediators, such as histamine, acetylcholine, leukotrienes and tachykinines, contribute to the bronchoconstriction (78). β_2-Agonists are classified by their selectivity, duration of action, affinity, potency and efficacy (table 1). Generally there are several agonists whose maximal response corresponds to the full response of the tissue (the

largest possible tissue response). These drugs are known as *full agonists*, and those whose maximal response falls short of the full response are known as *partial agonists*.

A major limitation of the β_2-agonists in use during the 1960s and 1970s was their short duration of action, typically 4-6 hours. *Formoterol* and *salmeterol* are both long-acting β_2-agonists and were developed in the 1980s (79). Both drugs have durations of effect exceeding 12 hours but their pharmacological and clinical profiles differ in several ways. Formoterol is moderately lipophilic and is taken into the cell membrane in the form of a depot, from where it progressively leaches out to interact with the active site of the β_2-adrenoceptor. The size of the depot is determined by the concentration or dose of formoterol given. Salmeterol is >10,000 times more lipophilic than salbutamol. It partitions rapidly (< 1 minute) into the cell membrane and then diffuses laterally through the cell membrane to approach the active site of the β_2-adrenoceptor through the membrane (80, 81). Salmeterol is a partial agonist at the β_2-adrenoceptor, achieving maximum effect after about 60 minutes while formoterol is almost a full agonist, achieving a more rapid onset of action (79). Experimental data show that salmeterol-receptor binding is only slowly reversible and non-competitive. To explain these findings, the "exo-site" hypothesis was proposed (82). According to this hypothesis, the long nonpolar tail of salmeterol anchors to an auxiliary binding site (exo-site) within the fourth domain of the β_2-adrenoceptor in such a way that the molecule is prevented from dissociating from the β_2-adrenoceptor, but the head can freely engage and disengage the active site of the receptor. The onset of salmeterol is therefore slower than that of formoterol, and the effects of salmeterol less dependent of dose than is formoterol (80). The existence of the "exo-site" mechanism is debated, though.

Table 1.

Summary of some pharmacological properties of selected β_2-agonists.

	Formoterol	Salmeterol	Salbutamol
Selectivity ratio (β_1: β_2 receptors)	1:120	1:85 000	1:1375
Affinity for β_2-receptor *(Ki)* (nM)	76	53	2500
Onset of action	2-3 min	30 min	2-3 min
Duration of bronchodilatory effect	>12 hours	>12 hours	4-6 hours
Efficacy (%)	100	63	86
Lipid solubility	Moderate	High	Low

β_2-agonists and inflammation

Although the major action of β_2-agonists on airways is relaxation of airway smooth muscles, they also exert several effects mediated through the activation of β_2-adrenoceptors expressed on other cell types. β_2-Adrenoceptors are present in submucosal glands, vascular endothelium, ciliated epithelium, mast cells, circulating inflammatory cells such as esoinophils and lymphocytes, Clara cells, type II pneumocytes and parasympathetic ganglia (83). It is well known that increases in intracellular cAMP promote endothelial barrier integrity. Formoterol is effective in inhibiting plasma leakage in the airways through an action on β_2-adrenoceptors on the endothelial cells of post-capillary venules, the major site of plasma leakage (84, 85).

Human lung mast cells express β_2-adrenoceptors, although in rather low density, and β_2-agonists inhibit the release of bronchoconstrictor mediators, including cysteinyl-leukotrienes and histamine (86, 87). Receptors are expressed on human neutrophils and β_2-agonists inhibit the release of reactive oxygen species (88). At least some of the β_2-agonists' effects on inflammatory cells appear to be independent of β_2-adrenoceptor activation, and may, for salmeterol, result from the stabilization of cell membranes (89).

β_2-Agonists exert anti-inflammatory activity (72, 74, 90-92) and reduce airway smooth muscle proliferation (93). Although β_2-agonists may have anti-inflammatory properties *in vitro*, most clinical trials have failed to convincingly demonstrate anti-inflammatory activities of these agents during chronic agonist therapy, based on changes in the number of activated mast cells, esoinophils, and macrophages in the airway (83). There are studies though, demonstrating anti-inflammatory effects of inhaled formoterol (94, 95) and salmeterol (96) in patients with asthma. A clinical favourable effect was seen in asthma patients treated with formoterol, with regard to a significant decrease in eosinophils and the epithelial expression of NF-κB (a transcription factor for proinflammatory genes), but these changes were not accompanied by reduced immunoreactivity for adhesion molecules or cytokines (95). Therapeutic use of β_2-adrenergic agonists to reduce plasma leakage in chronic airway diseases is limited, because vascular endothelium becomes desensitized by these agents (97).

There has been concern that regular use of β_2-agonists may result in deterioration of asthma control. A study designed to compare the effects of add-on salmeterol twice daily with placebo over 28 weeks in a randomised, double-blind, parallel-group fashion, with the intention to enrol 60,000 asthmatic patients, was halted prematurely because preliminary data revealed an increased mortality associated with regular use of salmeterol (98). The study was not matched for the use of inhaled corticosteroids, and a re-examination of the study revealed that the fatal or near-fatal asthma attacks predominantly occurred in patients who were randomized to the salmeterol group without being on treatment with inhaled corticosteroids (99). A study of long-acting β_2-agonist monotherapy demonstrated that markers of inflammation remained controlled in those patients on inhaled corticosteroids, whereas in those on salmeterol alone a deterioration in sputum eosinophils, eosinophil cationic protein, exhaled NO and methacholine sensitivity was observed (100). The adverse outcomes associated with long-acting β_2-agonists as monotherapy have been due to masking of inflammation rather than a toxic effect of the drugs (99). Careful examination of the reports which have caused concern in regard of safety does not reveal any evidence of an increased risk associated with the appropriate use of long-acting β_2-agonists when combined with inhaled corticosteroids (99).

Glucocorticosteroids

Endogenous glucocorticoids regulate normal reactions to stress. Thus, many of the physiologic and pharmacologic effects of glucocorticoids may be secondary to modulation of the action of numerous intercellular and intracellular mediators, including hormones, prostaglandins, lymphokines, and bioactive peptides (101). Glucocorticosteroids are among the most widely used drugs worldwide and are effective in many inflammatory and immune diseases. One of the most common use of glucocorticosteroids is in the treatment of asthma, where inhaled glucocorticosteroids have become first-line therapy and by far the most effective anti-inflammatory treatment (102). The target receptor for the glucocorticosteroid (GS) is the intracellular glucocorticosteroid receptor (GR). Under resting conditions, the inactive GR is largely located in the cytosol of the cell, associated with multichaperone proteins. The GS molecule penetrates the cell membrane and then binds to the GR through the

GS-binding domain (103). This induces dissociation of the chaperone proteins, and the formation of an active GS-GR complex. The complex forms a dimer and translocates from the cytosol to the nucleus of the cell, where it binds to specific DNA sequences (glucocorticoid response elements [GRE]) in the promoter region of target genes. (103). Alternatively, the active GS-GR complex, as a monomer can interact directly with intracellular transcription factors, such as activating protein-1 or NF-κB, through a protein-protein interaction to attenuate pro-inflammatory processes mediated by transcription factors (103, 104). There is also evidence that glucocorticoid-mediated repression of inflammatory genes involves post-transcriptional and/or translational mechanisms. Thus, the repression of inflammatory genes, including cyclooxygenase (COX)-2, IL-8, and inducible nitric-oxide synthase, involves mechanisms that regulate mRNA stability (104).

Interactions between glucocorticosteroids and β_2-agonists

Glucocorticosteroids can modulate β_2-receptors and their function by several mechanisms: protection against desensitization and the development of tolerance, increased efficiency of receptor coupling, and protection against inflammation-induced receptor downregulation and uncoupling (103). Glucocorticosteroids may enhance β_2-adrenergic receptor function by stimulating the transcription of β_2-adrenoceptor protein via binding to GRE in the promoter region of the β_2-receptor gene (figure 2). Dexamethasone has been shown to increase β_2-receptor mRNA in human peripheral lung, and β_2-adrenoceptor-stimulated adenylyl cyclase activity and cAMP accumulation increase after glucocorticoid treatment (105). β_2-agonists in turn may induce glucocorticoid receptors (GRs) nuclear localization and enhance GR binding to its specific target DNA sequences (101, 106).

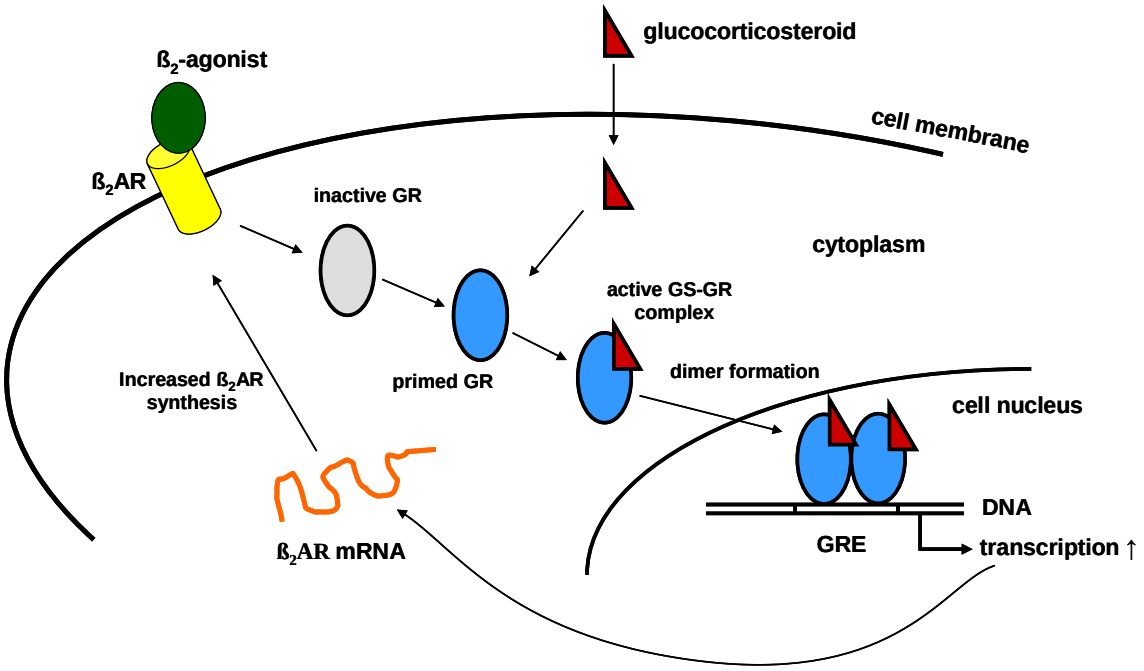

Figure 2. Proposed mechanism of action of the combination of a β_2-agonist and a glucocorticosteroid (GS). Binding of the β_2-agonist to the β_2-adrenoceptor (β_2AR) induces a conformational change (”priming”) of the glucocorticosteroid-receptor (GR) making it more sensitive, i.e. less GS is required for activation. GR forms a dimer that enters the cell nucleus and binds to the glucocorticoid responsive element (GRE) within the promoter region of the β_2-adrenoceptor gene. The result is an upregulation of β_2-adrenoceptor mRNA and protein.

Bronchial hyperresponsiveness

"Bronchial hyperresponsiveness" or "airway hyperresponsiveness" describes an exaggerated airway-narrowing response to many environmental triggers, such as allergen and exercise, and is characteristic of asthma. Bronchial hyperresponsiveness is measured with bronchial provocations using *direct* or *indirect* stimuli. Direct stimuli (methacholine, histamine) act directly on receptors on airway smooth muscle (muscarinic receptors, H_1 receptors). Methacholine is more commonly used than histamine as provocative stimulus. Methacholine challenge has a high sensitivity to identify bronchial hyperresponsiveness, and a negative test is often used to exclude asthma (107). One of the disadvantages with pharmacological bronchial provocation challenges, e.g. methacholine, is that even a number of non-asthmatics respond, i.e. the specificity is low (108) (figure 3). With the methacholine provocation method used in our studies, $PC_{20}FEV_1$ can be defined in almost 80% of healthy subjects (109).

The indirect stimuli act through one ore more intermediate pathways, most commonly acting via release of mediators from inflammatory cells. Indirect stimuli include physical stimuli (exercise, hyperventilation, cold air, non-isotonic aerosols, mannitol etc.) and certain chemical stimuli (adenosine monophosphate (AMP), propranolol, bradykinin, and tachykinins). The physiologic stimuli (dry air and hypertonic or hypotonic aerosols) may induce bronchoconstriction in subjects with asthma but elicit little if any effect in normal subjects (110), i.e. the specificity is high. It has been suggested that indirectly acting bronchial stimuli might better reflect the degree of airway inflammation than directly acting stimuli (111). Because naturally occurring asthma is associated with symptoms on exposure to indirect-acting stimuli, assessment of indirect airway hyperresponsiveness is clinically more relevant than direct challenges (112).

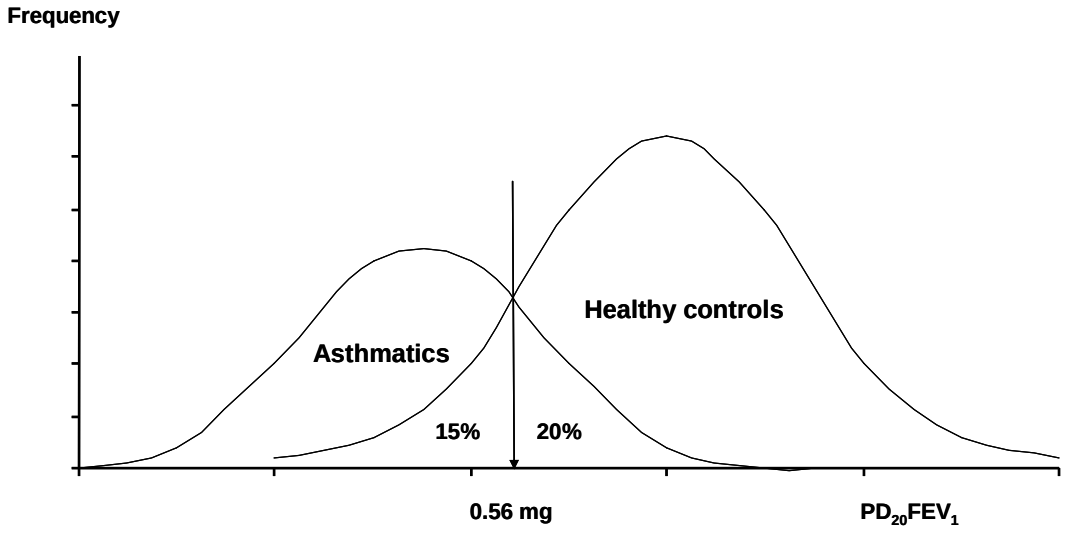

Figure 3. The distribution of bronchial responsiveness to methacholine in 203 healthy and 102 asthmatic subjects. Reference values for bronchial responsiveness to methacholine have been defined in our laboratory. The cut-off level for bronchial hyperresponsiveness was defined as based on the distribution of log $PD_{20}FEV_1$ for healthy and asthmatic subjects. The cut-off level was set at the point where the two distributions met, and was found to be 0.56 mg, corresponding to the 15th percentile in the healthy subjects and the 80th percentile in the asthmatic subjects. (Modified from Ehrs et al 2006)

Asthma

Asthma is one of the most common chronic diseases, with approximately 300 million individuals affected worldwide (www.ginasthma.com). Asthma is a clinical syndrome characterized by episodic reversible airway obstruction, increased bronchial responsiveness, and airway inflammation. Asthma results from complex interactions among inflammatory cells, their mediators, airway epithelium and smooth muscle, and the nervous system. Chronic inflammation of the airway wall is thought to be the primary factor that maintains the development of lesions that promote remodelling, excessive production of mucus and airway hyper-responsiveness that leads to acute and chronic limitations in airflow (113). Airway remodelling in asthma characteristically involves alterations in the bronchial epithelium, with shedding of columnar epithelial cells and goblet cell metaplasia leading to mucus plugging. The most prominent remodelling changes are observed beneath the altered epithelium: sub-basement membrane thickening involving deposition of interstitial collagen and smooth muscle hyperplasia (10). The classical IgE-associated allergic asthma phenotype starting in childhood is the most widely studied. In these patients, airway cells have a predominant T_H2 cytokine profile, i.e. producing IL-4, IL-5, IL-9 and IL-13 (15). In allergic asthma an increase in eosinophils in the tissues, blood, and bone marrow is a prominent feature. In severe persistent asthma though, there is an increase in neutrophil number both in sputum and peripheral blood (114) and the number of neutrophils is associated with asthma exacerbations (101, 115). Sputum neutrophilia combined with normal numbers of eosinophils is also a characteristic of non-atopic asthmatics, who respond less well to treatment with inhaled corticosteroids (116). Asthma presenting later in life is more often of the non-allergic, neutrophilic phenotype. A proportion of asthma patients develop disease which is relatively or totally refractory to glucocorticoid therapy, but this steroid resistance does not necessarily imply severe disease (117).

Aspirin intolerant asthma is a well defined clinical syndrome where asthma and chronic nasal problems such as rhinosinusitis and recurrent polyps are associated with intolerance to non-steroidal anti-inflammatory drugs (NSAIDs). Aspirin intolerant asthma affects 5-10% of asthmatic adults (118) who often suffer from a particularly severe form of asthma with extrapulmonary symptoms which in severe cases can progress into shock and respiratory arrest at exposure to NSAID (119). The hypersensitivity to common NSAIDs is not due to immunological reactions but rather the inhibitory effect of these drugs on cyclooxygenase (COX) (118, 120, 121). The effect of COX inhibitors in these patients is believed to be related to abnormal dependency on the anti-inflammatory action of prostaglandin (PG) E_2, resulting in mast cell activation on removal of PGE_2 by COX inhibitors (121). There are currently no biomarkers for aspirin intolerant asthma, but lysine-aspirin bronchoprovocation is used in clinical praxis for identification of this subgroup of asthma. This provocation method is safer than oral provocation and has a high degree of repeatability (119).

COPD

Chronic obstructive pulmonary disease (COPD) is characterized by airflow limitation that is not fully reversible, and usually progressive. The symptoms are cough, sputum production and dyspnea. Chronic obstructive pulmonary disease is currently the fourth most common cause of death, and is expected to be the third-leading cause of death in the year 2020 (122).

Cigarette smoking is undoubtedly the main cause of COPD in the population, although other inhaled noxious particles and gases may contribute (123).

Cough and sputum production may precede the development of airflow limitation, and some patients develop airflow limitation without chronic cough and sputum production (3). The diagnosis of COPD is based on lung function measurement. A post-bronchodilator ratio of forced expiratory volume in one second (FEV_1)/vital capacity (VC) or forced vital capacity (FVC) below 0.7 is required for the diagnosis (123). To avoid overestimation of the prevalence of COPD in elderly subjects, the 65/65 rule has been introduced in Sweden which implies that a post-bronchodilator FEV_1/VC ratio <0.65 is required for the COPD diagnosis in individuals older than 65 years (124).

Patients with COPD exhibit airway inflammation characterized by an increased presence of neutrophils and macrophages in the airways, and increased levels of IL-8 and TNF in induced sputum and bronchoalveolar lavage fluid correlate with the increased neutrophil accumulation (125-127). Airway neutrophilia is more pronounced in severe COPD than in mild disease (124). Although eosinophils and mast cells are important effector cells in asthma, neither has been ascribed a prominent role in COPD (11). It has been demonstrated that stable patients with a history of frequent exacerbations have increased induced sputum levels of IL-6 and IL-8, and that sputum levels of IL-6 further increase at COPD exacerbations (128).

Treatment of asthma and COPD

Treatment of asthma

β_2-Adrenoceptor agonists and glucocorticosteroids are cornerstones in asthma treatment. β_2-Adrenoceptor agonists are widely used as "reliever" medications in the treatment of acute bronchospasm (short-acting β_2-agonists), and long-acting β_2-agonists as an adjunct to anti-inflammatory therapy. Inhaled corticosteroids (ICS) reduce the levels of markers of airway inflammation in asthma patients, but these effects are lost once treatment is discontinued (129). The anti-inflammatory effects of ICS are expressed clinically by improved lung function, less asthma symptoms, and decreased rescue medication requirement. β_2-Adrenoceptor agonists and glucocorticosteroids used in combination are more effective than either alone (130-135). Clinical guidelines recommend adding a long-acting β_2-agonist to ICS therapy in patients with moderate-to-severe asthma (129), and β_2-agonists are now often dispensed in combination with inhaled steroid, for example as budesonide/formoterol or fluticasone/salmeterol.

Treatment of COPD

Current pharmacologic therapy lacks the ability to alter the progressive decline in lung function that is characteristic for COPD (136) but smoking cessation improves the natural course of COPD. The pharmacological treatment of COPD has three main purposes: to relieve symptoms, to prevent exacerbations and to be an aid in smoking cessation (124). To relieve the symptoms, long-acting bronchodilators are used, mainly an anticholinergic (tiotropium) and β_2-agonists (formoterol, salmeterol). Both long-acting β_2-agonists and tiotropium are effective bronchodilators in patients with COPD, but based on current literature, tiotropium provides greater benefit than long-acting β_2-agonists (136). There is added benefit of combination therapy of tiotropium and formoterol (137), and it is likely that a combination of long-acting anticholinergics and long-acting β_2-agonists will emerge as the therapy of choice

in the future. Inhaled glucocorticosteroids are used in COPD in combination with long-acting β_2-agonists mainly to prevent exacerbations, and have been shown to reduce all-cause mortality in COPD (130, 138). Acetylcysteine prevents exacerbations and days of illness (139, 140), but has also been shown to reduce the exacerbation rate only in patients not treated with inhaled corticosteroids (141). Pharmacological treatment may be supportive in smoking cessation programmes, and for this purpose nicotin replacement therapy, bupropion and varenicline are currently available (124).

Aims of the thesis

The general aim of this thesis was to elucidate the effect of long-acting β_2-agonists and glucocorticosteroids on airway inflammation and bronchial responsiveness. Specific aims were

- to study how long-acting β_2-agonists and glucocorticosteroids influence IL-6 and IL-8 release induced by organic dust in airway epithelial cells *in vitro,* and a possible interaction between these classes of drugs

- to study how a long-acting β_2-agonist and a glucocorticosteroid influence IL-6, IL-8 and GRO-α release from neutrophils, neutrophil chemotaxis, and chemokine receptor expression on neutrophils *in vitro*

- to examine a possible protective effect of a long-acting β_2-agonist on enhancement of bronchial responsiveness induced by exposure to airborne organic dust, and whether this effect is modified by a glucocorticosteroid or a COX-inhibitor

Material and Methods

The methods are briefly summarized below, detailed descriptions are provided in the individual papers.

Material

Cells

For the *in vitro* studies airway epithelial cells and isolated peripheral blood neutrophils were used. Two types of epithelial cells were used: alveolar type II cells from the human lung carcinoma epithelial cell line A549 (American Type Culture Collection, Rockville, USA) (study I) and primary bronchial epithelial cells isolated from bronchial tissue obtained from patients who underwent lobectomy or pulmectomy at the Karolinska University Hospital in Stockholm, Sweden (study I and II). Neutrophils (study III) were isolated from peripheral blood from non-smoking, non-allergic healthy donors.

Stimuli

Organic dust

Different samples of settled organic dust collected approximately 1.2 m above the floor in swine confinement buildings were used for stimulation of cells (study I and II). A freshly prepared suspension was used in every experiment. The dust was dissolved in cell culture medium at a concentration of 1 mg/ml and sonicated for 10 minutes. The dust suspension was then added to the respective well on the cell growth plates to a final concentration of 100 μg/ml.

Lipopolysaccharide

Commercially available lipopolysaccharide (LPS) extracted from Escherichia coli was used for stimulation of neutrophils at a concentration of 1 μg/ml (study III).

Cytokines and antibodies

Recombinant human IL-6, IL-8, TNF and GRO-α were used in the enzyme-linked immunosorbent (ELISA) assays (study I, II, III and IV) and chemotaxis experiments (study III). Monoclonal anti-human capture antibodies and biotinylated anti-human polyclonal detection antibodies were used in the ELISA assays. For the blocking experiments in the neutrophil migration assays (study III), monoclonal anti-human CXCR1 and monoclonal anti-human CXCR2 were used. For detection of CXCR1 and CXCR2 receptors by flow cytometry (study III), phycoerythrin (PE)-conjugated monoclonal mouse anti-human CXCR1 and CXCR2 antibodies and a PE-conjugated mouse IgG_{2A} isotype control were used.

Drugs

The following drugs were generous gifts: formoterol (study I, II and III) from AstraZeneca AB, Lund, Sweden; salmeterol (study I and IV), fluticasone proprionate (study IV) and placebo (study IV) from GlaxoSmithKline, Uxbridge, Middlesex, UK; sotalol (study I) from NM Pharma, Stockholm, Sweden; and ciprofloxacin (study I) from Bayer, Wuppertal, Germany. Budesonide (study II and III), propranolol (study I, II and III) and ibuprofen (Nycomed) (study IV) were purchased.

Chemicals

(Study III)

The acetomethoxy derivate of calcein (calcein AM) was used for labelling viable neutrophils to make them fluorescent for counting in a fluorescence plate reader.

A mixture of sodium diatrizoate and polysaccharide (Lymphoprep®) was used for separating neutrophils from lymphocytes by Ficoll centrifugation.

Methods

Subjects

All studies were approved by the local ethics committee and all subjects gave their informed consent to participate in the study. All subjects participating in study III and IV were healthy non-smokers with no history of allergic disease, asthma or other airway diseases.

Isolation of primary bronchial epithelial cells (PBEC)

(Study I and II)

Establishment of the PBEC was modified after van Wetering et al. (142). Bronchial tissues were obtained from patients who underwent lobectomy or pulmectomy due to lung cancer at the Karolinska University Hospital in Stockholm, Sweden. A piece of a central, macroscopically normal, human bronchus was excised and immediately put in ice-cold PBS buffer, trimmed, washed, and incubated with protease for 2 hours at 37 ºC. Epithelial cells were then gently scraped off from the luminal surface, washed and cultured in serum-free keratinocyte medium in 6-well plates at 37 ºC in humidified atmosphere of 5% CO_2 in air. During the first week of culture ciprofloxacin was added to the medium to assure the absence of *Mycoplasma*. After reaching confluence, the cells were trypsinized, centrifuged, dissolved in freezing medium and stored in liquid nitrogen.

Isolation of peripheral blood neutrophils

(Study III)

Heparinized whole blood (60 ml) was collected from healthy human donors, layered over a density gradient (Lymphoprep®) and spun at 600 g for 25 minutes. The polymorphonuclear cell (PMN) rich fraction was removed, washed with D-PBS and red blood cells were lysed

with distilled water. The neutrophils were washed twice with D-PBS buffer and resuspended in cell growth medium with 5% fetal bovine serum.

Neutrophil migration

(Study III)

The chemotaxis was performed as described by Frevert et al (143) with minor modifications. Calcein-labelled neutrophils were incubated for 20 min with formoterol, budesonide, budesonide and formoterol, anti-CXCR1, anti-CXCR2 or the combination of anti-CXCR1 and anti-CXCR2. Following the incubation, the neutrophils were directly added to the upper surface of a polycarbonate filter on the chemotaxis plate. The wells below the filter were filled with the applicable doses of IL-8 or GRO-α as chemoattractants, with or without drugs at the same concentration as in the neutrophil suspensions. Following incubation for 1 hour at 37 ºC the non-migrated cells and the filter was removed and the fluorescence of the migrated cells were read in a multi-well fluorescent plate reader.

Blood samples

(Study IV
Peripheral blood samples were allowed to coagulate at room temperature for 1 hour before centrifugation at 1000 *g* for 10 min. Serum was dispensed in aliquots kept at -70 ºC until analysis. Each sample underwent only one freeze-thaw cycle before assay.

Measurements of cytokines

Cell supernatants (study I, II, III), serum and sputum (study IV) were collected, centrifuged, aliquoted, and stored at -70 ºC. They underwent only one freeze-thaw cycle before analysis. IL-6, IL-8 and GRO-α concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available antibody pairs. TNF was analysed using commercial high sensitivity sandwich enzyme immuno-assay kits.

Measurements of receptor expression

(Study III)

Neutrophils were incubated with phycoerythrin (PE)-conjugated anti-CXCR1, anti-CXCR2 or mouse IgG2A as isotype control for 30 min on ice in the dark. They were then washed with PBS and analyzed by flow cytometery. Results were expressed as relative mean fluorescence intensity (rMFI) calculated as the ratio between the value for CXCR1 or CXCR2 and the isotype antibody value.

Lung function

(Study IV)

Spirometry (forced expiratory volume in 1 second, FEV_1 and vital capacity, VC) was measured using a wedge spirometer (Vitalograph®; Medical Instrumentation, Buckingham, UK) according to recommendations of the American Thoracic Society (144). Local reference

values were used (145, 146). Peak expiratory flow (PEF) was measured with a mini-Wright peak flow meter (Clement Clarke Ltd, London, UK).

Bronchial responsiveness

(Study IV)

Bronchial responsiveness was assessed by methacholine challenge by use of a drying device (147). After inhalation of the diluent (0.9% saline), increasing concentrations of methacholine were inhaled at 6 min intervals, starting at 0.5 mg/ml until FEV_1 had decreased by 20% or the highest concentration (64 mg/ml) was reached which corresponds to a $PD_{20}FEV_1$ of 25.5 mg.

Exhaled NO

(Study IV)

Exhaled nitric oxide (NO) was analysed with chemiluminiscence after reaction with ozone (NIOX®; Aerocrine, Stockholm , Sweden) and was assessed according to the recommendations of the American Thoracic Society (148).

Exposure to organic dust and endotoxin assessment

(Study IV)

The subjects were exposed to organic dust during a 3 hours stay in a swine confinement building, assisting the farmer in guiding pigs through a weighing box. This procedure generates a high concentration of airborne particles. In order to monitor the dust exposure, the subjects carried equipment with portable pumps and dust samplers. To sample inhalable (<10 μm) dust, IOM samplers (SKC Ltd, Blandford, England) were used. For sampling of respirable (<5 μm) dust, plastic cyclone samplers (Casella London Limited, Bedford MK42 7JY, England) were used. The sampling was performed at an airflow of 2.0 L/min for the IOM samplers and 1.9 L/min for the cyclone samplers. The filters in the dust samplers were weighed, extracted with sterile water and analyzed for endotoxin concentration by the use of a kinetic version of Limulus amebocyte lysate assay.

Sputum

(Study IV)

Sputum induction was performed according to in‘t Veen *et al*. with minor modifications. After inhalation of 400 μg salbutamol, sputum was induced by inhalation of increasing concentrations of saline using an ultrasonic nebuliser (De Vilbiss Ultraneb 2000: De Vilbiss Healthcare Worldwide, Somerset, PA). After each concentration the subjects blew the nose and rinsed the mouth with water, and were asked to cough deeply and expectorate sputum.

Results

Paper I

Effect of formoterol and salmeterol on IL-6 and IL-8 release from airway epithelial cells

The aim of this study was to find out whether long-acting β_2-agonists (formoterol and salmeterol) are capable of influencing cytokine release induced by organic dust from a swine confinement building.

Primary bronchial epithelial cells (PBEC) and A549 cells were characterized with immunostaining. Both cell types were positively stained with two different anti-cytokeratin antibodies unique for cells of epithelial origin, and negatively stained with antibodies directed against vimentin present in fibroblasts. Further characterization of PBEC demonstrated that the onset of IL-6 release in cells stimulated with organic dust from a pig house (100 µg/ml) preceded that of IL-8. Dose-response curves obtained after 24 hours incubation with dust at 0.1-100 µg/ml showed a significant release of IL-6 at the highest dust concentration only, while the release of IL-8 was significant at all dust concentrations (figure 4).

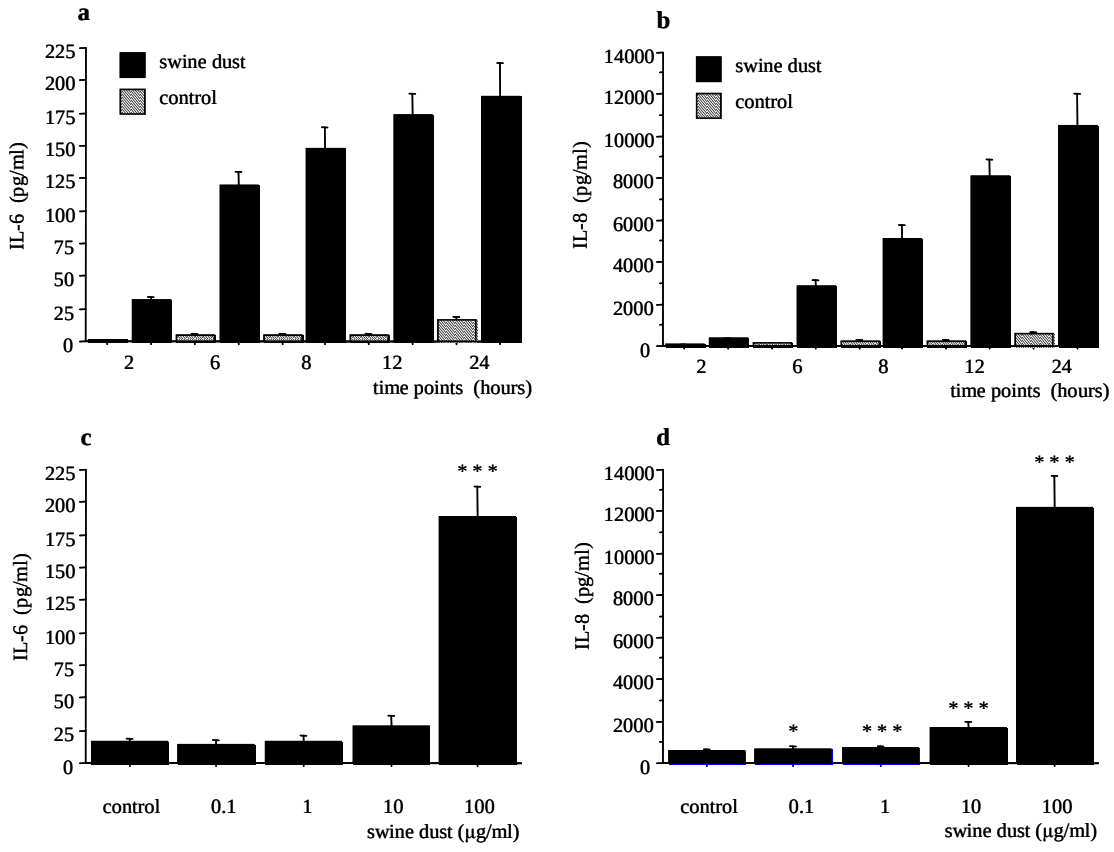

Figure 4. Time- (panels a and b) and dose-responses (panels c and d) of basal and dust-induced release of IL-6 and IL-8 in PBEC. The dust induced a significant (p<0.0001) enhancement of IL-6 (a) and IL-8 (b) release at all time points compared to the non-stimulated situation. The dust induced IL-6 and IL-8 release in a dose-response manner (0.1-100 µg/ml) with a steep increase of the response between 10 and 100 µg/ml (panel c, d). Data are expressed as mean and SEM. *p<0.05 and ***p<0.0001 compared with non-stimulated (negative) control.

A549 cells and PBEC were incubated for 24 hours with or without organic dust (100 µg/ml) from a swine house and formoterol or salmeterol, and cytokine release was assessed. In *non-stimulated A549 cells* formoterol did not affect the IL-6 release, whereas the IL-8 increased in a dose-response manner (figure 5a, b). Salmeterol increased both IL-6 and IL-8 release. Sotalol did not block the effect on IL-6 and IL-8 release induced by the two β-agonists. In *non-stimulated PBEC* formoterol and salmeterol increased the IL-6 and IL-8 release, with a peak at 10^{-8} M (figure 5c, d). The effect of formoterol and salmeterol was blocked with propranolol.

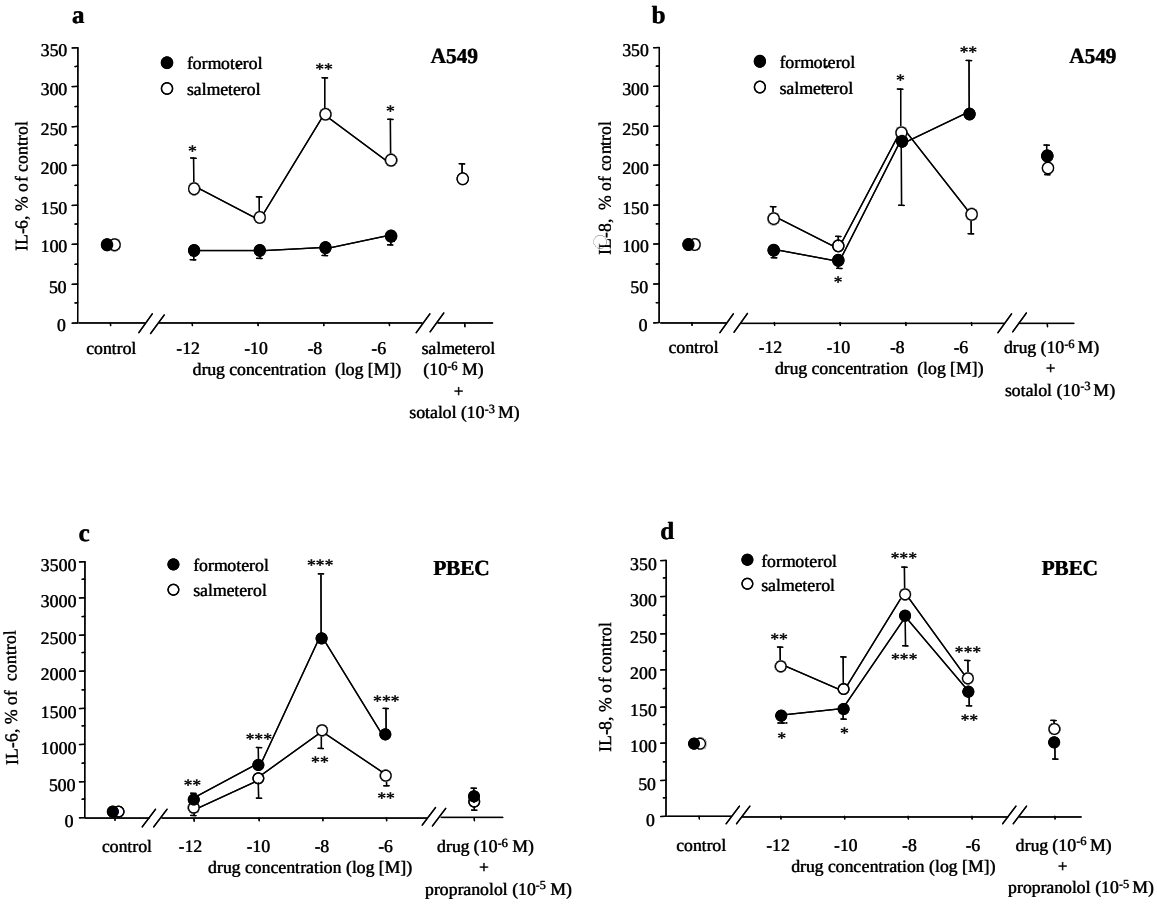

Figure 5. Dose-response curves for IL-6 (panel a, c) and IL-8 (panel b, d) release induced by formoterol and salmeterol (10^{-12}-10^{-6} M) in non-stimulated A549 cells (panel a, b) and primary bronchial epithelial cells (PBEC; panel c, d). The cytokine release is expressed as percent of control, i.e. non-stimulated cells in medium without drugs. Control values for A549 were 17.2 pg/ml (IL-6) and 163 pg/ml (IL-8. Corresponding control values for PBEC were 14.3 pg/ml (IL-6) and 460 pg/ml(IL-8). Data are expressed as mean and SEM. *<0.05, ** p<0.01 and ***p<0.001 compared with control.

In *dust-stimulated A549 cells* formoterol induced an enhancement of IL-6 and IL-8, while salmeterol did not influence the dust-induced IL-6 and IL-8 release (figure 6a, b). The effect of formoterol was not blocked with sotalol or propranolol. In *dust-stimulated PBEC* both formoterol and salmeterol enhanced dust-induced IL-6 release, the dose response curves being

bell-shaped (figure 6c). Formoterol and salmeterol induced a slight enhancement of dust-induced IL-8 release in PBEC although no dose-response relationship was observed (figure 6d). Propranolol blocked the β_2-agonist-induced enhancement of dust-induced IL-6 release.

In summary, formoterol and salmeterol induced IL-6 and IL-8 release and added to the effect of organic dust. The effects of formoterol and salmeterol were blocked with a β-blocker in PBEC but not in A549 cells. Only salmeterol induced IL-6 release, whereas both β_2-agonists induced IL-8 release in non-stimulated A549 cells, and only formoterol enhanced dust-induced IL-6 and IL-8 release in A549 cells.

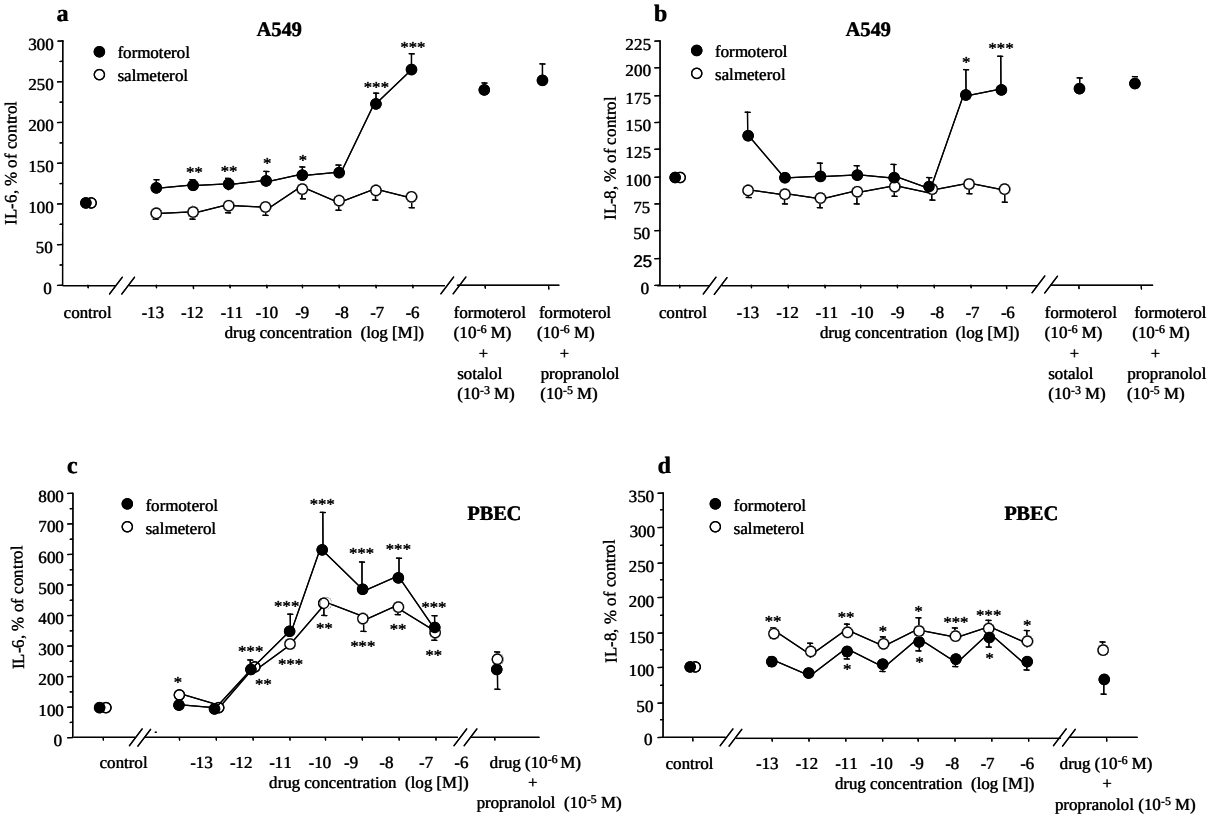

Figure 6. Dose-response curves for IL-6 (panel a, c) and IL-8 (panel b, d) release induced by formoterol and salmeterol (10^{-13}-10^{-6} M) in dust-stimulated A549 cells (panel a, b) and PBEC (panel c, d). The cytokine release is expressed as percent of control, i.e. cells stimulated with dust, without drugs. Control values for A549 were 118 pg/ml (IL-6) and 2230 pg/ml (IL-8). Corresponding values for PBEC were 316 pg/ml (IL-6) and 14.600 pg/ml (IL-8). * $p<0.05$ and ** $p<0.01$, and *** $p<0.001$ compared with control.

Paper II

Effect of budesonide and formoterol on IL-6 and IL-8 release in primary bronchial epithelial cells

The aim of this study was to explore possible interactions between a glucocorticosteroid (budesonide) and a long-acting β_2-agonist (formoterol) on pro-inflammatory cytokine (IL-6, IL-8) release. Primary bronchial epithelial cells (PBEC) were incubated for 24 hours with organic dust from a swine house and the effect of formoterol and budesonide on cytokine release was measured. Budesonide inhibited the release of IL-6 and IL-8 in a dose-response manner (figure 7). Formoterol enhanced dust-induced release of IL-6 but not IL-8. The dose-related inhibition of budesonide was sustained in the presence of formoterol, although there was approximately a 30% attenuation of the steroid effect in the presence of the β_2-agonist. The addition of formoterol made a left-shift of the budesonide dose-response curve. The formoterol effect on IL-6 release was blocked with propranolol.

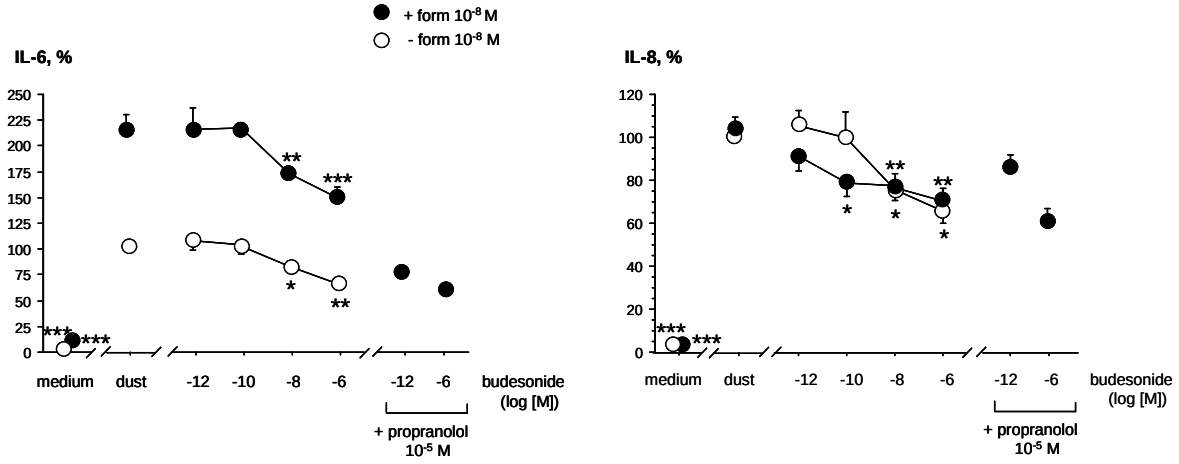

Figure 7. Effect of budesonide and formoterol on dust-induced IL-6 and IL-8 release. Dust (100 µg/ml) stimulated PBEC were incubated for 24 hours with budesonide10^{-12}-10^{-6} M, with or without formoterol 10^{-8} M. The levels of IL-6 differ at all budesonide concentrations ($P<0.001$) while the IL-8 curves are not significantly separated at any budesonide concentration. Results are presented in percentage of cytokine production induced by dust alone. Values after dust exposure (100%) correspond to 246 pg/ml for IL-6 and 16119 pg/ml for IL-8. Each point represents the mean ± SEM. *p<0.05, **p<0.01, and ***<0.001: within-group comparisons with positive control (dust).

Paper III

Effect of formoterol and budesonide on chemokine release, chemokine receptor expression and chemotaxis in human neutrophils

The aims of this *in vitro* study was to elucidate the effect of formoterol and budesonide on mechanisms of neutrophil migration. Drug effect on chemokine/cytokine release (IL-8, GRO-α, IL-6), regulation of chemokine receptors (CXCR1, CXCR2), and migration towards IL-8 and GRO-α in isolated human blood neutrophils from 10 healthy, non-smoking, non-allergic subjects (5 female, 5 male) were studied.

Non-stimulated or LPS (1 μg/ml) stimulated neutrophils were incubated for 8 hours with formoterol and/or budesonide and cytokine release was assessed. Formoterol or budesonide did not influence IL-6-, IL-8- or GRO-α release in non-stimulated neutrophils. In LPS-stimulated neutrophils, formoterol enhanced the IL-6- and IL-8 release in a dose-response pattern but did not affect the GRO-α release (figure 8a, c, e). Budesonide inhibited IL-6 and GRO-α release in a dose-response manner (figure 8b, f). A similar pattern, though not significant, was observed for budesonide effect with regard to IL-8 release (figure 8d). Formoterol enhanced the effect of budesonide on GRO-α release but not on IL-6 and IL-8 release. Formoterol-induced effects were blocked with propranolol.

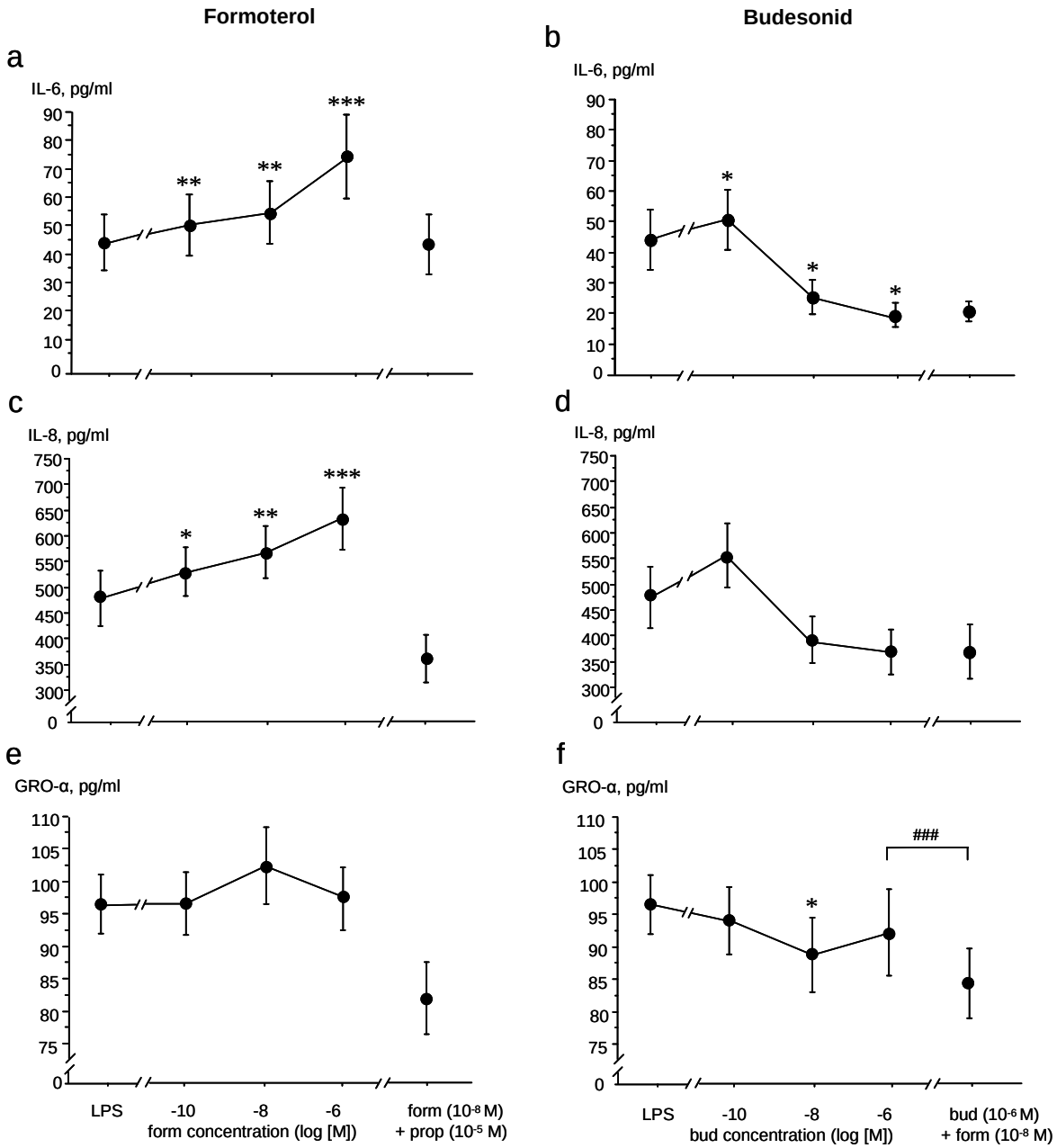

Figure 8. Effect of formoterol (a, c, e) and budesonide (b, d, f) on IL-6, IL-8 and GRO-α release from LPS-stimulated neutrophils from 10 healthy subjects. Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 indicate comparison with values after LPS stimulation.

Formoterol upregulated both CXCR1 and CXCR2 expression, whereas budesonide upregulated the expression of CXCR2 only (figure 9). Maximal drug effect was found already at the lowest concentration, and no further increase was obtained at higher concentrations. Formoterol did not add to the effect of budesonide, and the formoterol effects were blocked with propranolol. IL-8 decreased the expression of CXCR1 and CXCR2 by 52% and 76% respectively while GRO-α increased the CXCR1 expression by 13% and decreased the CXCR2 expression by 60% (figure 10).

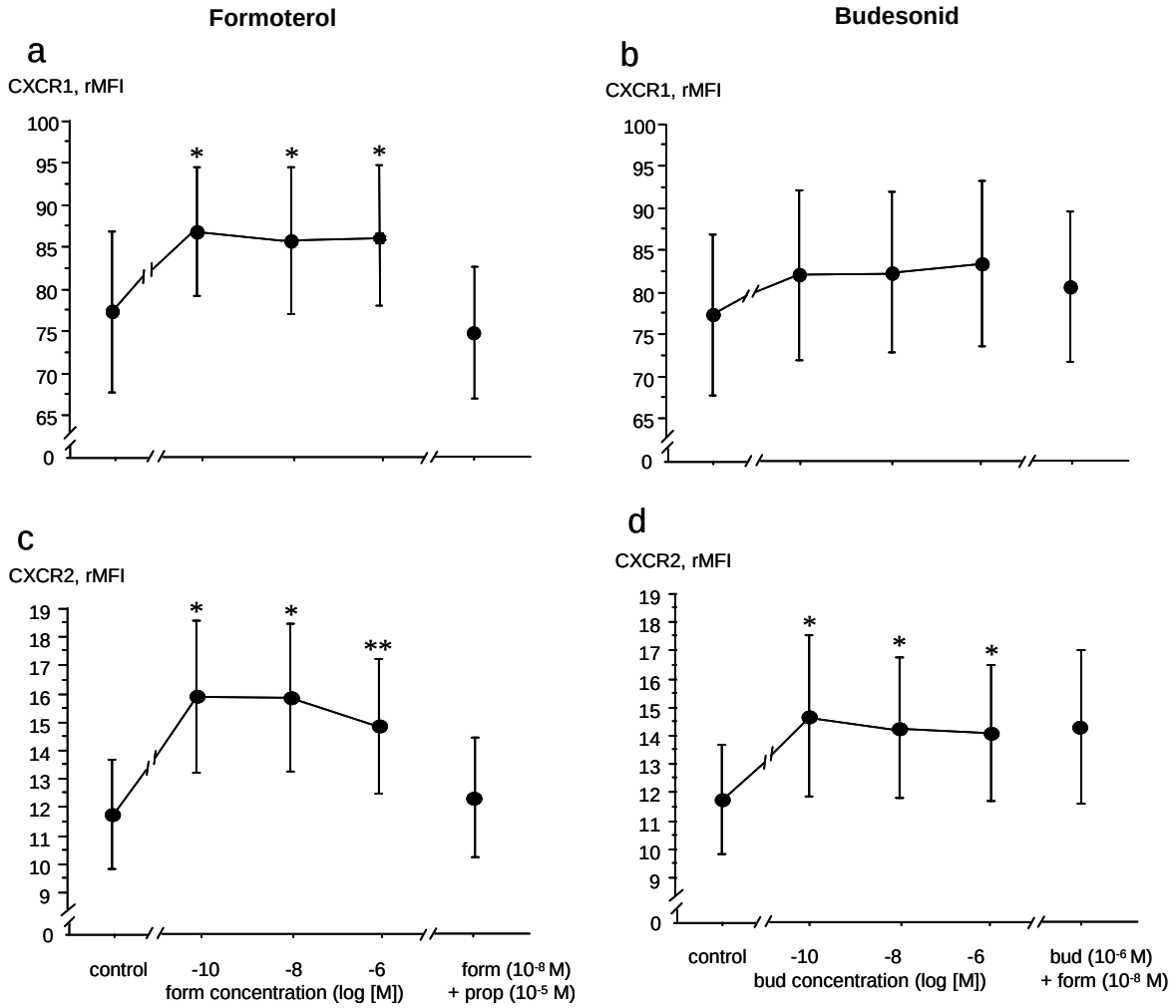

Figure 9. Effect of formoterol (a, c) and budesonide (b, d) on CXCR1 (a, b) and CXCR2 (c, d) expression on neutrophils from 10 subjects. Results are expressed as relative mean fluorescence intensity (rMFI) and presented as mean ± SEM. *P<0.05, **P<0.01 indicate comparison with control values.

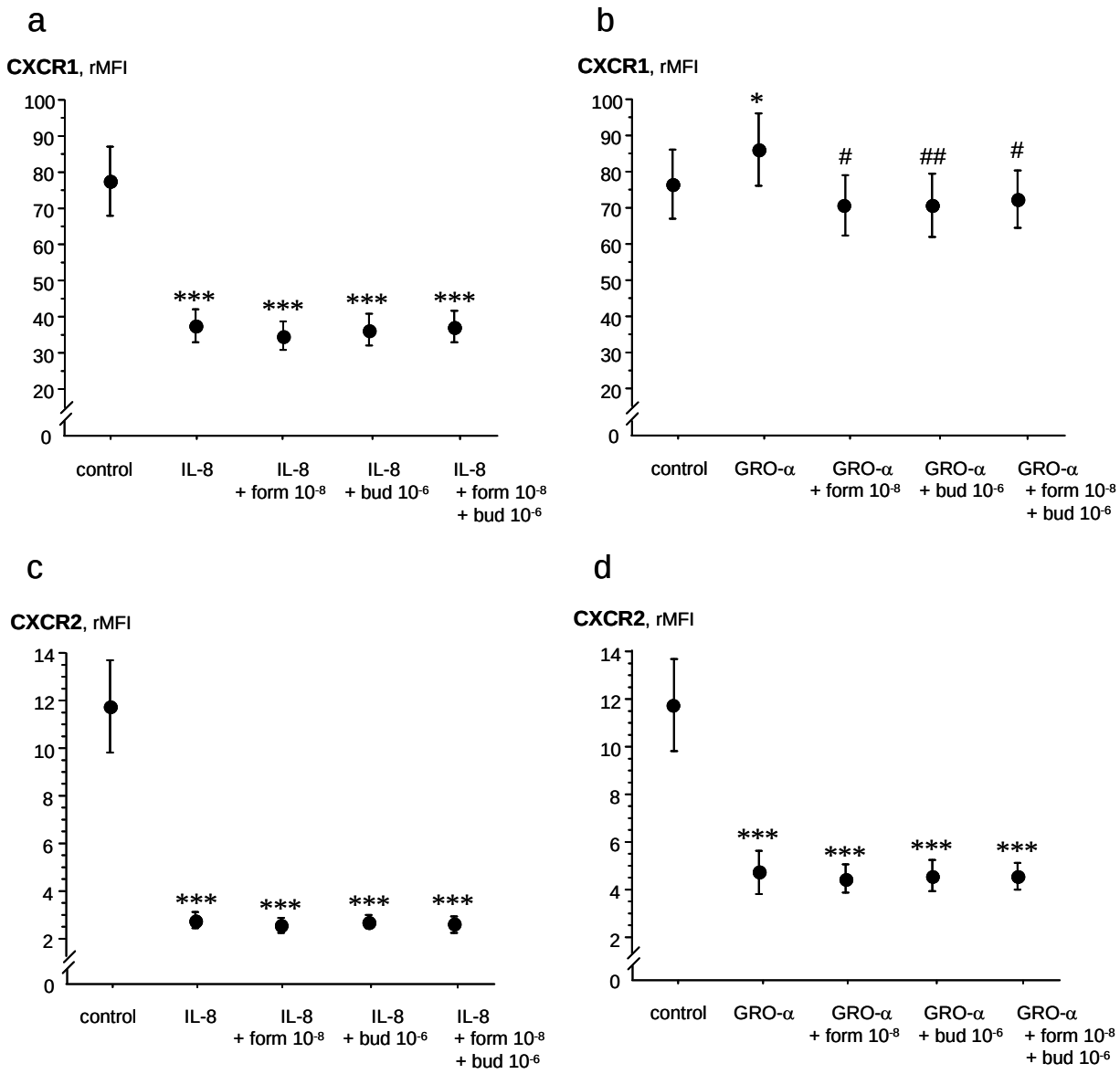

Figure 10. Effect of formoterol and budesonide on CXCR1 (a, b) and CXCR2 (c, d) down-regulation induced by IL-8 (a, c) and GRO-α (b, d) on neutrophils from 10 subjects. Results are expressed as relative mean fluorescence intensity (rMFI) and presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 indicate comparison with control values. #P<=0.05, ##P<0.01indicate comparison with GRO-α without drugs.

In the chemotaxis assays, IL-8 was a more efficient chemoattractant for neutrophils than was GRO-α, the migration rate being 43% and 17% of maximum migration. Neither formoterol nor budesonide, alone or in combination, influenced chemotaxis. The migration towards IL-8 was reduced by 32% in the presence of antibodies against both CXCR1 and CXCR2 while blocking only one of the receptors did not significantly influence migration. No significant blocking of migration with CXCR1 and CXCR2 antibodies was observed when GRO-α was used as chemoattractant.

Paper IV

Fluticasone and ibuprofen do not add to the effect of salmeterol on organic dust-induced airway inflammation and bronchial hyper-responsiveness

The main aim of this study was to examine whether a glucocorticosteroid (fluticasone) or a cyclo-oxygenase-inhibitor (ibuprofen) influences the protective effect of salmeterol on the increased bronchial responsiveness and the acute inflammatory response in healthy subjects after exposure in a pig barn. Twelve healthy, non-smoking, non-atopic subjects were included in this randomized, double-blind cross-over-designed study. The subjects were exposed to organic dust for 3 hours in a swine confinement building at four occasions separated by a 2-3 weeks wash-out period. During one week prior to each exposure, the subjects were treated with inhaled fluticasone, oral intake of ibuprofen, or inhalation/oral intake of placebo (table 2). Lung function, bronchial responsiveness to methacholine and inflammatory markers (IL-6 in blood; IL-6, IL-8 and TNF in sputum; exhaled NO) were evaluated before and after the organic dust exposure. The subjects indicated five general and seven airway specific symptoms on a visual analogue scale (VAS) before and 7 hours after exposure.

Table 2.

Treatment regimens

	Treatment during one week prior to exposure				A single dose, inhaled one hour prior to exposure	
	Inhalation		**Oral intake**		**Inhalation**	
	Fluticasone	**Placebo**	**Ibuprofen**	**Placebo**	**Salmeterol, 50 μg**	**Placebo**
Placebo/placebo		2 inh b i d		1 tabl b i d		2 inh
Placebo/salmeterol		2 inh b i d		1 tabl b i d	2 inh	
Fluticasone/salmeterol	500 μg b i d			1 tabl b i d	2 inh	
Ibuprofen/salmeterol		2 inh b i d	600 mg b i d		2 inh	

The results in the present study did not confirm our previous findings of a lack of protection of salmeterol against exposure-induced increase in bronchial responsiveness (149). We therefore conducted a retrospective analysis of pooled data from four previous studies of bronchial responsiveness before and after exposure in a pig barn in healthy subjects in order to find out how baseline pre-exposure bronchial responsiveness influences the increase in bronchial responsiveness induced by exposure in a pig barn. The aim of this separate, retrospective, analysis was to better understand the discrepancy between the present and previous results.

The maximal increase of post-exposure body temperature did not differ between treatments. There was a difference in exposure-induced symptoms between the different periods and, in general, exposure induced most symptoms during the fluticasone/salmeterol treatment period. Cough was the most prominent symptom irrespective of treatment.

Exposure in the pig house caused a slight but significant impairment of FEV_1 and VC following placebo/placebo treatment (table 3). The fall in FEV_1 and VC tended to be more pronounced after fluticasone/salmeterol than in placebo/salmeterol and ibuprofen/salmeterol.

Table 3.

FEV_1 and VC 2 weeks before and 7 hours after exposure in a pig barn. Mean ± SEM.

	Pre-exposure No medication	Post-exposure Change in % of pre-exposure				
	(% of predicted value)	Placebo/ placebo	Placebo/ salmeterol	Fluticasone/ salmeterol	Ibuprofen/ salmeterol	Difference between groups
FEV_1	97,9± (8.8)	-6.6±3.9***	-1.0±3.5 †††	-3.6±5.6 *†	-3.2±6.2	F=4.9 P=0.003
VC	95.4± 8.1	-3.7±4.4 **	-2.1±3.1 *	-4.4±4.4 **§	-2.5±4.9	F=6.0 P=0.0007

n=12 except for placebo/salmeterol treatment where n=11
* p<0.05, ** p<0.01, *** p<0.001 indicate comparison with pre-exposure value.
† p<0.05, ††† p<0.001 indicate comparison with placebo/placebo.
§ P<0.05 indicates comparison with placebo/salmeterol.

A significant decrease in PEF was found after placebo/placebo treatment only, and post-exposure PEF was significantly lower after placebo/placebo than after the other treatments (figure 11).

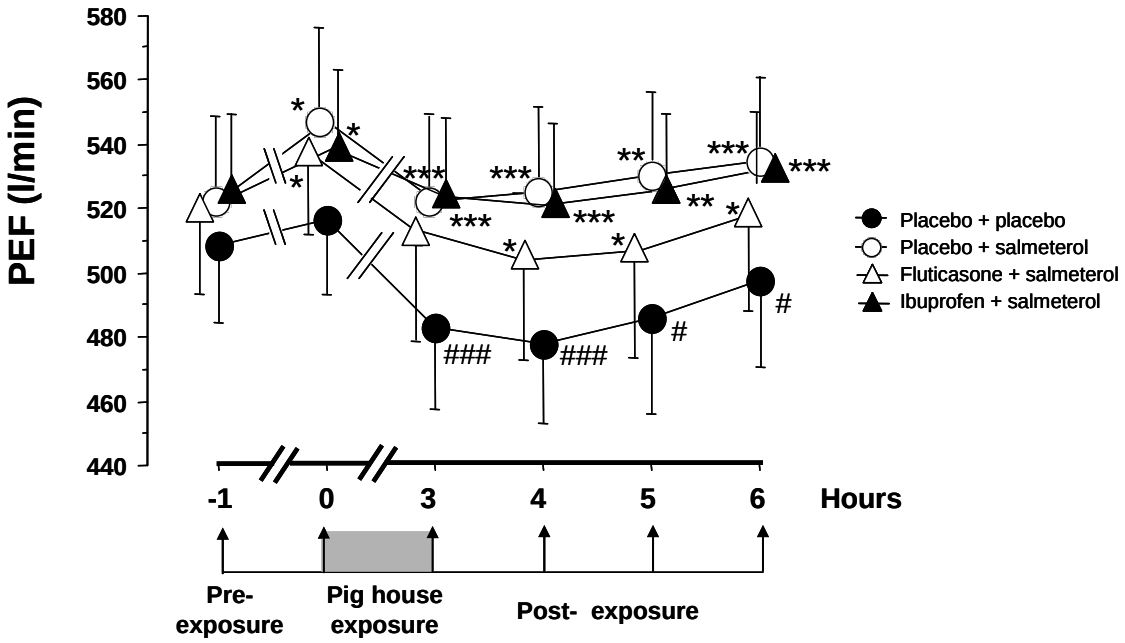

Figure 11. Peak expiratory flow (PEF) before and after 3 hours exposure in a pig barn. n=12 except for placebo/salmeterol treatment where n=11. Mean and SEM. There was no significant difference between the three periods when salmeterol was inhaled prior to exposure. *p<0.05, ** p<0.01, *** p<0.001 indicate comparison with placebo/placebo. # p<0.05, ### p<0.001 indicate comparison with one hour post-drug, pre-exposure value, i e at time point 0 hours.

Exposure in the pig barn induced a significant enhancement of bronchial responsiveness to methacholine after placebo/placebo, fluticasone/salmeterol and ibuprofen/salmeterol but not after placebo/salmeterol when compared with pre-exposure values (figure 12a). Compared with placebo there was a significant protection in all other three arms. Post-exposure $PD_{20}FEV_1$ did not differ between the three periods with pre-exposure salmeterol inhalations. Pig house exposure caused increased levels of exhaled NO after placebo/placebo and fluticasone/salmeterol treatment (figure 12b). Placebo/salmeterol and ibuprofen/salmeterol treatment resulted in significantly lower exhaled NO levels compared with placebo/placebo treatment while fluticasone/salmeterol treatment did not differ significantly from placebo/placebo. Post-exposure IL-6 in serum increased after all periods with no significant differences between treatments (figure 12c).

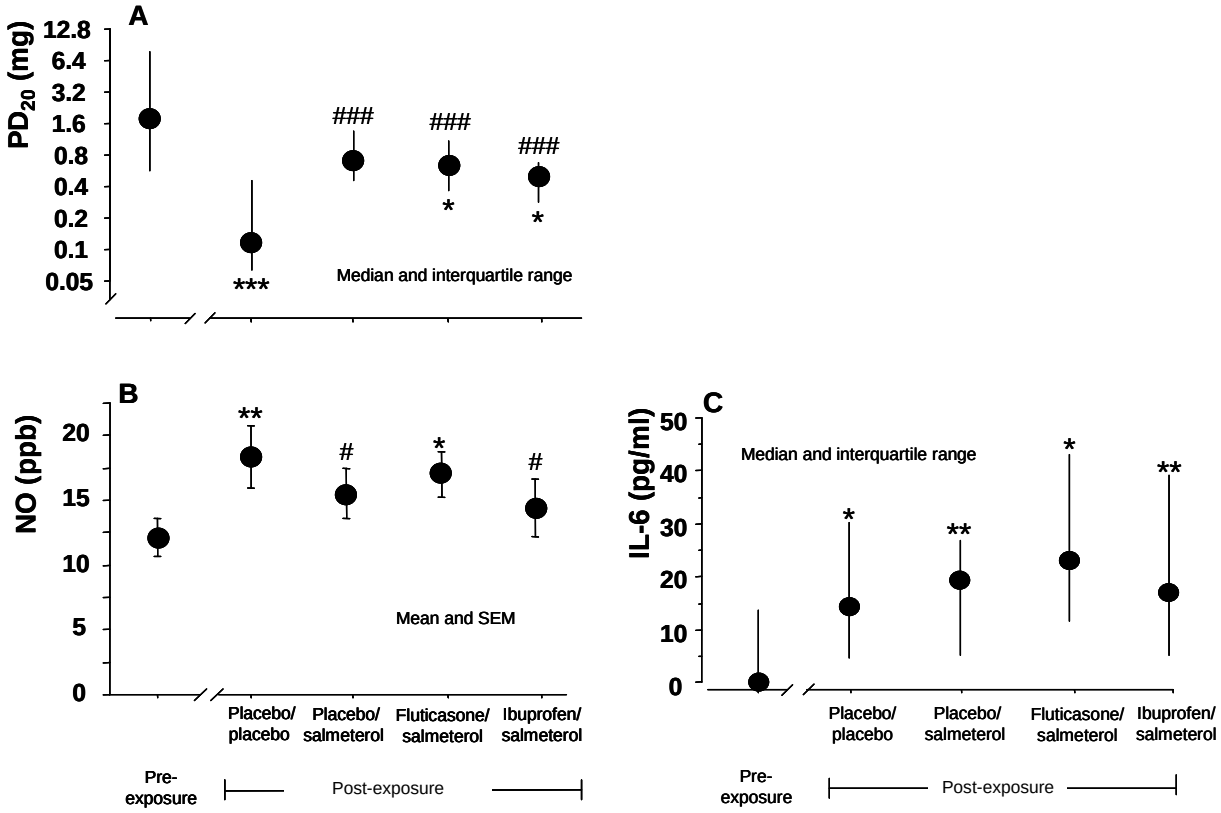

Figure 12. Bronchial responsiveness to methacholine, levels of exhaled nitric oxide (NO) and IL-6 serum levels before and after exposure in a pig barn. n=12 except for placebo/salmeterol treatment where n=11. $PD_{20}FEV_1$ before exposure was 1.86 (0.5**6**-7.16) mg. Pre-exposure level of exhaled NO was 12.4±1.60 ppb. Pre-exposure blood level of IL-6 was 1.5 (1.5-11.6) pg/ml. ∗ $p<0.05$, ∗∗ $p<0.01$, ∗∗∗ $p<0.001$ indicate comparison with pre-exposure values. # $p<0.05$, ### $p<0.001$ indicate comparison with placebo/placebo treatment.

In sputum the total number of cells and the number of neutrophils increased after exposure. Sputum cell count did not differ between treatments (figure 13). Exposure induced increase of IL-6, IL-8 and TNF levels in sputum irrespective of treatment with no significant differences between treatments (figure 14).

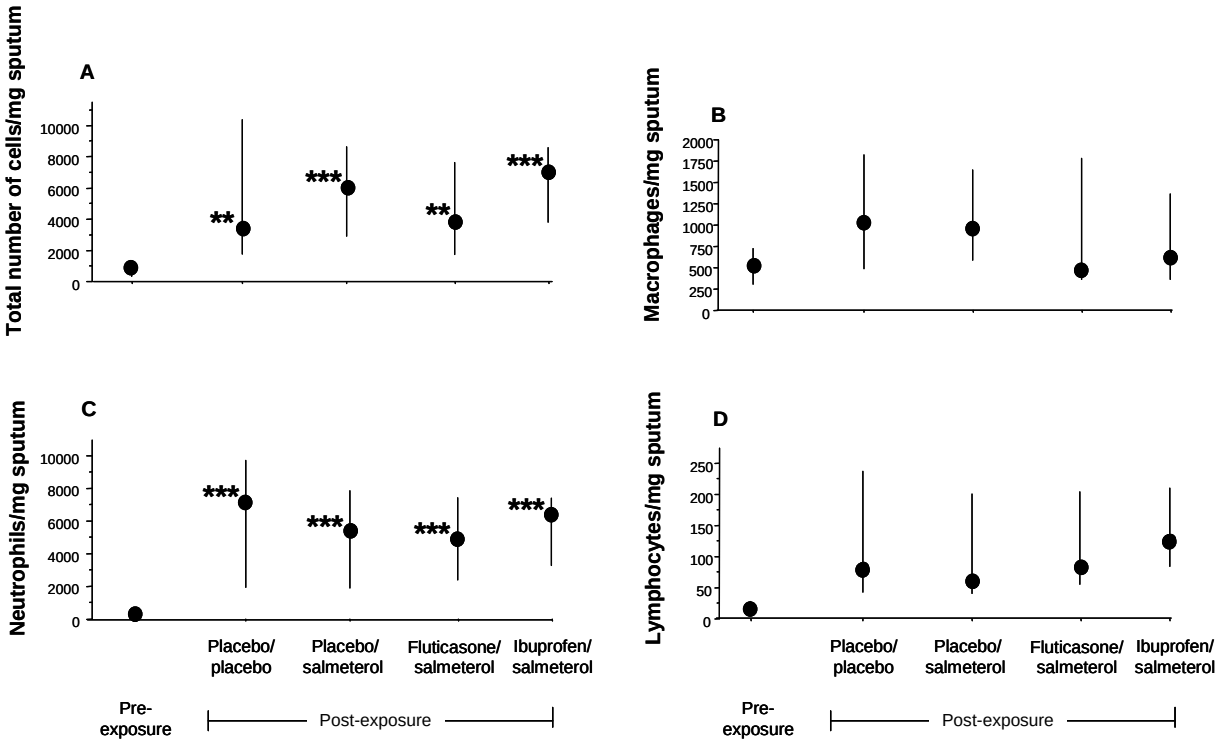

Figure 13. Cells in sputum before and after exposure in a pig barn. n=11.Pre-exposure values (median and 25th to 75th percentiles): Total cell number 804 (482-1119)cells/mg sputum, macrophages 515 (298-713) cells/mg sputum, neutrophils 78 (44-379) cells/mg sputum and lymphocytes 14 (7.6-26) cells /mg sputum. No differencebetween treatments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ indicate comparison with pre-exposure values.

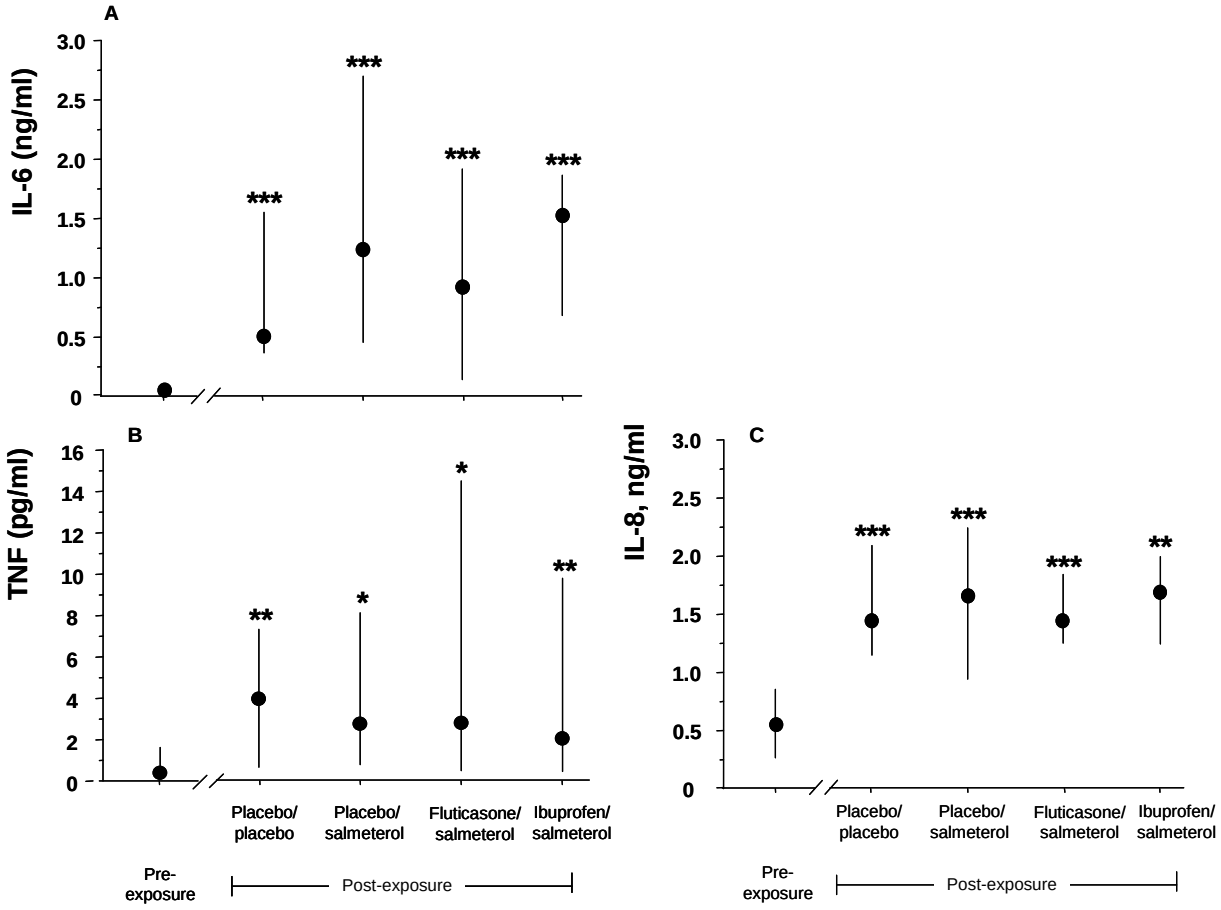

Figure 14. Cytokines in sputum before and after exposure in a pig barn. n=12 for IL-6 and IL-8 except for the placebo/ salmeterol period where n=11. Pre-exposure values (median and 25th to 75th percentiles): IL-6 28 (12-59) pg/ml, IL-8 541 (302-821) pg/ml, TNF 0.30 (0.30-1.6) pg/ml. * p<0.05, ** p<0.01, *** p<0.001 indicate comparison with pre-exposure values.

Bronchial responsiveness (retrospective data)

There was a weak correlation ($r^2 = 0.29$; $y = 0.12x + 2.72$) between post-exposure increase in bronchial responsiveness, calculated as doubling of the $PD_{20}FEV_1$, and pre-exposure bronchial responsiveness (figure 15a), whereas the correlation between pre-exposure $PD_{20}FEV_1$ and the pre- and post-exposure difference in $PD_{20}FEV_1$ was high ($r^2 = 0.995$; $y = 0.94x – 0.10$) (figure 15b). This excellent correlation is explained by the fact that post-exposure bronchial responsiveness ends up at a similar level in all subjects irrespective of pre-exposure $PD_{20}FEV_1$-level (figure 15c). There is, however, a correlation between pre-and post-exposure bronchial responsiveness and those with a high pre-exposure $PD_{20}FEV_1$ is also exhibiting a slightly higher $PD_{20}FEV_1$ after exposure (figure 15d).

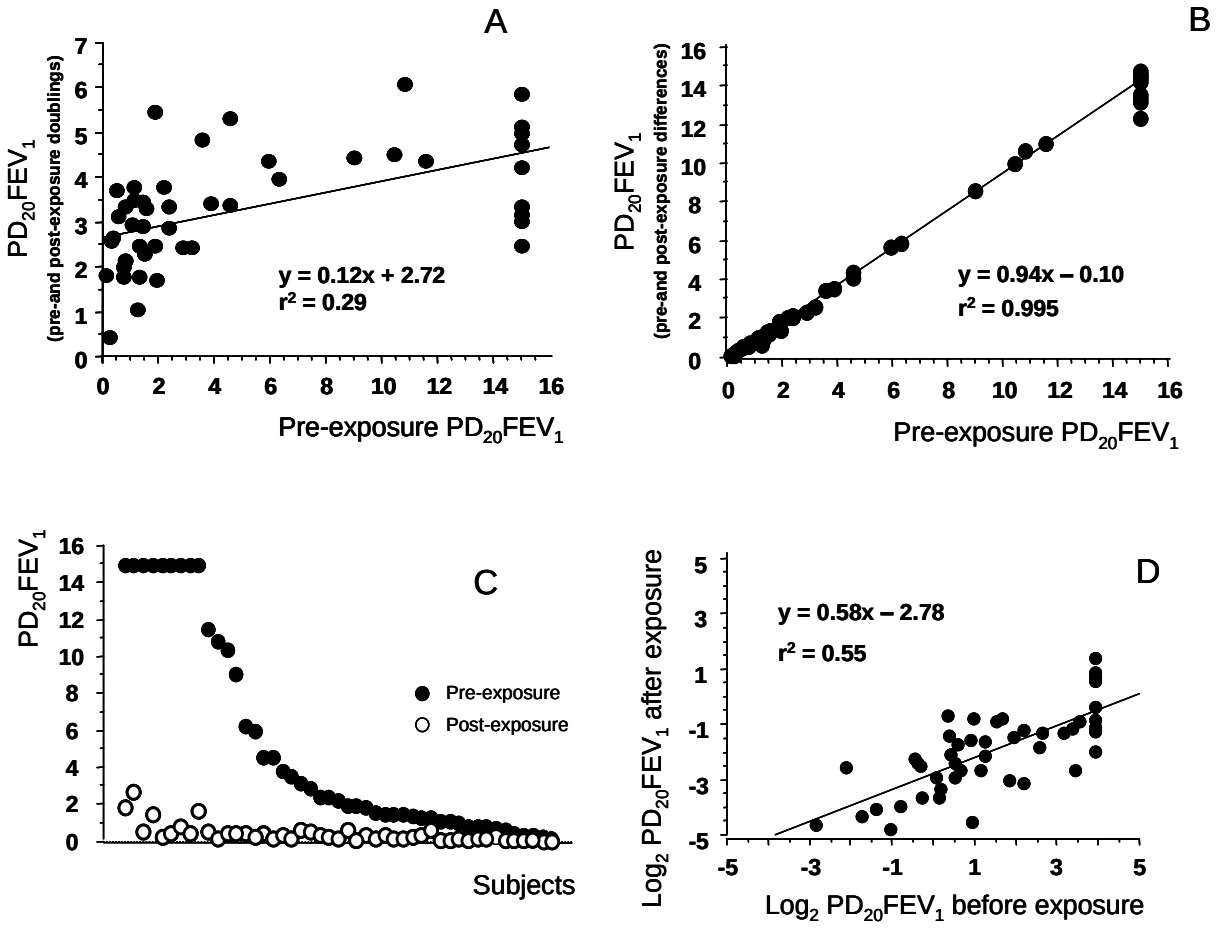

Figure 15. A. Relation between pre-exposure $PD_{20}FEV_1$ and exposure-induced doubling enhancement of bronchial responsiveness in 47 healthy subjects. r = 0.54. B. Relation between pre-exposure $PD_{20}FEV_1$ and the difference between pre- and post-exposure $PD_{20}FEV_1$ in 47 healthy subjects. r = 0.997. C. $PD_{20}FEV_1$ before and after 3 hours of exposure in a pig barn in 47 healthy subjects. Filled circles represent pre-exposure and open circles post-exposure $PD_{20}FEV_1$ in the same subject along a vertical line. Pre-exposure $PD_{20}FEV_1$ was <0.56 mg in 5 subjects. Post-exposure $PD_{20}FEV_1$ was >0.56 mg in 8 subjects. D. Relation between pre-and post-exposure $PD_{20}FEV_1$ in 47 healthy subjects. r = 0.76. Data are log_2 transformed.

Discussion

β_2-Agonists induced IL-6 and IL-8 release in non-stimulated PBEC and A549 cells. Furthermore, the β_2-agonists added to the stimulating effect of swine house dust on IL-6 and IL-8 release in A549 cells and PBEC, and to the stimulating effect of LPS on the release of IL-6 and IL-8, but not GRO-α, in neutrophils. The β_2-agonist effects were blocked by a β-blocker in PBEC and in the neutrophils, but not in the A549 cells indicating that the drug effects observed in A549 cells were not β_2-adrenoceptor mediated. Thus, although A549 cells possess β_2-adrenoceptors (150) and are capable of increasing intracellular levels of cyclic AMP following stimulation with forskolin (151), the coupling between the receptor and the effector in A549 cells seems to fail as far as IL-6 and IL-8 release are concerned (figure 16).

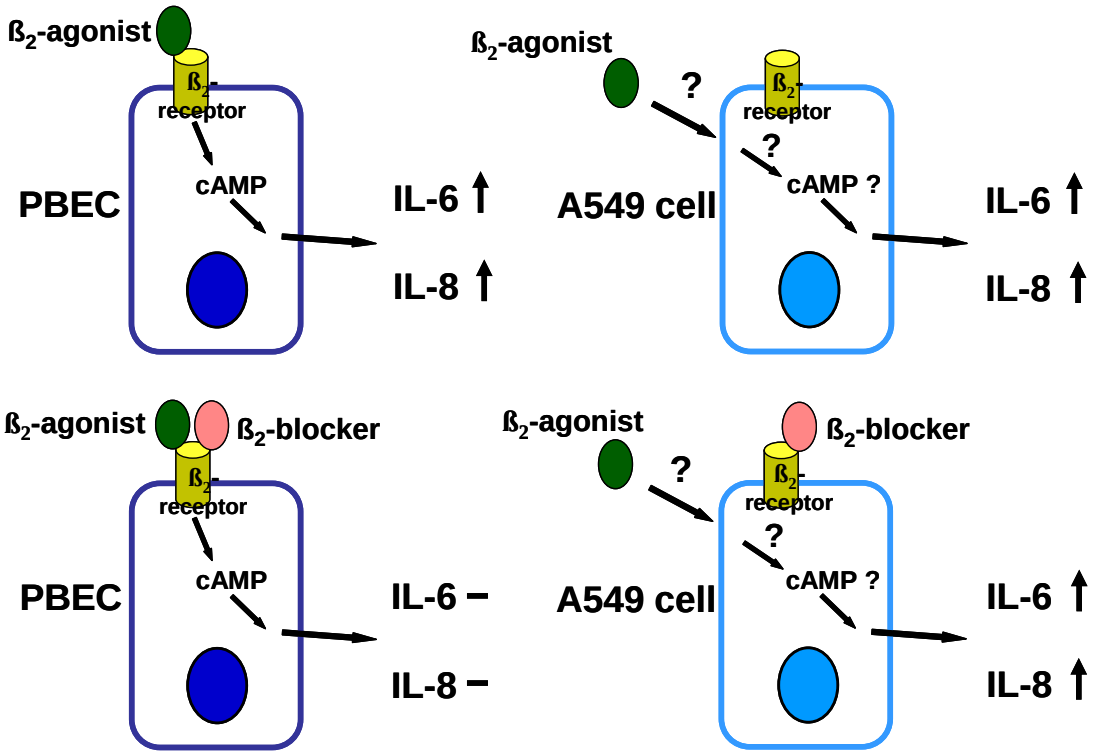

Figure 16. The long-acting β_2-agonists formoterol and salmeterol induced IL-6 and IL-8 release in primary bronchial epithelial cells (PBEC) and A549 cells. ↑ = increased release, – = unchanged release. These effects were blocked with a β-blocker (propranolol) in PBEC but not in A549 cells indicating that the β_2-agonists effects observed in A549 cells were not β_2-adrenoceptor mediated. Thus, what signalling pathway is used, and whether cAMP is the second messenger in A549 cells in this context is not clear.

The signal transduction induced by β_2-adrenoceptor activation may be altered in this transformed cell line (A549) while being intact in normal bronchial epithelium (PBEC) and peripheral blood neutrophils. There was almost no difference between formoterol and salmeterol induced IL-6- and IL-8-release in PBEC, whereas the difference between these drugs was pronounced in A549 cells. The reason for this discrepancy may be that the effects

were β_2-adrenoceptor mediated in PBEC, thus using a common signalling pathway (with cAMP as second messenger), while the drug effects in A549 cells seem to be mediated by other signalling pathways, likely different for formoterol and salmeterol. The cell membrane-stabilizing effect demonstrated for salmeterol but not for formoterol (89) could be another explanation for the discrepancies in drug effect on IL-6 and IL-8 release. Whether this discrepancy between formoterol and salmeterol is specific for A549 or also exists in normal human type II alveolar cells is not clear. It seems reasonable though, to assume variations in drug responsiveness of different epithelial cell types *in vivo* as well.

In dust-stimulated PBEC it was shown (study I) that the onset of IL-6 release preceded that of IL-8, a finding in accordance with a previous study (152). In that study mRNA expression for IL-6 peaked at 1-1.5 hours and then levelled off, whereas the IL-8 mRNA expression remained elevated. The cytokine levels assessed in our *in vitro* studies reflect the cumulative release induced by the β_2-agonists during 24 hours. As time kinetic experiments were not performed, it could not be decided whether cytokines were released continuously during the incubation or if the release levelled off with time.

The reason for the bell-shaped dose-response relationship observed for the β_2-agonists in airway epithelial cells is not clear. Desensitization due to long exposure time may limit the effect of the agonists, the cell type and the outcome measures (153). This would likely result in a reduced cumulative cytokine release during 24 hours of incubation, and should be more pronounced at higher drug concentrations. These bell-shaped dose-response curves for the β_2-agonist were not observed in the experiments on neutrophils (study III), which may be due to the shorter incubation time used (8 instead of 24 hours).

Our findings regarding the influence of β_2-agonists on chemokine release *in vitro* are in agreement with earlier findings demonstrating a β_2-agonist-induced increase in IL-8 release from TNF-stimulated human bronchial epithelial cells (72, 73) and airway smooth muscle cells (74). There are other findings, though, showing a suppressive effect of formoterol and salmeterol on the secretion of IL-8 from TNF-stimulated A549 cells (154). The reason for the discrepancy between that study and the others is not clear.

An enhancement of β_2-adrenoceptor agonists on cytokine production and release induced by pro-inflammatory agents may possibly have clinical implications and contribute to the explanation why β_2-agonists not always have a beneficial effect during asthma exacerbations. Bronchial biopsies from formoterol treated atopic asthmatics show a significant decrease in eosinophils and the epithelial expression of activated NF-κB, but no reduced immunoreactivity for cytokines (95). However, salmeterol has been shown to reduce the numbers of neutrophils in bronchial biopsy samples (155) and IL-8 in bronchoalveolar lavage fluid from asthmatics (156). Several data indicate that long-acting agents like salmeterol and formoterol increase cAMP in neutrophils and therefore inhibit adhesion, accumulation, activation, and induce apoptosis. The final result is a possible reduction in the number and activation status of neutrophils in airway tissue and in the airway lumen (81). Both *in vitro* and *in vivo* studies are somewhat contradictory, indicating a great complexity in this field. The implication is that even though β_2-agonists lead to an increased release *in vitro* of cytokines considered as pro-inflammatory, these drugs may not have pro-inflammatory effects *in vivo*.

The cytokine/chemokine release was attenuated by a glucocorticosteroid (budesonide) in PBEC (study II) and in peripheral blood neutrophils (study III). Budesonide was less potent in inhibiting IL-6 and IL-8 release in PBEC than has been previously demonstrated in dust-stimulated A549 cells (63). The inhibition of IL-6 and IL-8 release with budesonide 10^{-6} M was 37% and 41% respectively in PBEC compared to 80% and 72% in A549 cells incubated

with budesonide 10^{-9} M. Dexamethasone has been shown to be a less effective inhibitor of defensine-induced IL-8 release in PBEC than in A549 cells (142). It is not clear whether the alveolar epithelium is more sensitive to steroids than is bronchial epithelium *in vivo*, but if this is the case, it might implicate that even smaller amounts of inhaled steroids reaching the very distal parts of the airways could induce biological effects.

One of the main aims of these *in vitro* studies was to explore a possible synergism/additive effect between a glucocorticosteroid and a β_2-agonist. This could, however, not be demonstrated except from the effects on GRO-α release from LPS-stimulated neutrophils where formoterol added to the inhibitory effect of budesonide (study III). Although synergistic effects between β_2-agonists and glucocorticosteroids have been shown (74, 101, 106), there are other *in vitro* findings rather supporting antagonistic actions between β_2-agonists and glucocorticosteroids, both on molecular and cellular levels (157, 158). An additive inhibitory effect with formoterol and budesonide was observed on GM-CSF, but not on IL-8 production in IL-1β-stimulated human lung fibroblasts (92). Previous findings have thus come to different results, and whether an additive/synergistic or even an antagonistic effect between β_2-agonists and glucocorticosteroids is obtained may differ in different cell types and is likely depending on which inflammatory markers have been assessed.

Formoterol upregulated both CXCR1 and CXCR2 expression, whereas budesonide upregulated the expression of CXCR2 only. The formoterol effects were blocked with propranolol indicating β-adrenoceptor mediated effects. The β_2-agonist and the glucocorticoid did not influence the downregulation of CXCR1 and CXCR2 induced by their respective agonists, IL-8 and GRO-α. We found that GRO-α upregulated CXCR1, a phenomenon that has been demonstrated also by others (159). This upregulation of CXCR1 expression by GRO-α was abolished by formoterol and/or budesonide. Considering that CXCR1 (but not CXCR2) is thought to be important in the respiratory burst and the activation of phospholipase D at inflammatory sites (160), this finding might be of clinical importance.

Despite the clear effects of formoterol and budesonide on chemokine release and chemokine receptor expression, no influence of these drugs on chemotaxis was demonstrated. Our results partly challenge previous findings showing that β_2-agonists reduce neutrophil chemotaxis (161-163). Cyclic AMP has been shown to be a potential inhibitor of neutrophil migration, and stimulation of adenylyl cyclase or inhibition of phosphodiesterase attenuates chemotaxis of neutrophils (164, 165). On the other hand, Straub et al found a stimulation of monocyte chemotaxis by β_2-agonists (166). The inhibitory effects of cAMP on chemotaxis have been shown to be dependent upon the elevating agent and the chemoattractant as indicated in a previous study (167). Thus, it is far from clear how β_2-agonists influence cell migration *in vitro*, and the situation is of course even more complicated *in vivo*. Furthermore, cell density may influence migration rate as shown in a study on mouse macrophages where a certain density of cells was required for migration; as the density of cells increased a larger fraction migrated, suggesting that the cells interact (168). After activation, chemokine receptors become either partially or totally desensitized to repeated stimulation with the same or other agonists. Desensitization may be critical for maintaining the capacity of the cell to sense a chemoattractant gradient (169).

The concentrations of formoterol and budesonide used in our *in vitro* studies are similar to what have been found in the airways during treatment in a clinical setting (170-172). Formoterol has been shown to reduce sputum IL-8 and neutrophil numbers in mild asthmatic patients (173). One might speculate that β_2-agonist-induced IL-8 release from activated neutrophils makes them less inclined to migrate towards chemokines released by other cell types in the airways, either by homologous receptor desensitization, or by diminishing the

chemoattractant chemogradient. No influence of formoterol on IL-8 release in non-stimulated neutrophils was observed in our study, and formoterol did not influence the chemotaxis which in this context is logical since only non-stimulated (i.e. not stimulated with LPS) neutrophils were used in our chemotaxis assays. *In vivo* there are several agents, e.g microbial factors, with the potency to activate neutrophils. The results from our study indicate that activated neutrophils are more sensitive to β_2-agonist-induced cytokine/chemokine release than are non-stimulated neutrophils.

de Coupade et al found a gender difference in β_2-agonist effect on chemotaxis (161) where an inhibitory effect of isoprenaline in neutrophils from females, but not males, was demonstrated. However, this was not confirmed in our study as separate analysis of women and men did not reveal a gender difference, or even a trend towards a difference.

A significant reduction of the neutrophil migration towards IL-8 was observed only in the presence of antibodies against both CXCR1 and CXCR2. This is quite logical since IL-8 is a ligand for both receptors. The reason for the lack of a significant blocking of migration towards GRO-α might be that the migration rate when GRO-α was used as chemoattractant was only 17%, while the migration rate towards IL-8 was 43%. The low migration rate towards GRO-α limits the scope for observing blocking effects of the antibodies; due to small effects there is no space for detectable changes.

It is not clear whether the agonists (IL-8, GRO-α) bind to the same epitopes on the chemokine receptors as do the anti-CXCR1 and anti-CXCR2 antibodies used in study III. If receptor binding competition is the case, this might explain the decreased receptor antibody-labelling observed after incubation with IL-8 or GRO-α. However, previous binding experiments with a radiolabelled ligand showed that the rate of down-regulation of the IL-8 receptor is very rapid. The receptor internalization is an absolutely temperature-dependent process, and the internalization is completely inhibited at 4 °C (174). In the present study, incubation with the receptor agonists was performed at 37 °C while incubation with anti-CXCR1 and anti-CXCR2 antibodies was performed at low temperature (samples kept on ice in the refrigerator), thereby probably preventing the internalized receptors from recycling back to the cell surface. Thus, the antibody-labelling of chemokine receptors should reflect the real number of receptors not being internalized through homologous desensitization by IL-8 and GRO-α. CXCR2 is overall more likely to be internalized than CXCR1, and is vulnerable to degradation after internalization and thereby less likely than CXCR1 to be re-expressed following internalization (32).

Cytokines play a key role in the chronic inflammation of asthma and COPD, and blocking the actions of cytokines might be of clinical benefit. Chemokine receptors are an attractive therapeutic target, and several small-molecule inhibitors have been developed, of which some, including antagonists against CXCR2, are under consideration for trials in COPD (126).

The main aim of study IV was to further explore a previous, somewhat surprising, finding that a long-acting β_2-agonist (salmeterol) did not protect against enhanced bronchial methacholine responsiveness induced by exposure in a pig house (149). Pro-inflammatory cytokines, such as IL-1β and TNF attenuate the ability of cultured cells to relax in response to β_2-agonists, an effect that has been claimed to involve cyclo-oxygenase (COX)-2 (75, 77). According to this hypothesis COX-2 increases PGE_2 release, resulting in increased cAMP formation which in turn leads to PKA activation and thereby phosphorylation and desensitization of the β-adrenoceptor (75). As IL-1 and TNF are involved in the airway inflammatory reaction following exposure in a swine barn (42, 43, 50), we assumed that the previously described lack of protective effect of salmeterol against dust-induced bronchial hyper-responsiveness (149) may be caused by heterologous desensitization of airway β_2-adrenoceptors induced by

pro-inflammatory cytokines. If so, inhibition of COX-2 would counteract heterologous β_2-adrenoceptor desensitization leading to protection of a β_2-agonist against enhanced bronchial responsiveness induced by exposure in a pig house. We further hypothesized that a glucocorticoid or a COX-2 inhibitor synergistically interacts with salmeterol, offering a protection against dust-induced increased bronchial responsiveness and airway inflammation. However, as we were not able to confirm a lack of salmeterol effect, this hypothesis became redundant. One week pretreatment with ibuprofen did not modify the effect of salmeterol. Thus, the reason for a partial instead of a full bronchoprotective effect with salmeterol is not likely caused by heterologous desensitization.

Inhalation of a single dose salmeterol 1 hour prior to exposure offered a partial protection against increased bronchial responsiveness following exposure in a pig house. This protection was similar when salmeterol was given together with placebo, fluticasone and ibuprofen. In addition, one single dose of salmeterol, by itself or in combination with fluticasone or ibuprofen treatment, did not influence the cell and cytokine/chemokine (IL-6, IL-8, TNF) response assessed in induced sputum. The post-exposure increase in exhaled NO levels was attenuated by salmeterol preceded by 1 week of treatment with placebo or ibuprofen, but not with fluticasone.

The results of study IV did not confirm the previous findings by Ek et.al. (149) where salmeterol did not protect against exposure-induced increase in bronchial responsiveness. In this previous study those who were randomized to salmeterol treatment were slightly less responsive to methacholine prior to exposure than those who received placebo. It was therefore discussed whether the difference in pre-exposure bronchial responsiveness may have influenced the results. To eliminate this possible source of error we performed the present study using a cross-over design. We have previously shown that a wash out period of 1 week is enough to normalize increased bronchial responsiveness induced by exposure in a pig barn (175). We thus regard 2-3 weeks wash out, as has been adopted in study IV, to be sufficient to eliminate possible carry over effects from the previous exposure.

In order to better understand the reasons for this discrepancy regarding the protective effect of salmeterol on exposure-induced increase of bronchial responsiveness, we conducted a retrospective analysis of pooled data from four previous studies (60, 62, 176, 177). In these four studies, bronchial responsiveness was studied before and after exposure in 47 healthy subjects who were not treated or wearing protective devices during 3 hours of exposure in a pig barn. This analysis showed that $PD_{20}FEV_1$ after exposure in a swine barn is almost totally independent of pre-exposure $PD_{20}FEV_1$-level; all subjects end up at the same low post-exposure $PD_{20}FEV_1$ (figure 19). In the parallel group study by Ek et al., the salmeterol baseline $PD_{20}FEV_1$ was higher than was $PD_{20}FEV_1$ in the placebo group, and we found no difference of *the fall* in $PD_{20}FEV_1$ between the two groups, as those in the salmeterol group, who started at a higher pre-exposure $PD_{20}FEV_1$, also had a higher post-exposure $PD_{20}FEV_1$ compared with the placebo group. Without drug administration, pre- to post-exposure difference will become larger if pre-exposure $PD_{20}FEV_1$ is high. From these findings we concluded that the *absolute* post-exposure $PD_{20}FEV_1$ values and not the fall in $PD_{20}FEV_1$ should be compared. Our data clearly demonstrated that interventions altering bronchial responsiveness must be compared between groups with similar pre-challenge bronchial responsiveness or in a cross-over design.

The protection of salmeterol in the present study was not complete which is most likely due to the time interval between inhalation of the drug and the methacholine challenge (8 hours); the effect of salmeterol had probably weaned off at the time of the bronchial provocation. This interpretation is supported by a previous study in which the protective effect of salmeterol on

methacholine-induced bronchoconstriction was slightly diminished at 8 hours compared with 2 hours after drug inhalation (149). The uncomplete protection offered by salmeterol could also be related to the fact that salmeterol is a partial agonist and may therefore not be capable of complete protection against exposure-induced increase of bronchial responsiveness.

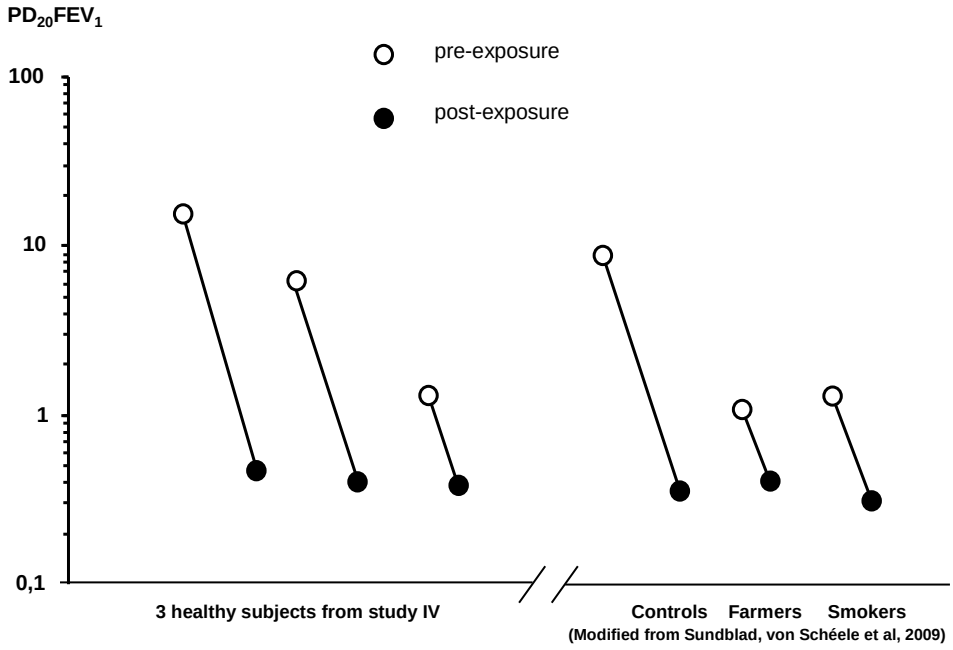

Figure 19. Pre- and post-exposure $PD_{20}FEV_1$ in 3 healthy subjects from study IV, and median $PD_{20}FEV_1$values from a study (62) on healthy controls, farmers and smokers (n=12 in each group). Following exposure to a strong pro-inflammatory stimulus such as organic dust in a pig barn, bronchial responsiveness is enhanced to a certain level irrespective of pre-exposure bronchial response. The pre-to post-exposure difference will become larger if pre-exposure $PD_{20}FEV_1$ is high.

The subjects exhibited a post-exposure neutrophilic airway inflammation and pre-treatment with fluticasone did not influence the inflammatory response. Neither the level of exhaled NO nor the number of neutrophils in sputum were decreased by fluticasone. The finding that pretreatment with fluticasone did not influence the neutrophilic inflammatory response observed in our experiment model is maybe not surprising considering that asthma patients with predominantly neutrophilic airway inflammation respond worse to steroid therapy than do patients with predominantly eosinophilic airway inflammation (116). Furthermore, a slight increase of exhaled NO following exposure was found only after placebo and fluticasone treatment, and was thus not attenuated by the steroid. This observation diverges from what has been described in asthmatics with eosinophilic asthma, where fluticasone treatment lowers the level of exhaled NO (178). The effect of inhaled steroids on exhaled NO varies depending on the type of airway inflammation (179).

None of the treatments in the present study influenced the elevation of body temperature and cytokine levels in blood and sputum observed after exposure. This is in contrast with a previous study where post-exposure plasma IL-6 levels and body temperature were significantly lower after fluticasone treatment than after placebo (56). This discrepancy is

likely to be explained by the different levels of airborne dust, which were considerably higher in the previous study performed in a swine confinement building with 700 pigs, compared to study IV which was performed in a building with 300 pigs. We have previously shown that 2 weeks of fluticasone inhalations did not inhibit or even attenuate the increase in bronchial responsiveness to methacholine following exposure in a swine barn (56). The conclusion must be that the pronounced, neutrophilic airway inflammation obtained in our experimental model, i.e. 3 hours exposure to organic dust in a pig barn, is steroid resistant in its main characteristics.

Exposure in a pig barn enhanced the levels of pro-inflammatory cytokines (IL-6, TNF) and chemokine (IL-8) in the airways, as assessed by analysis of induced sputum. The pharmacological intervention did not affect these inflammatory parameters. The sputum samples were solubilised with DTT (dithiothreitol), which disrupts sulphydryl bonds and therefore may alter proteins so that they are not recognized by antibodies. This is a particular problem with several cytokines and chemokines. Furthermore, proteases in sputum may degrade certain protein mediators (180). Proteases such as neutrophil elastase and metalloproteinases are produced mainly by neutrophils, and it could therefore be expected that the protease levels are increased in the airways after exposure in a pig barn. Another potential problem in the analysis of whole sputum samples is variable oropharyngeal contamination, potentially affecting the levels and reproducibility of various markers of inflammation. However, in a study by in ‘t Veen et al., it was shown that the repeatability of cellular and soluble markers of inflammation in induced sputum from patients with mild and moderate-to-severe asthma was satisfactory (181).

The pronounced neutrophilic inflammation and bronchial responsiveness observed in practically all of the more than 200 subjects who have participated in our dust exposure experimental model through the years, is quite resistant to different pharmacological treatment. We have previously shown that the increased bronchial responsiveness, observed after exposure to organic dust in a swine barn, is not influenced by pre-treatment with cromoglycate, although this therapy halved the influx of neutrophils and almost totally inhibited the airway release of tumour necrosis factor (TNF) (182). Arachidonic acid metabolites are produced in the airway reaction to swine dust exposure (183), but attempts to block the increase in bronchial responsiveness by the administration of zileuton, a leukotriene synthesis inhibitor, failed (60).

Caution is always required in the interpretation of *in vitro* studies and their extrapolation to mechanisms operating *in vivo*. Though formoterol and salmeterol have been shown to increase the release of pro-inflammatory cytokines *in vitro*, it is far from clear what the cellular and molecular effects *in vivo* are. A systematic search was recently performed on online databases for randomized controlled trials evaluating the anti-inflammatory effects of long-acting β_2-agonists in 32 studies and 1105 participants (184). This systematic review demonstrated that long-acting β_2-agonists do not decrease inflammatory cell numbers in the airways of patients with asthma and suggests that the clinical synergy between long-acting β_2-agonists and inhaled corticosteroids are not related to an anti-inflammatory effect of long-acting β_2-agonists. The clinical benefit of combining a long-acting β_2-agonists and a glucocorticosteroid is not questioned, though, by the authors of this review.

Treatment with β_2-agonists and glucocorticosteroids is of great clinical benefit for millions of people. Still, a better understanding of underlying inflammatory mechanisms *in vitro* and *in vivo* is crucial for development of new and more efficient therapeutic, not least considering the need for new treatment strategies in steroid-resistant severe asthma, and COPD.

Conclusions

- Organic dust from a pig barn was a strong stimulus for IL-6 and IL-8 release in primary bronchial epithelial cells (PBEC) and in human alveolar epithelial carcinoma cells (A549).

- The long-acting β_2-agonists formoterol and salmeterol enhanced both the basal and the organic dust-induced IL-6 and IL-8 release in PBEC and A549 cells in a dose-response manner. This effect seems to be mediated by specific β_2-adrenoceptor mechanisms in PBEC but not in A549 cells.

- Formoterol stimulated and budesonide inhibited IL-6, IL-8 and GRO-α release in LPS-stimulated isolated peripheral blood neutrophils from healthy, non-allergic donors.

- Formoterol upregulated both CXCR1 and CXCR2 expression, whereas budesonide upregulated the expression of CXCR2 only. Despite the effects of formoterol and budesonide on chemokine receptors, these drugs had no effect on chemotaxis towards IL-8 and GRO-α. Thus, the regulation of CXCR1 and CXCR2 does not necessarily correlate to the functional response (chemotaxis/migration) in neutrophils *in vitro*.

- One single dose of salmeterol partially protected against the increased bronchial responsiveness to methacholine in healthy, non-smoking, non-atopic subjects following organic dust exposure in a pig barn, but did not significantly influence the inflammatory response. One week pre-treatment with fluticasone or ibuprofen did not affect the protective effect of salmeterol on bronchial responsiveness, and did not influence the inflammatory response.

- Exposure to organic dust in a pig barn leads to an enhancement of bronchial responsiveness in healthy subjects to a certain maximal level which is similar in all subjects and unrelated to pre-exposure level of bronchial responsiveness. Interventions altering bronchial responsiveness must therefore be compared between groups with similar pre-challenge bronchial responsiveness or in a cross-over design.

- No additive/synergistic effects between β_2-agonist and glucocorticoid were observed, neither *in vitro* nor *in vivo*.

Sammanfattning (in Swedish)

Luftvägarna och lungorna exponeras för olika ämnen i omgivningsmiljön, somliga skadliga för celler och vävnader. Inflammatoriska luftvägssjukdomar är mycket vanliga i befolkningen. Cirka 10% av alla vuxna i västvärlden har astma, och global förekomst av kroniskt obstruktiv lungsjukdom (KOL) uppskattas till cirka 10% i befolkningen över 40 år. Behandling med anti-inflammatoriska läkemedel är central vid dessa sjukdomar och de vanligaste använda läkemedlen är β_2-agonister och glukokortikoider. Synergieffekter mellan dessa läkemedel är väl belagda i kliniska studier samt har även påvisats på cellulär och molekylär nivå *in vitro*.

Det är känt att lantbrukare, i synnerhet svinuppfödare, har en ökad förekomst av inflammatoriska luftvägssjukdomar, och att akut exponering för höga halter organiskt damm ger upphov till symptom såväl hos svinskötare som hos personer som vanligen inte vistas i lantbruksmiljö. Denna kunskap ligger till grund för vår forskargrupps försöksmodell där friska, tidigare oexponerade försökspersoner under 3 timmar utsätts för organiskt damm i svinstall. Akut exponering i svinhusmiljö ger upphov till en kraftig luftvägsinflammation, ökad bronkiell reaktivitet och influensaliknande symptom med feber, muskelvärk, illamående, trötthet och hosta. Organiskt damm insamlat i svinstall är ett potent stimulus för frisättning av pro-inflammatoriska cytokiner och kemokiner i cellkultur.

Huvudsyftet med de studier som ligger till grund för denna avhandling var att närmare studera effekten av β_2-agonister och glukokortikoider på inflammatoriska mekanismer *in vivo* och *in vitro*. De läkemedel som använts är formoterol och salmeterol (långverkande β_2-agonister) samt budesonid och flutikason (glukokortikoider).

I det första delarbetet studerades hur formoterol och salmeterol påverkar cytokin- och kemokinfrisättning från ostimulerade och svindamms-stimulerade luftvägsepitelceller *in vitro*. Två olika sorters epitelceller användes; A549, en human alveolär adenocarcinom-cellinje, samt primära bronkiella epitelceller (PBEC) isolerade från bronkialvävnad erhållen från patienter som genomgått lungoperation. Formoterol och salmeterol ökade frisättningen av IL-6 och IL-8 både i ostimulerade och dammstimulerade epitelceller. Denna läkemedelseffekt hämmades av β-blockerare (propranolol, sotalol) i PBEC men ej i A549-celler. Detta tyder på olika mekanismer för β_2-agonisteffekt i bronkiella och alveolära epitelceller, och att effekterna i A549-celler inte var β_2-adrenoceptormedierade.

I det andra delarbetet studerades effekten av formoterol och budesonid på IL-6 och IL-8-frisättning från dammstimulerade PBEC. Budesonid hämmade frisättningen av både IL-6 och IL-8 i ett dos-responsförhållande medan formoterol stimulerade frisättningen av IL-6 men inte IL-8. Budesonids hämmande effekt kvarstod i närvaro av formoterol, men ingen synergieffekt mellan läkemedlen observerades.

I det tredje delarbetet studerades effekten av formoterol och budesonid på kemokin/cytokinfrisättning, kemokinreceptoruttryck samt kemotaxis i isolerade humana blodneutrofiler *in vitro*. Formoterol ökade och budesonid hämmade frisättningen av IL-6, IL-8 och GRO-α från LPS-stimulerade neutrofiler. Formoterol uppreglerade uttrycket av kemokinreceptorerna CXCR1 och CXCR2, medan budesonid uppreglerade CXCR2 enbart. Trots klara effekter på kemokinfrisättning och kemokinreceptoruttryck påvisades ingen effekt av β_2-agonist och glukokortikoid på kemotaxis.

I det fjärde delarbetet exponerades 12 friska försökspersoner för organiskt damm under 3 timmar i ett svinstall i samband med vägning av grisar, ett arbetsmoment som innebär att stora

mängder damm virvlar omkring i luften. Varje försöksperson exponerades vid 4 olika tillfällen med 2-3 veckors mellanrum, och behandlades med olika läkemedel inför varje exponering. I denna studie i cross-over design fann vi att engångsdos av salmeterol delvis skyddar mot den ökade bronkiella reaktivitet som erhålls efter exponeringen. Det inflammatoriska svaret i luftvägarna (invandring av inflammatoriska celler och frisättning av pro-inflammatoriska substanser) påverkades ej av salmeterol. Behandling med flutikason eller ibuprofen under en vecka innan exponering påverkade inte effekten av salmeterol. En retrospektiv studie med sammanslagning av data från fyra tidigare studier med samma exponeringsmodell gjordes också. Slutsatsen av denna var att exponering av friska försökspersoner för organiskt damm i svinstall leder till en ökning av bronkiell reaktivitet upp till en viss nivå som är nästan helt oberoende av individuellt utgångsvärde (dvs. före exponering).

Slutsatserna från dessa studier är

- att organiskt damm från svinstall är ett potent stimulus för frisättning av pro-inflammatoriska cytokiner och kemokiner från luftvägsepitelceller och neutrofila granulocyter *in vitro*

- att formoterol och salmeterol ökade medan budesonid minskade frisättningen av pro-inflammatoriska cytokiner och kemokiner från luftvägsepitelceller och neutrofila granulocyter *in vitro*

- att formoterol och budesonid ökade uttrycket av kemokinreceptorer på neutrofila granulocyter men trots detta inte hade någon effekt på neutrofil kemotaxis *in vitro*

- att engångsdos av salmeterol innan exponering av friska försökspersoner for organiskt damm i ett svinstall delvis skyddade mot ökning av bronkiell reaktivitet

- att interventionsstudier av bronkiell reaktivitet i denna typ av försöksmodell måste göras som jämförelse mellan grupper med samma utgångsvärde för bronkiell reaktivitet, eller hellre, i cross-over design

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